Evolutionarily conserved TRH neuropeptide pathway regulates growth in *Caenorhabditis elegans*

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In vertebrates thyrotropin-releasing hormone (TRH) is a highly conserved neuropeptide that exerts the hormonal control of thyroid-stimulating hormone (TSH) levels as well as neuromodulatory functions. However, a functional equivalent in protostomian animals remains unknown, although TRH receptors are conserved in proto- and deuterostomians. Here we identify a TRH-like neuropeptide precursor in *Caenorhabditis elegans* that belongs to a bilaterian family of TRH precursors. Using CRISPR/Cas9 and RNAi reverse genetics, we show that TRH-like neuropeptides, through the activation of their receptor TRHR-1, promote growth in *C. elegans*. TRH-like peptides from pharyngeal motor neurons are required for normal body size, and knockdown of their receptor in pharyngeal muscle cells reduces growth. Mutants deficient for TRH signaling have no defects in pharyngeal pumping or isthmus peristalsis rates, but their growth defect depends on the bacterial diet. In addition to the decrease in growth, *trh-1* mutants have a reduced number of offspring. Our study suggests that TRH is an evolutionarily ancient neuropeptide, having its origin before the divergence of protostomes and deuterostomes, and may ancestrally have been involved in the control of postembryonic growth and reproduction.

thyrotropin-releasing hormone | *C. elegans* | neuropeptide | molecular evolution | growth regulation

After Harris's initial proposal on the hypothalamic control of pituitary secretion (1), it took almost two decades to identify the first hypophysiotropic molecule. In 1969 the groups of Schally and Guillemin isolated the tripeptide pQHP-NH_2 (2, 3), named "thyrotropin-releasing hormone" (TRH). The sequence of TRH is fully conserved across all vertebrates, indicating that strong evolutionary pressure has acted to preserve its structure (4). In all vertebrate phyla TRH is synthesized from a larger precursor protein (preproTRH) that contains five to eight copies of the TRH sequence (4). Following the explosion of genome and transcriptome sequence data, preproTRH was identified in chordate species lacking a bona fide pituitary, e.g., cephalochordates (5), and in the genomes of more ancient deuterostomes, including echinoderms (6, 7). In contrast to vertebrate (pQHP-NH_2) and chordate (pQSP-NH_2) tripeptide TRHs, most predicted echinoderm TRHs are tetrapeptides (pQ[W/Y][Y/F/P][T/G/A]-NH_2) (8). Like vertebrate TRH, they are small peptides with an N-terminal pyroglutamate, a C-terminal amide (-NH_2) group, and amino acids with aromatic or cyclic side chains at the second and third positions. TRH therefore is widely distributed throughout the deuterostomian lineage of the Animal Kingdom, suggesting an ancient origin for this neuropeptide hormone.

In mammals, hypothalamic TRH is the prime regulator of the set point of thyroid-stimulating hormone (TSH) synthesis and secretion by the anterior pituitary thyrotrophs (9). TSH secretion stimulates the thyroid gland to produce the thyroid hormones (THs) thyroxine (T_4) and triiodothyronine (T_3). The hypothalamus–pituitary–thyroid (HPT) axis is essential for growth, metabolism, and energy homeostasis (10–12). THs have pleiotropic effects on vertebrate development, with amphibian metamorphosis being the most spectacular example (9). THs can also induce metamorphosis in urochordates, *Amphioxus*, and echinoderms (5, 13, 14). Although this function of THs likely originated early in deuterostomian evolution (15), the prime role of TRH in controlling TH levels seems to have evolved more recently in vertebrates. In fish and amphibians, TRH has no or only a minor effect on the production of TSH but allows the secretion of growth hormone (GH), prolactin (PRL), and α-melanocyte–stimulating hormone (α-MSH) (9, 16). In nonmammalian vertebrates corticotropin-releasing hormone (CRH), a prime regulator of stress responses, is a potent inducer of TSH secretion (16). These effects raise the question as to what the ancient function of TRH might have been. In addition to its role in the HPT axis, mammalian TRH has pleiotropic neuromodulatory functions in the control of arousal, sleep, food intake, thermogenesis, locomotor activity, and immunity (reviewed in refs. 17 and 18). TRH is also secreted from nonhypophysiotropic neurons in the CNS and from a number of peripheral organs, including the pituitary, thyroid, placenta, gallbladder, and testis (17, 18).

Like most neuropeptides, TRH acts through G protein-coupled receptors (GPCRs) named "TRH receptors" (TRHRs). Orthologs of mammalian TRHRs are conserved in all major deuterostomian lineages (19, 20). Phylogenetic analyses across bilaterians also revealed orthologs of deuterostomian TRHRs in Protostomia, in both lophotrochozoan and ecdysozoan species. TRHR orthologs were identified in the annelid *Platynereis dumerilii* and in the genomes and transcriptomes of mollusks, arthropods, and *Caenorhabditis elegans* (19–21). However, with the exception of *Platynereis* and molluscan EFLGamides, no ortholog of the vertebrate TRH precursor was found in any protostomian genome (21, 22). Therefore, it has been proposed that the HPT axis of vertebrates,

Significance

The hypothalamic neuropeptide TRH (thyrotropin-releasing hormone) is one of the major endocrine factors that regulate vertebrate physiology. For decades the general assumption has been that TRH neuropeptides are not present in protostomes, at least not in ecdysozoans, despite the presence of TRH receptor orthologs in these phyla. Here we identify a TRH-related neuropeptide–receptor pathway in the nematode *Caenorhabditis elegans*. TRH-like neuropeptides activate the *C. elegans* TRH receptor ortholog in cell-culture cells. Using RNAi and CRISPR/Cas9 reverse genetics, we discovered that TRH-related signaling in the pharyngeal system promotes *C. elegans* growth. Our study provides evidence of a functional TRH neuropeptide–receptor pathway in invertebrates, suggesting that TRH signaling had evolved in a bilaterian ancestor more than 700 million years ago.

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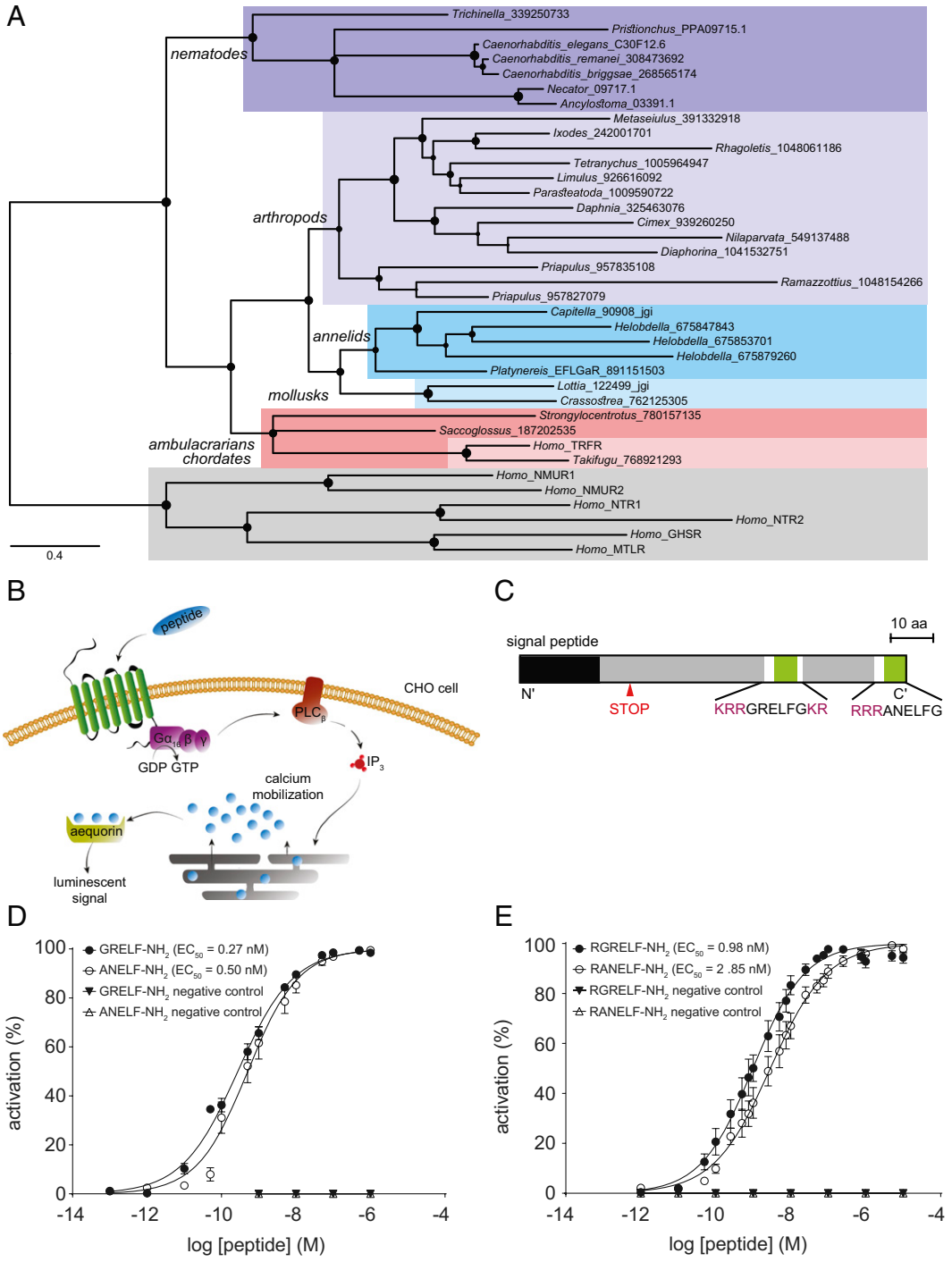

Fig. 1. *C. elegans* TRH-1 neuropeptides activate the TRHR ortholog TRHR-1. (*A*) Tree-like representation of the phylogenetic relationship between *C. elegans* TRHR-1, protostomian, and deuterostomian TRHRs. Sequences are identified using their genus or full scientific name (for *Caenorhabditis* species) and their GenBank accession number (by default), JGI (for *Lottia* and *Capitella* sequences), or WormBase identifiers (for nematode sequences). Groups of sequences are colored according to their phyletic distribution, indicated in italic from top to bottom as nematodes, arthropods, annelids, mollusks, ambulacrarians (echinoderms and hemichordates), and chordates. Human neuromedin U (Homo_NMUR1/2), neurotensin (Homo_NTR1/2), ghrelin (Homo_GHSR), and motilin (Homo_MTLR) receptors were used as outgroup to root the tree, because this group of sequences was previously shown to be most related to bilaterian TRHRs (19, 20). Branches with a support value below 0.5 were collapsed, and circles associated with each branch were drawn with a diameter proportional to the branch values. (*B*) Luminescence-based calcium mobilization assay for measuring GPCR activation. The GPCR is expressed in CHO cells coexpressing aequorin and the promiscuous human $G\alpha_{16}$ subunit. Upon agonist binding, GPCR activation initiates the release of calcium from intracellular storage sites, which is monitored using the calcium-sensitive biosensor aequorin. (*C*) Domain structures of the *C. elegans* TRH-1 precursor. Predicted proprotein convertase sites are in purple; the arrowhead indicates the site of a premature stop codon in a *trh-1*–mutant allele, *lst1118*, generated by CRISPR/Cas9. C′, carboxyl terminus; N′, amino terminus. (*D* and *E*) TRH-1 neuropeptides dose-dependently activate TRHR-1 in a cellular assay described in *B*. The EC_{50} values (95% CI) are 0.27 nM (0.24–0.31 nM) for GRELF-NH_2, 0.50 nM (0.39–0.66 nM) for ANELF-NH_2, 0.98 nM (0.82–1.18 nM) for RGRELF-NH_2, and 2.85 nM (2.41–3.37 nM) for RANELF-NH_2. Cells transfected with an empty vector were used as a negative control. Dose–response data are shown as relative (%) to the highest value (100% activation) after normalization to the total calcium response. Error bars represent SEM; $n \geq 3$.

including TRH, has been lost in protostomes, at least in the ecdysozoan phyla (arthropods and nematodes) (21–24). In contrast, the conserved TRH precursors in deuterostomes and the occurrence of TRHR orthologs in both deuterostomes and protostomes, including in ecdysozoans, suggests that the evolutionary origin of TRH signaling dates back to a common ancestor of bilaterian animals. The most parsimonious hypothesis for the evolutionary conservation of TRHRs across protostomes suggests the existence of a conserved protostomian TRH system. Here, we identified a TRH-related neuropeptide pathway in the nematode *C. elegans*. Using RNAi and CRISPR/Cas9 reverse genetics, we demonstrate that *C. elegans* TRH-like signaling is required for growth and regulates body size.

Results

Identification of a TRH Precursor Ortholog in *C. elegans*. *C. elegans* has a single receptor ortholog of vertebrate TRHRs encoded by the gene C30F12.6 (19, 20). The protein is an orphan receptor of the rhodopsin GPCR family and was originally named "NMUR-4" based on its sequence similarity to mammalian neuromedin U receptors (NMURs). Phylogenetic analyses show that NMUR-4 is orthologous to vertebrate TRHRs (Fig. 1*A* and refs. 19 and 20). Therefore, we refer to NMUR-4 from here onwards as "TRHR-1." To identify the cognate ligand(s) of this receptor, we expressed TRHR-1 with the promiscuous G_q-like $G\alpha_{16}$ protein in Chinese hamster ovary (CHO) cells and monitored its activation by a bioluminescent calcium indicator (Fig. 1*B*). We challenged receptor-expressing cells with a synthetic library of more than 250 known and predicted *C. elegans* peptides, but none was able to activate TRHR-1.

The lack of TRHR-1 activation by known *C. elegans* peptides and the presence of orphan TRHR orthologs in Ecdysozoa and Lophotrochozoa led us to hypothesize that an as yet unknown family of TRH-like precursors might be present in these phyla and might encode the cognate ligand of TRHR-1. We therefore performed an in silico search for TRH-like neuropeptide precursors in *C. elegans*. Using a hidden Markov model (HMM)-based strategy (19, 20), we first generated a list of peptide precursor candidates in the *C. elegans* genome that showed conservation across protostomes. From this screening strategy the ELFamide precursor, encoded by the *C. elegans* gene C30H6.10, stood out as the most promising candidate, because (*i*) it was conserved across all the main protostomian phyla, and (*ii*) it yielded peptides with features similar to TRHs in terms of their small size (five or six amino acids) and biochemical characteristics (see below). The newly predicted ELFamide precursor has 96 amino acid residues including an N-terminal signal sequence (Fig. 1*C*). It contains two TRH-like progenitor sequences that are preceded by a triple basic site (KRR or RRR) (Fig. 1*C*). The presence of a C-terminal glycine residue in these peptide sequences is indicative of post-translational modification to an amide ($-NH_2$) group in the mature peptides (25). Based on its relationship to deuterostomian *trh* precursor genes, we propose designating C30H6.10 as the thyrotropin-releasing hormone 1 (*trh-1*) gene.

TRH-1 Neuropeptides Activate *C. elegans* TRHR-1. To determine whether the predicted TRH-like peptides are ligands of TRHR-1, we tested synthetic peptide replicates of GRELF-NH_2 and ANELF-NH_2 in our calcium bioluminescence assay (Fig. 1*B*). Because both peptides are preceded by a tribasic site in the TRH-1 precursor, we also tested the activity of RGRELF-NH_2 and RANELF-NH_2 peptides that are generated if cleavage occurs after the second arginine residue. All TRH-1 peptides activated TRHR-1 dose dependently and with nanomolar EC_{50} values, whereas cells transfected with a control vector showed no calcium responses (Fig. 1 *D* and *E*). TRHR-1 was not activated by scrambled TRH-1 peptides (Fig. 2 *A* and *B*) or by any other neuropeptide-like protein (NLP) or FMRFamide-like peptide (FLP) of *C. elegans*. TRH-1 peptides therefore specifically activate TRHR-1 *in cellulo*, suggesting that they are the cognate ligands of this receptor. We further studied TRHR-1 signaling in cells lacking expression of the $G\alpha_{16}$ subunit, which couples the receptor to the $G\alpha_q$ pathway regardless of its endogenous G-protein coupling. When expression of $G\alpha_{16}$ was omitted, TRH-1 peptides elicited strong calcium responses (Fig. S1*A*), indicating that TRHR-1 can couple to endogenous $G\alpha_q$ proteins in CHO cells and can signal through the calcium pathway.

Neuropeptide ligands have been identified for vertebrate and *Platynereis* TRHRs (22, 26). We tested whether TRH-like peptides of these species could activate *C. elegans* TRHR-1 in cell-culture cells (Fig. 1*B*). Both vertebrate TRH (pQHP-NH_2) and *Platynereis* EFLGamide (FSEFLG-NH_2) activated TRHR-1 at 10 μM (Fig. S1*B*). The predicted echinoderm TRH of *Strongylocentrotus purpuratus* (pQYPG-NH_2 and ref. 6) elicited no calcium response (Fig. S1*B*). *C. elegans* TRH-1 peptides also activated the human and *Platynereis* TRHRs at concentrations down to 1 μM (Fig. 2 *C* and *D*). Because ligand–receptor couples coevolve, cross-activation of TRHRs by TRH-like peptides from different species is expected to require high ligand concentrations. Taken together, these results support the evolutionary conservation of a TRH-related signaling system in *C. elegans*.

C. elegans TRH-1 peptides have five to six amino acid residues. To determine residues that are crucial for activation of TRHR-1, we tested a series of analogs in which amino acids of TRH-1 were replaced by alanine residues (Fig. S1 *C–E*). Substitution of the N-terminal glycine, arginine, or asparagine residues had nearly no effect on peptide activity. By contrast, the activity of TRH-1 peptides was strongly reduced when residues of the C-terminal ELFamide sequence were replaced by alanine (Fig. S1 *C–E*). Hence the ELF-NH_2 tripeptide motif, which is conserved in all TRH-1 peptides, is important for activating TRHR-1, although it is not sufficient to elicit full receptor activation (Fig. S1*B*).

Comparison of TRH-Like Neuropeptides in Protostomia. In addition to *C. elegans* TRH-1, our in silico search for TRH precursor orthologs identified TRH-like sequences in the genomes of several protostomes, including annelids, mollusks, arthropods, and other nematode species (Fig. 3). Like deuterostome TRHs, all protostomian TRH-like peptides have short sequences and a C-terminal amide group. All listed TRH-like peptides contain five to eight amino acids in protostomes, four residues in echinoderms, and three amino acids in vertebrates. The C-terminal E-[L/F]-[L/F/V]-[G/Ø]-NH_2 motif is common to all protostomian TRH-like sequences. On the other hand, all deuterostomian TRHs share a pyroglutamate residue (pQ) that can be generated from an N-terminal glutamine or glutamic acid residue. In protostomes, the glutamic acid residue is not situated at the N terminus, suggesting that the pyroglutamate modification most likely arose later in evolution. Furthermore, all TRH precursors yield multiple related peptides, ranging from two copies in *C. elegans* to more than 20 variants in annelids and *Branchiostoma*.

***C. elegans* TRH-1 and TRHR-1 Are Expressed at the Neuromuscular Pharynx.** To gain hints on putative functions of TRH-1 signaling, we determined the expression patterns of *trh-1* and *trhr-1*. Transgenic *C. elegans* expressing translational fosmid-recombineered GFP transgenes or a polycistronic *Ptrhr-1::trhr-1::SL2::gfp* reporter transgene showed *trhr-1* expression only at the pharynx (Fig. 4*A* and Fig. S2 *A* and *B*). The pharynx is a neuromuscular feeding organ that consists of three functional parts: the corpus, subdivided into an anterior procorpus and a posterior metacorpus, the isthmus, and the terminal bulb (27). More specifically, TRHR-1 localized to pharyngeal muscle cells of the metacorpus (pm4) and the isthmus (pm5).

Reporter transgenes for *trh-1*, including the *trh-1* promoter region and the gene's genomic or cDNA sequence, showed similar expression patterns in the pharyngeal nervous system that is separated

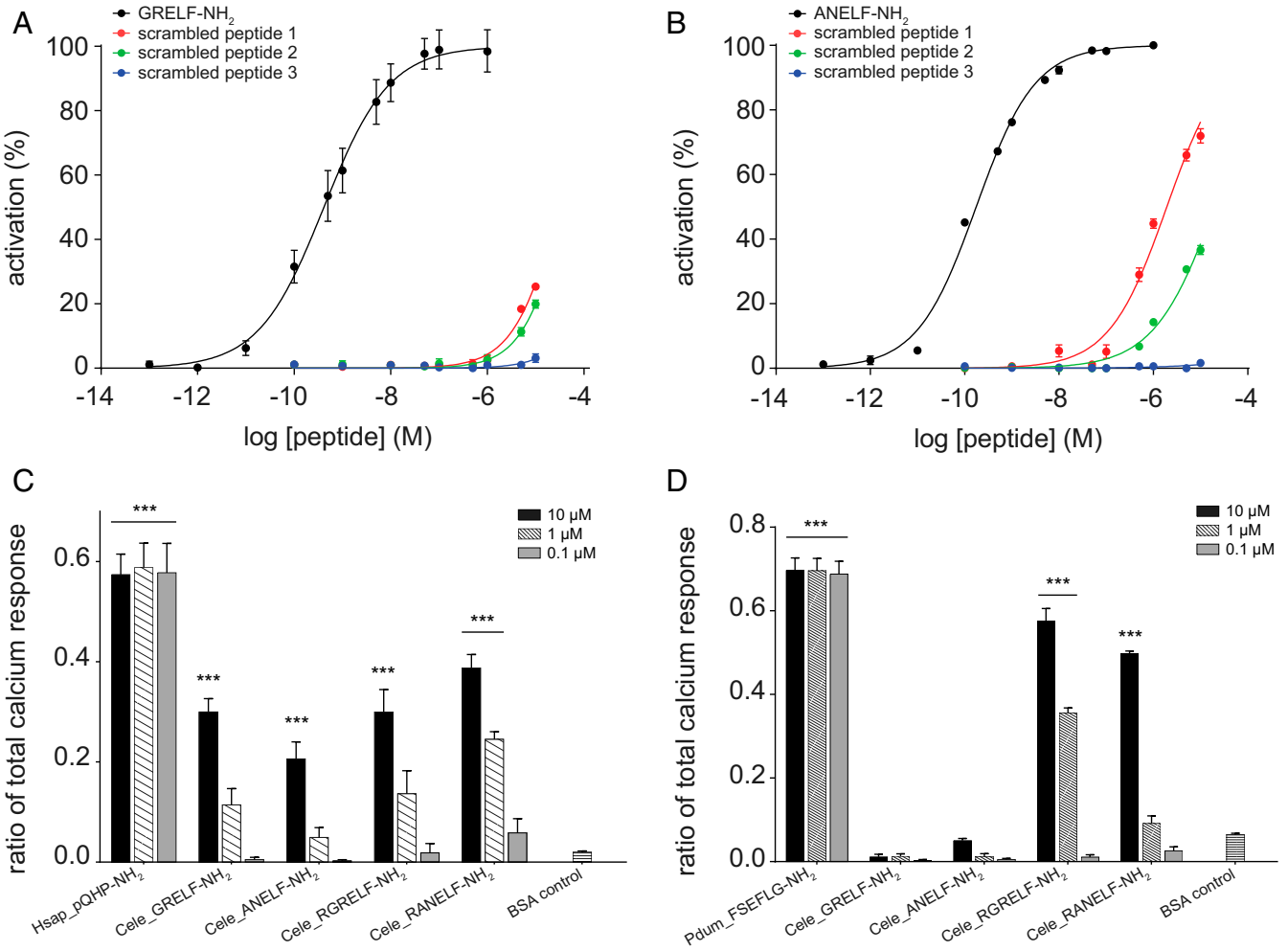

Fig. 2. Different TRHR orthologs are activated by TRH-1 peptides *in cellulo*. (*A* and *B*) *C. elegans* TRHR-1 expressed in CHO cells (as described in Fig. 1*B*) is activated by TRH-1 peptides but not by scrambled peptides of GRELF-NH_2 (peptide 1: RELFG-NH_2; peptide 2: EFLRG-NH_2; peptide 3: EGFLR-NH_2) (*A*) and ANELF-NH_2 (peptide 1: LFAEN-NH_2; peptide 2: NELAF-NH_2; peptide 3: FNEAL-NH_2) (*B*). (*C* and *D*) TRH-1 peptides activate human (*C*) and *Platynereis* (*D*) TRHRs at micromolar concentrations in cell culture cells. Human TRH and *Platynereis* EFLGamide were used as positive controls. Statistical significance of peptide-evoked responses compared with the BSA control (***$P < 0.001$) was determined by two-way ANOVA and Sidak multiple comparison test. Data are shown as the ratio of peptide-evoked responses to total calcium responses. Error bars represent SEM; $n \geq 3$.

from the CNS by a basal lamina but is thought to secrete signals to the pseudocoelomic fluid (28). We identified *trh-1*–expressing cells as the pharyngeal motor neurons M4 and M5 based on their position and morphology (Fig. 4*B* and Fig. S2*C*). M4 directly innervates the pharyngeal muscle pm5 and controls isthmus peristalsis; the function of the motor neuron M5 is unclear (27, 29).

CRISPR/Cas9-Induced Knockout Mutants of *trh-1* Have Normal Pharyngeal Pumping and Peristalsis Rates. The pharyngeal motor neuron M4, which expresses *trh-1*, is essential for feeding because it controls peristalsis of the posterior isthmus muscles of the pharynx (30, 31). We therefore hypothesized that TRH-1 neuropeptides might regulate isthmus peristalsis. Because no null mutants of *trh-1* were available, we used CRISPR/Cas9-mediated genome editing to introduce mutations in the *trh-1* gene. We isolated a loss-of-function *trh-1* allele, *lst1118*, with an 8-bp indel (Fig. 1*C*). The mutation caused a frameshift and premature stop codon immediately downstream of the signal peptide sequence, which should target the aberrant *trh-1* transcripts for nonsense-mediated decay and fully eliminate *trh-1* function.

We examined the isthmus peristalsis rate of *trh-1* mutants by feeding worms red fluorescent *Escherichia coli* OP50 bacteria, allowing direct visualization of the peristaltic contractions of the pharyngeal isthmus (32, 33). There was no difference in the isthmus peristalsis rates of wild-type and *trh-1*–mutant worms fed OP50 (Fig. 4*C*). The rates of pharyngeal pumping while feeding on *E. coli* OP50 were similar as well (Fig. 4*D*). These results suggest that TRH-1 neuropeptides have no obvious effects on isthmus peristalsis or on pharyngeal pumping.

The TRH-1/TRHR-1 Pathway Promotes Growth in *C. elegans*. Although the role of TRH-like peptides in protostomes has remained unknown, TRH signaling in vertebrates regulates several aspects of physiology, such as development, growth, reproduction, and locomotor activity (9, 34–39). We investigated if TRH-1 neuropeptides mediate *C. elegans* locomotion by measuring the forward speed of worms on a bacterial lawn and off food. In both cases, *trh-1* mutants crawled at a speed similar to that of wild-type worms (see Fig. S3 *A* and *B*).

In vertebrates, TRH promotes growth as a stimulatory factor of GH secretion (9). To determine whether *trh-1* regulates growth in *C. elegans*, we quantified worm sizes by measuring the length and width of formaldehyde-fixed or anesthetized worms and approximated their volume as a cylinder. We synchronized a population of

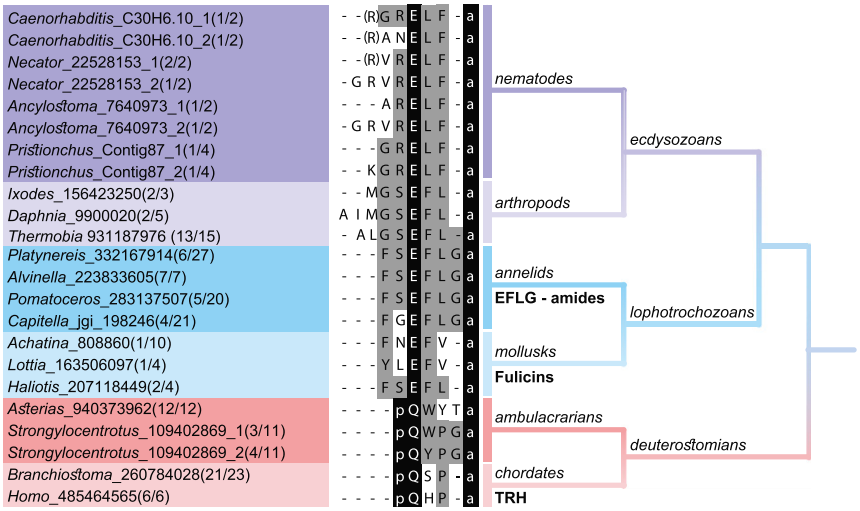

Fig. 3. Amino acid sequence alignment of TRH-like neuropeptides of representative protostomians and deuterostomians. Identical amino acids are highlighted in black, and similar amino acids are highlighted in gray (pQ is a posttranslationally modified glutamate or glutamine). Sequences were placed and colored according to their phyletic group, following the scheme in Fig. 1*A*. Names of TRH-like peptides that have already been identified and named in some species (EFLGamides in *Platynereis dumerilii*, fulicins in *Achatina fulica*, and TRH in chordates) are indicated in bold. The C-terminal amide (-NH_2) groups are indicated by "a" and pyroglutamate residues as "pQ." Sequences can be identified by their names, either through GenBank (for most sequences), JGI (for *Capitella*), or the Contig number (for *Pristionchus*). The number of peptides with a sequence identical to the one shown and the total number of TRH-like peptides encoded by the precursor are indicated in parentheses at the end of the names. For example, there are four copies of the FGEFLGa *Capitella* peptide in the precursor (GenBank ID: 198246), which codes for 21 peptides in total. For each species, only the most abundant peptide sequence or two examples of peptides in the precursor are represented. Protostomian sequences share a common core signature E-[L/F]-[L/F/V], and deuterostomian sequences harbor a common N-terminal pyroglutamate residue.

worms by embryonic starvation, leading to developmental arrest at the first larval stage (L1), and quantified the body volume every 24 h after feeding until the fifth day of adulthood. The body size of *trh-1* mutants was smaller than that of wild-type worms 24 h after L1 arrest. This size defect increased and persisted in subsequent larval and adult stages (Fig. 5*A* and Fig. S4 *A* and *B*). The *trh-1* mutants showed a reduced growth rate from early larval stages to adulthood (Fig. S4 *C* and *D*). The largest difference was seen around 48 h after the L1 arrest, corresponding to the fourth larval (L4) stage and its transition to adulthood. The body size defect was most pronounced at the adult stage (Fig. 5*A*). Therefore 1-day-old adult worms were used in further experiments. *trh-1* mutants had a significantly smaller body length and width, resulting in a reduced body volume (Fig. 5 *B*–*D*). Restoring *trh-1* expression under the control of its promoter rescued the size defect (Fig. 5*D*); hence TRH-1 neuropeptides are required for normal body size. Overexpression of the neuropeptide significantly increased the body volume of wild-type worms, suggesting that *trh-1* is sufficient to induce growth (Fig. 5*D*).

We next investigated the role of TRHR-1 in growth regulation. Although a strain carrying a *trhr-1* mutant allele is available (RB1284), we found that a genomic sequence of the reported deletion is still present in the strain's background and that it is unlikely to be a *trhr-1*–null mutant. We therefore applied RNAi using *trhr-1* promoter-driven sense and antisense (*sas*) transgenes (40). Knockdown of *trhr-1* significantly decreased body volume (Fig. 5*D*). To verify that TRH-1 and its receptor function together in vivo, we combined knockdown of *trhr-1* with overexpression or knockout of *trh-1*. The body size of *trh-1(lst1118); trhr-1(RNAi)* worms was similar to that of *trh-1(lst1118)* mutants or *trhr-1(RNAi)* worms (Fig. 5*E*). *trh-1* overexpression increased the body size of wild-type worms, and knockdown of *trhr-1* suppressed this effect (Fig. 5*E*). Taken together, these results indicate that TRH-1 regulates growth through the activation of its receptor TRHR-1.

A reduction in body volume may result from slower development caused by impaired TRH-1 signaling. However, *trh-1* overexpression increased the body size of wild-type worms. The size defect of *trh-1* mutants also persisted at least 5 days into adulthood (from 72 h onwards; Fig. 5*A*), suggesting that growth, and not development, is affected. Corroborating this finding, *trh-1* mutants displayed little to no delay in reproductive maturation (Fig. S3*C*). As with wild-type worms, egg-laying of *trh-1* mutants started within 72 h after synchronized L1 larvae were fed and reached its maximum at the second day of adulthood (Fig. S3*C*). Although reproductive maturation was normal, the number of viable offspring was significantly reduced by 17% in *trh-1* mutants compared with wild-type worms (Fig. S3*D*). This defect may indicate a role of *trh-1* in regulating *C. elegans* reproduction. However, reintroducing a wild-type copy of *trh-1* cDNA that restored the growth defect failed to rescue the reduced brood size of the mutant strain (Fig. S3*D*).

Pharyngeal TRH-1 Signaling Controls Body Size. Both TRH-1 neuropeptides and their receptor are expressed at the neuromuscular pharynx. To determine the cellular basis of TRH-1–mediated growth, we transgenically restored *trh-1* gene expression in specific neurons in worms otherwise lacking *trh-1* function. Although no cell-specific promoter is known to drive expression in the M5 neuron, we used the *ceh-28* promoter to restore *trh-1* expression only in the pharyngeal motor neuron M4 (41). M4-specific expression of *trh-1* partially rescued the reduced body size of *trh-1* mutants (Fig. 5*F*), indicating that *trh-1* is required in M4 to promote growth. The partial rescue effect suggests that growth regulation likely depends on *trh-1* functions in M4 and M5 neurons. The growth defect of *trh-1* mutants was fully rescued when we restored *trh-1* expression under the control of *trh-1* or *glr-8* promoters (Fig. 5 *D* and *G*), which drive expression in both M4 and M5 neurons (in addition to other neurons driven by the *glr-8* promoter) (Table S1) (42). To determine the cellular focus of *trhr-1*, we performed a tissue-specific knockdown of the receptor in pharyngeal muscle cells by expressing sense and antisense transgenes under control of the *myo-2* promoter (43). Knockdown of *trhr-1* in the pharyngeal

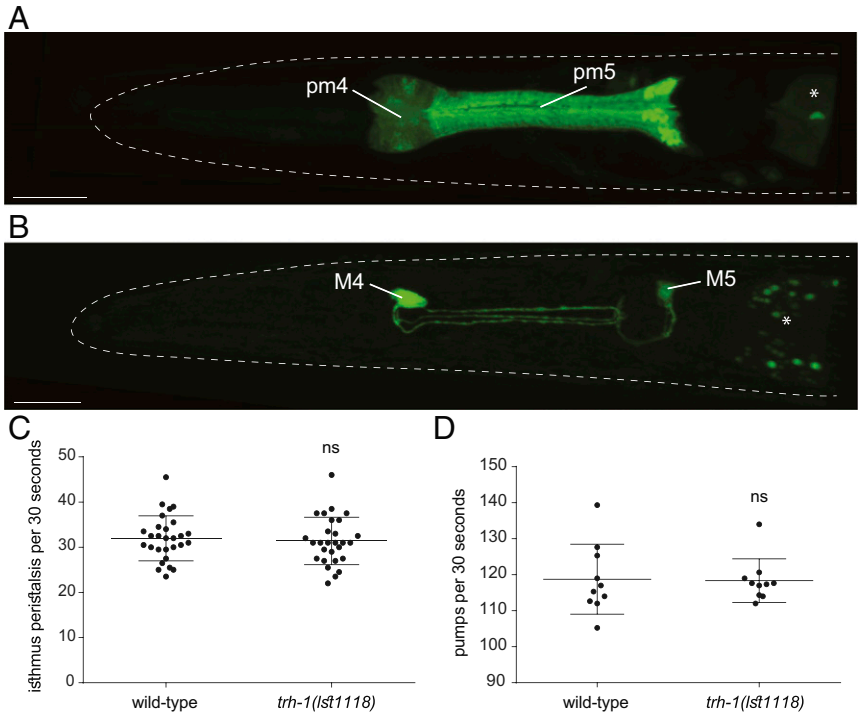

Fig. 4. (*A* and *B*) Representative confocal Z-stack projections of the head region showing expression of a *gfp* reporter transgene containing the promoter and cDNA sequences of *trhr-1* (*A*) and *trh-1* (*B*). Asterisks mark fluorescence in the intestine resulting from the coinjection marker *Pelt-2*::*mCherry*. (Scale bars, 10 μm.) (*C* and *D*) *trh-1(lst1118)* mutants fed *E. coli* OP50 have normal isthmus peristalsis ($n = 22$) (*C*) and pharyngeal pumping rates ($n = 10$) (*D*). Data were analyzed by a two-tailed unpaired *t* test (ns, not significant: $P > 0.05$). Error bars represent SD.

muscles resulted in a significantly decreased body volume, comparable to the reduced size of the *trh-1* mutant (Fig. 5*H*).

TRH-1 Signaling Regulates Growth Independently of TGF-β Signaling and Depending on Bacterial Diet. DBL-1, a ligand of the TGF-β superfamily, is a major growth regulator in *C. elegans*. Among other cells, it is synthesized in the *trh-1*–expressing neuron M4 (44, 45). Similar to *trh-1* mutants, worms deficient in *dbl-1* signaling hatch as L1 larvae indistinguishable in length from wild-type L1s and display a continuously reduced body size throughout development (46). To determine whether *trh-1* and *dbl-1* act in the same growth-regulatory pathway, we measured the body volume of *trh-1;dbl-1* mutants. Double-mutant worms were significantly smaller than *trh-1* and *dbl-1* single mutants (Fig. 6*A*), suggesting that *trh-1* regulates body size independently of DBL-1/TGF-β signaling.

C. elegans body size also depends on the type of bacteria and quality of the food (47–49). Because growth is regulated by TRH-1/TRHR-1 signaling in the pharyngeal system, we hypothesized that its regulation of body size might be food-dependent. We therefore compared the body volume of wild-type and *trh-1*–mutant worms grown on the standard laboratory food source *E. coli* OP50 with those fed *E. coli* HB101, a high-quality food source which better supports growth (47–50). Although *trh-1* mutants grown on OP50 have a growth defect, their body volume is similar to that of wild-type worms when they are fed *E. coli* HB101 bacteria (Fig. 6*B*). For both bacterial strains, *trh-1* mutants showed normal pharyngeal pumping and isthmus peristalsis rates (Fig. 4 *C* and *D* and Fig. S5), indicating no obvious defects in food intake.

Discussion

In this study we identified a functional TRH-like neuropeptide system in *C. elegans*. Our findings expand our understanding of the evolutionary history of the TRH neuropeptide family, for which we identified similar peptides in all major protostomian phyla of the Ecdysozoa (nematodes and arthropods) and Lophotrochozoa (annelids and mollusks).

Phylogenetic analyses of TRHRs show a clear conservation of this receptor family in Protostomia and Deuterostomia (19, 20), including the *C. elegans* TRHR ortholog NMUR-4, which we named TRHR-1. In silico studies and peptidomics previously identified 31 FLP and 52 NLP precursors in *C. elegans* (51, 52), but none of the corresponding peptides show sequence similarity to deuterostomian TRHs or were able to activate TRHR-1 in cell-culture cells. Given the presence of orphan protostomian TRHR orthologs (19–21), we hypothesized that an as yet unknown family of neuropeptide precursors in these phyla might encode the cognate ligand of TRHR-1, and we performed an in-depth in silico search. Our search identified the *trh-1* precursor (C30H10.6) as the most promising candidate, because this precursor shows a phylogenetic distribution similar to protostomian TRHRs and encodes two peptides with TRH-like sequence features in terms of length and biochemical properties. We found that TRH-1 neuropeptides (R)GRELF-NH_2 and (R)ANELF-NH_2 are cognate ligands of TRHR-1 in vivo and activate *C. elegans*, *Platynereis*, and human TRHRs *in cellulo*, supporting the homology of TRH-1 to deuterostomian TRH precursors.

Based on the lack of evidence for TRHR ligands in protostomes, except in *Platynereis* (22), it was proposed that TRH neuropeptides have been lost in this lineage, or at least in Ecdysozoa (21–23, 53). Our in silico search revealed the presence of TRH-like peptides in nematodes, arthropods, annelids, and mollusks. Various lines of evidence suggest that they belong to the TRH neuropeptide family. (*i*) The conservation of their precursor proteins across Ecdysozoa and Lophotrochozoa matches the evolutionary conservation of TRHR orthologs in these phyla. (*ii*) Similar to vertebrate preproTRH, all the identified protostomian precursors contain multiple copies of TRH-like peptides. (*iii*) The encoded peptides are short amidated sequences (five to eight amino

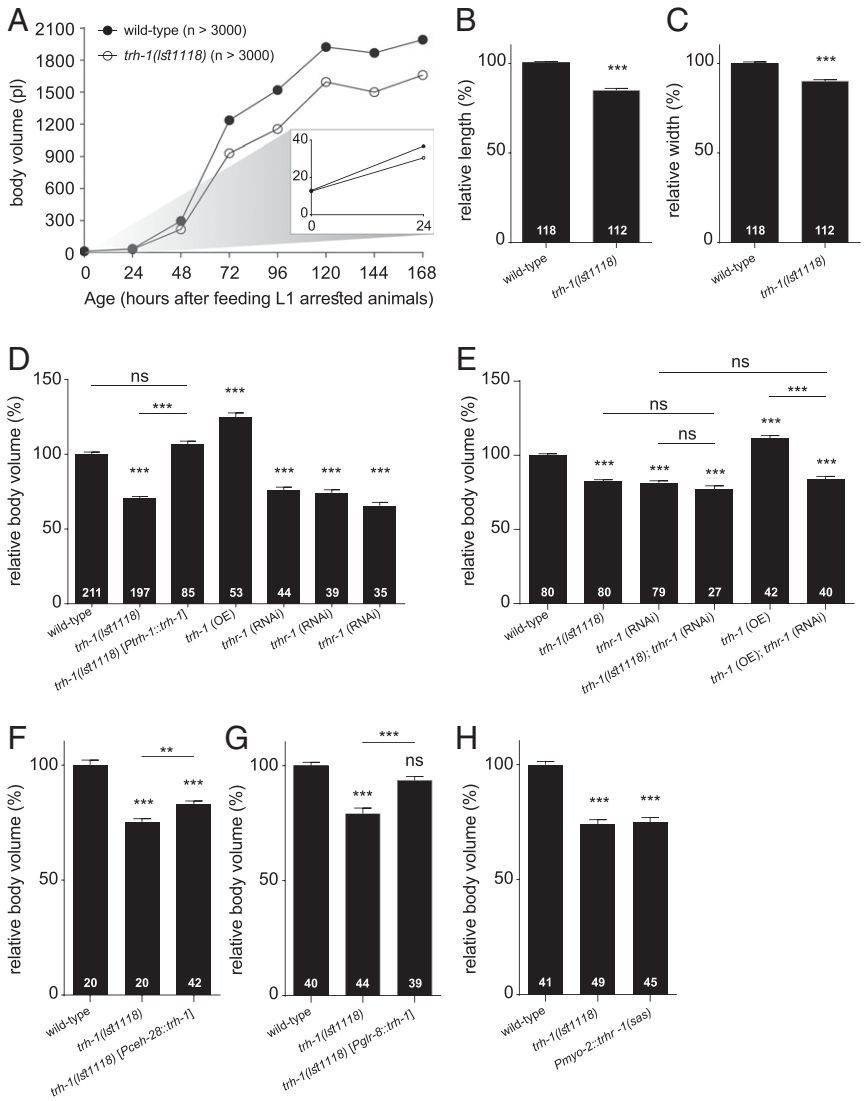

Fig. 5. TRH-1/TRHR-1 signaling promotes growth in *C. elegans*. (*A*) Growth curves of wild-type and *trh-1(lst1118)* mutants fed *E. coli* OP50. Each time point represents the average body volume of at least 3,000 worms. The first time point corresponds to the time when L1 larvae, synchronized by embryonic starvation, were fed. Worms reach the adult stage between 48 and 72 h. *trh-1(lst1118)* mutants show a reduced body volume compared with wild-type worms from 24 h after feeding (*Inset*) to the adult stage. (*B* and *C*) Body length (*B*) and width (C) of 1-day-old adults. (*D*) Body volume of 1-day-old adults. Expression of *trh-1* under the control of its promoter sequence (*Ptrh-1::trh-1*) rescues the small body size of *trh-1(lst1118)* mutants, and worms overexpressing (OE) *trh-1* have a larger body size than wild-type worms. Similar to *trh-1(lst1118)* mutants, RNAi-mediated knockdown of *trhr-1* decreases body volume in three independent knockdown strains. (*E*) *trh-1* and *trhr-1* function together in vivo. The body size of *trh-1(lst1118);trhr-1(RNAi)* worms is similar to that of *trh-1(lst1118)*–mutant and *trhr-1(RNAi)* worms. *trh-1* overexpression increases body size of wild-type worms, but this effect is suppressed by knockdown of its receptor *trhr-1*. (*F*) M4-specific expression of *trh-1* from the *ceh-28* promoter (*Pceh-28::trh-1*) partially rescues the reduced body size of *trh-1(lst1118)* mutants. (*G*) Expression of *trh-1* under the control of the *glr-8* promoter sequence (*Pglr-8::trh-1*) rescues the small body size of *trh-1(lst1118)* mutants. (*H*) TRHR-1 signaling in pharyngeal muscles mediates growth. Tissue-specific knockdown of *trhr-1* in pharyngeal muscles reduces body volume. Data are shown relative to the body volume, length, or width of wild-type worms (100%). Statistical significance (compared with wild-type worms unless indicated otherwise) was determined using one-way ANOVA and Sidak multiple comparison test (*B* and *E–H*) or a two-tailed unpaired *t* test (*C* and *D*). Error bars represent SEM; *n* is shown in each bar. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significant.

acids), like TRHs, that show the highest degree of similarity for the C-terminal tripeptide motif E-[L/F]-[L/F/V]. (*iv*) This core signature shares the acidic residue of deuterostomian TRHs (pyroglutamate in all deuterostomes and glutamic acid in all protostomes), whereas the hydrophobic residue at position two is also found in deuterostomian echinoderm TRHs. (*v*) Neuropeptides encoded by the *C. elegans* TRH-1 precursor activated the *C. elegans*, *Platynereis*, and human TRHRs in our cellular assays. The ELFamide motif of TRH-1 peptides, which resembles the protostomian core signature, is crucial for receptor activation. In *Platynereis* the EFLGamide peptides were recently shown to activate the TRHR ortholog in this species (22). Our results are in line with this study. We identified the annelid EFLGamides and the related fulicin peptides in mollusks as homologs of the *C. elegans* TRH-1 peptides. Based on these lines of evidence we suggest that the annelid EFLGamides and molluscan fulicins, together with the related peptides that we identified in nematodes and arthropods, are all part of the TRH neuropeptide family (Fig. 3).

Our search in public databases for TRH-like peptides and receptors indicates that this signaling system is present in major protostomian groups including annelids, mollusks, arthropods,

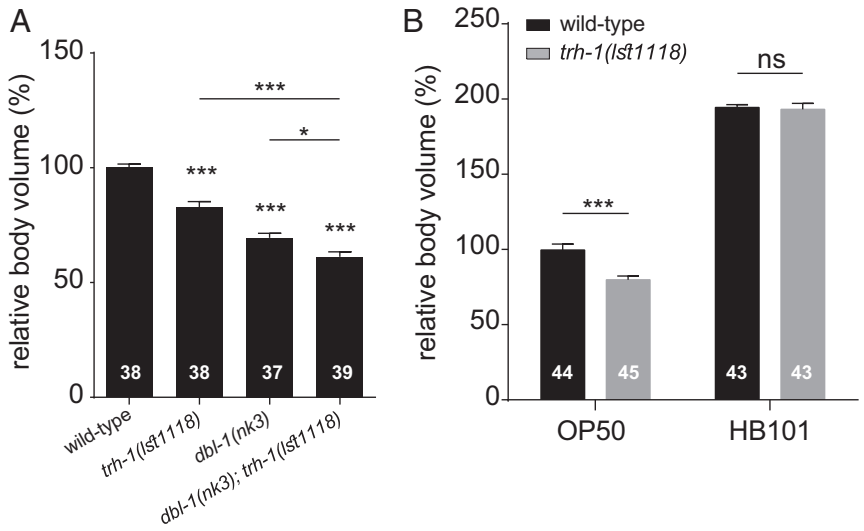

Fig. 6. TRH-1 signaling promotes growth independently of DBL-1 signaling. (*A*) Body volume of 1-day-old adults fed *E. coli* OP50. (*B*) The growth-stimulatory effects of TRH-1 neuropeptides depend on the bacterial diet. The body volume of *trh-1(lst1118)* mutants is similar to that of wild-type worms when they are fed a high-quality food source, *E. coli* HB101. Data are shown relative to the body volume of wild-type worms (100%). Statistical significance (compared with wild-type worms unless indicated otherwise) was determined using one-way ANOVA and Sidak multiple comparison test (*A*) or a two-tailed unpaired *t* test (*B*). Error bars represent SEM; *n* is shown in each bar. $*P < 0.05$; $***P < 0.001$; ns, not significant.

and nematodes. However, we could retrieve only a low number of TRHR sequences in insects that were restricted to the Hemiptera (*Nilaparvata*, *Cimex*, and *Diaphorina*) and Diptera (*Rhagoletis*) groups. Furthermore, we found a TRH-like precursor in only one insect species, the Firebrat (*Thermobia domestica*). These findings suggest that the TRH system has been lost in many insect groups. TRHRs share strong sequence similarity with ecdysis-triggering hormone (ETH) receptors, which are well conserved in insects. However, phylogenetic studies indicate that they are not orthologous to TRHRs. ETH receptor orthologs are present in non-arthropod species, including in some deuterostomes, and coexist with TRH-like peptides and TRHRs in some arthropods (e.g., *Ixodes* and *Daphnia*) (54). These findings suggest that the TRH and ETH systems were both established in an early bilaterian and potentially evolved from a common ancestral receptor-encoding gene (19, 20). Differential loss of the TRH or ETH system may explain the conservation of only one of the two systems in some arthropods.

The role of TRH-like neuropeptides in protostomes is largely unknown. A function was previously described for fulicin peptides in the African giant snail, *Achatina fulica*. Fulicin and fulyal, a fulicin gene-related peptide, modulate female and male copulatory behavior (55–57). Both peptides, which are TRH-like, show an excitatory effect on contractions of the vagina and the oviduct and potentiate contraction of the penis retractory muscle. TRH-1 peptides also may have a role in regulating hermaphrodite reproduction in *C. elegans*. We found that *trh-1* mutants produce fewer offspring. However, reintroducing wild-type copies of *trh-1* cDNA failed to rescue the brood-size defect in the mutant strain, although it rescued other phenotypic defects. A possible explanation might be that the reproductive phenotype is highly sensitive to *trh-1* expression levels. Alternatively, brood size may be regulated by *trh-1* at the germline, where the expression of transgene arrays with repetitive gene or cDNA copies is silenced (58).

Using CRISPR/Cas9 gene editing and RNAi, we found that TRH-1/TRHR-1 signaling regulates body size in *C. elegans*. TRH-1 neuropeptides are required for promoting growth and regulating body size through activation of their receptor TRHR-1. The *trh-1* mutants are smaller than wild-type worms throughout the larval and adult stages, but they have no delay in reproductive maturation, indicating that TRH-like signaling promotes growth rather than development. The reduced growth rate of *trh-1* mutants was most pronounced around the period of larval-to-adult transition, although a reduction is already seen at earlier larval stages. This temporal pattern for growth rate and body size defects resembles that of DBL-1/TGF-β and feeding-defective mutants (46, 59, 60), but we found no indications that *trh-1* is involved in feeding or in interacting with DBL-1/TGF-β signals.

We showed that TRH-1 peptides are synthesized and required for growth in pharyngeal motor neurons, including M4 and most likely M5. These motor neurons innervate or are in close apposition to the pharyngeal muscles that express the peptide's receptor TRHR-1. Expression of *trhr-1* localizes to a subset of muscles that constitute the metacorpus (pm4) and isthmus (pm5) and are involved in sequestering and regulating the flow of food in the pharynx (28). Tissue-specific knockdown confirmed that TRHR-1 signaling in pharyngeal muscle cells regulates growth. Although reported previously (61), we did not observe intestinal expression of *trhr-1* using a translational reporter transgene. This discrepancy could be explained by the absence of *cis*-acting regulatory sequences in the transcriptional reporters used previously (61).

How might TRH-like signaling regulate growth in *C. elegans*? TRHR-1 acts in the pharyngeal muscles, which regulate body size through their function in feeding and development of the pharynx (60, 62–64). Knockout of *trh-1* caused no obvious defects in pharynx morphology. The frequency of the two main motions that constitute feeding—pharyngeal pumping and isthmus peristalsis—was also unaffected, although we cannot rule out the possibility that TRH signaling might have subtle modulatory effects on food intake. Interestingly, TRH-1 neuropeptides and their receptor are expressed together with signaling components of the DBL-1/TGF-β pathway, which plays a major role in the regulation of body size. Our results indicate that *trh-1* regulates body size independently of *dbl-1*. Because TRH-1 mediates growth by signaling to the pharyngeal muscles, we hypothesized that its regulation of body size might be food-dependent. Worms are usually cultured on the laboratory food source *E. coli* OP50. HB101 bacteria are considered a high-quality *E. coli* food source, which better supports growth (47–50). Although *trh-1* mutants fed OP50 are growth-defective, they have a normal body volume when

growing on HB101. *E. coli* HB101 bacteria are larger and have a different nutrient composition, containing higher levels of monounsaturated fatty acids and carbohydrates than OP50 (47, 50). This finding suggests that the body size defect of *trh-1* mutants might result from a reduction in the efficiency with which nutrients in the OP50 strain are absorbed.

Vertebrate TRH is a releasing factor of growth-stimulating hormones, including GH and THs (9). Evidence for the existence of these hormones and their receptors in nematodes is lacking (23). However, THs or TH receptors have been described for some mollusks, flatworms, echinoderms, and nonvertebrate chordates and may be conserved regulators of postembryonic development (5, 65, 66). These findings suggest that the evolutionary origin of the TH system likely dates back to a common ancestor of bilaterians, although its function may have been lost or taken over by other endocrine factors in Ecdysozoa (23, 24, 67, 68). Like many invertebrates, *C. elegans* has a homolog of vertebrate glycoprotein receptors, which include TSH receptors. The ortholog FSHR-1 is involved in innate immunity, stress responses, and germline fertility (69–71). The *C. elegans* genome also encodes two genes, *flr-2* and T23B12.8, for α and β glycoprotein subunits (72, 73). It will be interesting to see whether TRH-like signaling regulates the release of invertebrate glycoproteins, similar to its function in vertebrates, or whether its role in these species points to a different ancestral function.

Taken together, our results lend further support to the ancestral origin of TRH neuropeptides predating the split of protostomian and deuterostomian animals. Our genetic analysis of the *C. elegans* TRH-like system suggests that this pathway may be a conserved regulator of growth and reproductive behaviors. The protostomian TRH-like peptides that we identified in this study provide a molecular basis for further investigations of whether these functions are shared by TRH in other invertebrates and whether TRH signaling may have ancestrally regulated animal growth and reproduction.

Materials and Methods

In Silico Search for TRH Precursors. We first performed an in silico search for neuropeptide precursors in *C. elegans* using a screening strategy described in ref. 20. From this list we extracted potential peptide precursors that had not been associated with a receptor and did not belong to known nematode-specific families of peptide precursors, such as most RFamide precursors. We then used BLAST and degenerate motif search to search the GenBank, Joint Genome Institute (JGI), and nematode databases [www.pristionchus.org (74)] for precursor sequences from protostomian lineages that contained peptides resembling each of the *C. elegans* candidate peptides. We retained *C. elegans* candidates with a phylogenetic distribution spanning the arthropod, annelid, and molluscan lineages as expected from phylogenetic TRHR analysis. From that screening strategy the ELFamide precursor, encoded by the *C. elegans* gene C30F12.6, stood out as the most promising candidate, because it was clearly conserved across all the main protostomian phyla and yielded peptides which had features similar to TRH peptides in terms of length ($3 \leq N \leq 8$) and biochemical characteristics (N-terminal pyroglutamate in deuterostomians and conserved glutamic acid close to the C terminus in protostomians).

Aequorin Bioluminescence Assay. CHO cells expressing the luminescent calcium indicator aequorin and (unless otherwise stated) the human $G\alpha_{16}$ subunit were transfected with pcDNA3.1/receptor plasmid. Two days post-transfection, cells were loaded with coelenterazine H (Invitrogen) for 4 h at room temperature. Luminescence after the addition of peptides dissolved in BSA medium was measured for 30 s at 469 nm with a Mithras LB940 (Berthold Technologies) reader.

***C. elegans* Strains and Maintenance.** The wild-type (N2) and NU3 *dbl-1(nk3)* strains were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. The LSC1118 *trh-1(lst1118)* strain was created by CRISPR/Cas9 and contains an 8-bp indel, causing a frameshift and premature stop codon immediately after the signal peptide sequence (Fig. 1*C*). Details of mutant and transgenic strains can be found in Table S2. The guide RNA (gRNA) sequences used to target *trh-1* and the corresponding 3′ protospacer adjacent motif (PAM) sequences are listed in Table S3. All strains were cultivated at 20 °C on nematode growth medium (NGM) plates seeded with *E. coli* OP50 bacteria, unless otherwise indicated.

Body Size Quantification. Worm volumes were measured in two different ways. (*i*) For growth curves, images of synchronized worms fixed in 4% (wt/vol) formaldehyde were captured. For every sample, the average length and width of at least 1,500 particles were calculated by a RapidVUE Particle Shape and Size Analyzer (Beckman Coulter) (75). Two independent experiments were performed, and each sample was run three or four times (technical replicates). (*ii*) For rescue, overexpression, and RNAi experiments, worm length and width (just behind the pharynx) were measured from images of anesthetized day 1 adult worms with ImageJ on at least two independent days. In both methods, worm volume was approximated using the cylinder formula $V = \pi L(D/2)^2$, where L is the length of the worm, and D represents its diameter (width). Worm growth rates were modeled by regressing the length and width data obtained by the RapidVUE Particle Shape and Size Analyzer using a sigmoid (logistic) function. The regression was further used to compute the increase in length and width over time.

Detailed descriptions of all methods and reagents and extended experimental procedures are provided in *SI Materials and Methods*.

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