FGFs have traditionally been associated with cell proliferation, morphogenesis, and development; yet, a subfamily of FGFs (FGF19, -21, and -23) functions as hormones to regulate glucose, lipid, phosphate, and vitamin D metabolism with impact on energy balance and aging. In mammals, Klotho and beta-Klotho are type 1 transmembrane proteins that function as obligatory co-factors for endocrine FGFs to bind to their cognate FGFRs (FGFRs). Mutations in Klotho/beta-Klotho or fgf19, -21, or -23 are associated with a number of human diseases, including autosomal dominant hypophosphatemic rickets, premature aging disorders, and diabetes. The Caenorhabditis elegans genome contains two paralogues of Klotho/beta-Klotho, klo-1, and klo-2. klo-1 is expressed in the C. elegans excretory canal, which is structurally and functionally paralogous to the vertebrate kidney. KLO-1 associates with EGL-15/FGFR, suggesting a role for KLO-1 in the fluid homeostasis phenotype described previously for egl-15/fgfr mutants. Altered levels of EGL-15/FGFR signaling lead to defects in excretory canal development and function in C. elegans. These results suggest an evolutionarily conserved function for the FGFR-Klotho complex in the development of excretory organs such as the mammalian kidney and the worm excretory canal. These results also suggest an evolutionarily conserved function for the FGFR-Klotho axis in metabolic regulation.

Unlike the classical FGFs, the endocrine FGFs have low affinity for heparan sulfate and do not require heparan sulfate proteoglycans for efficient FGFR interaction. Instead, the endocrine FGFs are present in the circulation and require type 1 transmembrane proteins Klotho and beta-Klotho, as obligatory co-factors to bind and activate their cognate FGFRs (for reviews, see Refs. 4 and 5).

Klotho was originally identified as an aging suppressor gene (6–8). Overexpression of Klotho extends life span and disruption of Klotho in mice leads to shorter life span with multiple disorders resembling human premature aging syndromes. Beta-Klotho (KLB) is a Klotho homologue and loss-of-function mutations in Klb lead to increased synthesis and excretion of bile acids in mice (9).

Given the wide distribution of FGFRs, Klotho and beta-Klotho are expressed in a more restricted manner and by facilitating the active FGF/FGFR complex formation, specify the target tissues for endocrine FGF action. FGF21 and -23 have selective preference for Klotho or beta-Klotho, further adding to the tissue specific action. Therefore, despite the fgfrs being ubiquitously expressed and the endocrine FGFs circulating in the body, the endocrine FGFs play distinct physiological roles. FGF19 utilizes either Klotho or beta-Klotho as a cofactor to regulate cholesterol/bile acid synthesis in the liver (10), FGF21 requires beta-Klotho to control glucose and lipid homeostasis in adipose tissue (11, 12), and FGF23 is dependent on Klotho to mediate phosphate and vitamin D metabolism in the kidney (13, 14).

The nematode C. elegans has a single orthologue of vertebrate fgfrs, egl-15, and two homologues of fgf ligands, egl-17 and let-756. EGL-15/FGFR has two differentially expressed isoforms EGL-15 (5A) and EGL-15 (5B), due to alternative splicing of the fifth exon. The intracellular signaling cascades activated by EGL-15 are relatively well characterized and share a high degree of conservation with mammalian FGFRs (15, 16). Loss of function in egl-15 (5B)/fgfr or in let-756/fgf lead to larval lethality, but the exact cause of this lethality is not known. EGL-15/FGFR signaling is negatively regulated by a receptor phosphatase, CLR-1 (17) and by N-glycosylation of the extracellular domain of EGL-15 (18). Removal of either of the negative controls results in excess EGL-15 signaling, leading to accumulation of fluid in the animal pseudocoelom and clear appearance of the animals (Clr phenotype).

The morphogen role of EGL-17/FGF and EGL-15(5A)/FGFR for the migration and differentiation of sex myoblasts is relatively well characterized (19–21). Whereas in mammals...
the morphogen and endocrine functions of FGFs have diverged, the role of EGL-15/FGFR signaling in fluid homeostasis and the suggested action of LET-756 as a paracrine factor (15), raise the intriguing possibility of parallel morphogen and endocrine functions for the *C. elegans* FGFs EGL-17 and LET-756. The *C. elegans* excretory cell is a large H-shaped cell with structural and functional parallels with the mammalian kidney (22, 23). The excretory cell is responsible for exchange of solutes and water to eliminate waste and to maintain osmotic homeostasis. We show that altered levels of EGL-15 signaling lead to defects in the excretory canal morphology. Excess EGL-15 activity in EGL-15 N-glycosylation mutants leads to defects in fluid homeostasis, with no effect on gross excretory canal morphology, suggesting defects in the canal function.

We have identified the *C. elegans* orthologues of *Klotho/beta-Klotho*, *klo-1* and *klo-2*, and shown that parallel to *Klotho/beta-Klotho* regulating endocrine functions in mammals, *klo-1* and *klo-2* are expressed in the excretory canal, the intestine and the hypodermis, tissues which are responsible for ion homeostasis in *C. elegans*. *klo-1* expression is regulated by EGL-15 signaling. EGL-15 associates with KLO-1 in *vitro*, and *klo-1* overexpression of *klo-1* leads to defects in ion balance. Physiological stress causes developmental delay in mutants in KLO-1/KLO-2 or EGL-15 signaling. Taken together, these results suggest parallel endocrine and morphogen functions for the *C. elegans* FGFs and evolutionary conservation of FGFR/Klotho signaling in metabolic homeostasis.

**EXPERIMENTAL PROCEDURES**

**Strains—** *C. elegans* strains were maintained at 20 °C essentially as described (24) unless stated otherwise. Wild type strain used in this study is N2 var. Bristol. The following previously described mutant strains were used. LG I: CB3241; *clrl-1* (e1745ts). LG III: FF628; *let-756* (s2613) *unc-32* (e189), OH2638; *dpy-17* (e164) *let-756* (s2887) *unc-32* (e189) III; *oyls14 V*; *otEx1467* (let-756 (+); pch-22::GFP) (25). LG IV: MT4479; *soc-2* (n1774). LG X: NH2693; egt-1 (n1456/sz11), TC341; *egl-15* (n1456); *jtEx79* (egl-15 N401A, N407A, N433A, N440A; *myo-3*; *gfp*) (18). RB1549 carries an *ok1862* deletion allele isolated by the *C. elegans* Gene Knock-out Consortium. We determined the breakpoints of the *klo-2* (*ok1862*) allele and found it to be a 770-bp deletion and an adenosine insertion with genomic breakpoints 5′-ATTTCACTAGCCACTTTGGATA/TCTAGATACACTTTGCCGCTTTA-3′. *ok1862* leads to a premature stop codon and truncation of protein product after Ile129.

**DNA Constructs and Transgenics—** The plkil-1::gfp reporter gene construct contains a 1378-bp fragment and the plskil-1::gfp reporter gene construct contains a 238-bp fragment upstream of the ATG start codon of *klo-1*. The *plkilo-2::gfp* reporter gene construct contains a 538-bp fragment upstream of the ATG start codon of *klo-2*. The DNA fragments were amplified by PCR (plkil-1, 5′-gattctccagcagtagatatc-3′ and 5′-aaccgaagaacatgc-3′; plskil-1, 5′-tcacacacatggtatctg-3′ and 5′-aaccgaagaacatgc-3′; *plkilo-2*, 5′-tcttttttggtatctccttt-3′ and 5′-tgcagcagcagcagctcagc-3′) and Gateway cloned into a vector containing the 3′-UTR sequence of *unc-54*. DNA microinjection was performed as described previously (26). Transgenic lines were created in wild type (N2) background at 30 ng/μl together with *ptph-1::mCherry* or *pttx-3::RFP* as co-injection marker. For each DNA microinjection, at least three independent transgenic lines were analyzed. *p(l)kilo-1::GFP* was used to generate extrachromosomal arrays *jtEx109-jtEx113*, *p(s)kilo-1::GFP* was used to make transgenic lines *jtEx166-jtEx170*, and *plkilo-2::GFP* was used to generate extrachromosomal arrays *jtEx129-jtEx132*. *jtEx163-jtEx165* contain *plkilo-1::KLO-1* expression construct together with *ptph-1::mCherry*, and *jtEx166-jtEx169* contain *plkilo-1::KLO-1* expression construct together with *p(s)kilo-1::GFP*.

**Protein Purification and Western Blotting—** *C. elegans* were grown in liquid nitrogen, and frozen worm powder was solubilized in a buffer containing 1% Igepal (Nonidet P40; Sigma-Aldrich) in 50 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 1 mM p-nitrophenylsulfon fluoride, 2 mg/ml aprotinin (Sigma), and 1 X Protease Inhibitor Cocktail (Roche Diagnostics) were added to protect proteolysis. Protein lysates were cleared by centrifugation at 10,000 × g for 30 min at +4 °C. Proteins were immunoprecipitated with rat monoclonal anti-Klotho antibody (R&D Systems) or rabbit polyclonal anti-EGL-15 antibody, Crackle (a generous gift from professor Michael Stern). Immunoprecipitates were captured using protein A- or protein G-agarose beads (SigmaAldrich), samples were separated on a 10% SDS-PAGE and transferred into Amersham PVDF membrane (Millipore). Proteins were detected with anti-Klotho or Crackle antibodies, followed by anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (GE Healthcare) and ECL Chemiluminescence Detection kit (Biological Industries, Beit Haemek, Israel).

**Life Span and Physiological Stress Assays—** Life span assays were conducted as described previously (27). Age refers to days after adulthood, and *p* values were calculated using the Log-rank (Mantel-Cox) method. Response to physiological stress under 0 mM Mg<sup>2+</sup> and 0 mM Ca<sup>2+</sup> conditions was assayed essentially as described previously (28) with the following modifications. Eggs from each strain were transferred to control, 0 mM Mg<sup>2+</sup> or 0 mM Ca<sup>2+</sup> plates (50 eggs/plate) and grown at 23 °C in parallel, and the number of adults was counted every 12 h.

**Microscopy—** Fluorescent and differential interference contrast images were acquired using Zeiss AxiosCam MRm camera mounted on Zeiss Axioskop2 microscope equipped with ×10, ×20, ×40, and ×63 epifluorescence and differential interference contrast optics. Images were captured using Axiovision and further cropped and scaled using Adobe Photoshop CS4.

**RESULTS**

C50F7.10 and E02H9.5 Encode *C. elegans* Klotho/Beta-Klotho Homologues *klo-1* and *klo-2*—A Blast search of the *C. elegans* database identifies the sequences C50F7.10 and
E02H9.5 as the homologues of vertebrate Klotho/beta-Klotho, which we have named klo-1 and klo-2. The vertebrate Klotho/beta-Klotho contain ~1000 amino acids and consist of a signal peptide, two Klotho domains, KL1 and KL2, which share ~28% sequence identity, a transmembrane domain and a short cytoplasmic domain (29, 30) (Fig. 1B). The predicted C. elegans KLO-1 contains 479 amino acids and ClustalW analysis shows 33–35% sequence identity to vertebrate Klothos (Fig. 1D) and 29–30% identity to beta-Klothos (supplemental Fig. S1). The predicted KLO-2 contains 475 amino acids and bears 34–35 and 33–34% sequence identity to vertebrate Klothos and beta-Klothos, respectively (Fig. 1 and supplemental Fig. S1). KLO-1 and KLO-2 contain ~33–35% sequence identity to the first vertebrate Klotho domain (KL1) and 18–24% sequence identity to vertebrate KL2 domains. Klotho orthologues are present in other Caenorhabditis species (Caenorhabditis brenneri, Caenorhabditis briggsae, and Caenorhabditis remanei) and in Drosophila. The nematode and fruit fly Klotho genes encode proteins with a single KL domain with no predicted transmembrane domain, and phylogenetic analysis suggests that the nematode Klotho genes may represent ancestral forms of the vertebrate Klothos (Fig. 1E). Both human and mouse Klotho genes are alternatively spliced to produce a shorter, secreted isoform (29, 30), which resembles the nematode and fruit fly Klotho proteins containing only one KL domain. It is plausible that the C. elegans KLO-1 and KLO-2 are secreted and may mediate their function cell non-autonomously. The predicted KLO-1 protein lacks a putative secretion signal. We analyzed the 5’-ends of klo-1 mRNA using RT-PCR and confirmed that the predicted 5’-end of the klo-1 cDNA is correct (data not shown). From the protein sequence, it is thus not possible to predict KLO-1 protein localization.

klo-1 and klo-2 Are Expressed in Excretory System—klo-1 and klo-2 expression was analyzed using transgenic animals carrying transcripational GFP reporters driven by sequences upstream of the first exon of either gene. pklo-1::GFP expression is first observed in the developing intestine in embryos at ~270 min after first cell cleavage (Fig. 2C). pklo-1::GFP expression in the developing intestine persist throughout embryonic development. At the first larval stage, pklo-1::GFP expression is seen in the excretory canal where the expression continues in adults in all transgenic lines analyzed (Fig. 2, A, B, and F). pklo-2::GFP expression is similarly first detected in the developing intestine at ~270 min after first cell cleavage (Fig. 2E), and the expression of klo-2::GFP in the intestine persists in adults in all five transgenic lines analyzed (Fig. 2F). pklo-2::GFP expression is also observed in the hypodermis (epidermis) in one of the transgenic lines analyzed (Fig. 2, D and F). These findings suggest that the expression of klo-1 and klo-2 partially overlap. Both klo-1 and klo-2 are expressed in the intestine, klo-1 is expressed in the excretory canal, and klo-2 is expressed in the hypodermis. Together, these tissues comprise the C. elegans organs that are involved in osmoregulation and uptake and secretion of metabolites. These results also suggest that the function of Klotho and beta-Klotho in regulation of ion balance is evolutionarily conserved from nematodes to mammals.

egl-15/fgfr Signaling Is Required for klo-1 Expression in Excretory Canal—Vertebrate Klotho and beta-Klotho function as obligatory co-factors for endocrine FGFs to bind and activate their cognate FGF receptors. C. elegans contains a single homologue of vertebrate fgfs, egl-15, and two homologues of fgfs, egl-17 and let-756. Loss-of-function alleles of both egl-15/fgfr and let-756/fgfr are lethal at the first larval stage (L1), but the exact nature of this lethality remains unknown (for reviews, see Refs. 15 and 31). Hypomorphic alleles of egl-15 and let-756 are scrapy (scr). Excess EGL-15 activity leads to defects in osmoregulation and fluid accumulation within the animals, resulting in a clear (Clr) phenotype (17, 18). The C. elegans excretory canal is formed from a single excretory cell born midway through embryogenesis (for a review, see Ref. 32). The excretory cell undergoes morphogenesis to form an “H”-shaped tubular cell consisting of two excretory canals that run laterally on either side of the animal. At hatching, the posterior canals have reached about half of the length of the animal and continue to extend throughout the first larval stage. By the end of the first larval stage, the excretory canal ends have reached the tail of the animal, and after that, the canals grow as the worms grow in size. Laser ablation of either the excretory cell or the canal-associated neurons, which are thought to regulate the excretory system, leads initially to fluid accumulation and later to lethality demonstrating that the excretory canal is essential for viability (23). C. elegans survives without a functional excretory system until the excretory canals have fully developed in late first larval stage. Due to the tubular form and role in osmoregulation, the excretory canal is structurally and functionally equivalent to the vertebrate kidney. Given the plausible link between EGL-15/FGFR, KLO-1/Klotho, and regulation of fluid homeostasis, transgenic pklo-1::GFP expression in the excretory canals was used to assess canal morphology in mutants that lack EGL-15 signaling. egl-15 (n1456) is a loss-of-function (lf) allele and as homozygous leads to lethality at first larval stage (L1), egl-15 (lf) was maintained as heterozygous and L1 progeny were selected for analysis of pklo-1::GFP expression based on the scrapy phenotype of egl-15 (lf) homozygotes. Of the egl-15 (lf) progeny analyzed at late L1 stage 83% (n = 52) lacked expression of pklo-1::GFP despite carrying the co-injection marker present in the transgenic array (Fig. 3, D and F). This represents a statistically significant difference when compared with animals analyzed at later developmental stages (from L2 to young adults), and thus representing egl-15 (lf)/+ heterozygotes, of which only 30% (n = 40) of the progeny did not show pklo-1::GFP expression when the co-injection marker was present (Fig. 3; p < 0.001 by chi-square test). Expression of pklo-1::GFP was also absent in the gut of egl-15 (lf) mutants. These findings suggest that klo-1 is a EGL-15-responsive gene.

Similarly, pklo-1::GFP expression in the excretory cell was absent in null mutants of let-756/fgf. pklo-1::GFP expression in the excretory canal was absent in 93% of the scrapy L1 progeny (n = 69) of let-756 (s2887) mutants (Fig. 3E). Expression of pklo-1::GFP was also absent in the gut. Reducing the level of LET-756 in a hypomorphic allele of let-756 (s2631) had no effect on pklo-1::GFP expression in the excretory ca-
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A. C50F9.10/klo-1
   E02H9.5/klo-2
   ok1862
   0.5 kb

B.
   KLO-1
   KLO-2
   hKlotho
   hsKlotho
   479 aa
   475 aa
   1012 aa
   549 aa

C.
   -0.2 kb
   GFP
   p(s)klo-1::GFP
   p(l)klo-1::GFP
   -1.4 kb
   unc-54 3'UTR
   pklo-1::KLO-1
   pklo-2::GFP

D.

E.

hKLO1
mKLO1
D. rerio KL1
D. mel AAF49418
C. elegans KLO-1
C. remanei
C. brenneri
C. briggae
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Figure 1. Molecular characterization of klo-1 and klo-2, which encode the C. elegans Klotho homologues. A, schematic structure of klo-1 (CS07F7.10) and klo-2 genes (E02H9.5). Gray boxes, exons; black lines, mRNA-splicing pattern. The klo-2 deletion ok1862 is underlined (b), and klo-1 and klo-2 encode proteins with 479 and 475 amino acids (aa), respectively, with sequence homology to the vertebrate KL1 domains. Human Klotho contains 1012 amino acids and consists of a signal peptide (gray box), two Klotho boxes, KL1 (black box), and KL2 (dark gray box) with sequence homology to each other, a transmembrane domain (black box), and a short cytoplasmic tail (white). KLO-1 shares 34% amino acid identity to the human KL1. Vertebrate Klothos can be cleaved by ADAM10 and ADAM17 to release the extracellular domain from the membrane (arrow). The human Klotho mRNA can be alternatively spliced to produce a secreted 549-amino acid protein consisting mainly of the KL1 domain (hKL1). Truncation of KLO-2 caused by ok1862 mutation is illustrated with an asterisk. C, schematic illustration of klo-1 and klo-2 DNA constructs used in this work. D, sequence alignment of Klotho orthologues from C. elegans, KLO-1 and KLO-2, Drosophila (GenBank accession no. AAF49418), and KL1 domains of human (hKL1; GenBank accession no. AA23382) and mouse (mKL1; GenBank accession no. AA23382) Klothos. Phenylalanine 352 in human Klotho (marked by double asterisk) is conserved in all species (287 in C. elegans KLO-1). This nucleotide displays single nucleotide polymorphism associated with tumor susceptibility in humans. Missense mutations at His190 in human Klothos (boxed), E, Phylogenetic tree of Klotho orthologues from Caenorhabditis species (C. elegans, C. brenneri, C. briggsae, and C. remanei), Drosophila melanogaster (D. mel.), zebrafish (Danio rerio), mouse, and human. The nematode and fly proteins, which contain only one KL domain, may represent the ancestral forms of the vertebrate Klothos, which are composed of two KL domains.

Figure 2. Expression of pklo-1::GFP and pklo-2::GFP reporter genes. A and B, pklo-1::GFP is expressed in the excretory canal both in larvae and in adults. A, inset, higher magnification showing pklo-1::GFP expression in the excretory canals. B, pklo-1::GFP expression in the H-shaped excretory canals and a the excretory canal cell (arrowhead). Only one arm of the H-shaped canal is in the focal plane. C, pklo-1::GFP is first expressed in the developing intestine in embryos at ~270 min post first cell cleavage. D, expression of pklo-2::GFP in the hypodermis. E, expression of pklo-2::GFP in the developing intestine in embryos (270 min post fertilization). F, p(s)klo-1::GFP is expressed in the excretory canal (exc canal) and in the intestine in all transgenic lines analyzed, p(l)klo-1::GFP is expressed in the excretory canal in all lines analyzed and in the intestine in three of five lines analyzed, pklo-2::GFP is expressed in the intestine in all transgenic lines analyzed and in the hypodermis in one of the lines analyzed. Magnifications used were: ×400 (A and D), ×630 (B, C, E, and A, inset). Scale bars, 50 μm (A), 20 μm (B, D, and E), and 10 μm (A, inset and C).

Figure 3. klo-1 reporter expression is absent in mutants that lack EGL-15/LET-756 signaling. Shown are differential interference contrast (A and C) and epifluorescence images (B and D) ofegl-15(+/+); pklo-1::GFP control animals (A and B) and egl-15(+/+); pklo-1::GFP animals (C and D) at first larval stage. The same animal is shown for differential interference contrast and epifluorescence for each strain. B, pklo-1::GFP expression is seen in the excretory canals. The left canal is on focal plane; the right canal is out of focal plane. C and D, scravy progeny from heterozygous egl-15 (n1456)/+; pklo-1::GFP mothers were picked for analysis after hatching. pklo-1::GFP is not expressed in the progeny (D), although it expresses the co-injection marker (not shown). Note the large cyst where the excretory cell body and the beginning of the excretory canals are normally positioned (arrowheads in C). E, percentage of worms expressing pklo-1::GFP. egl-15 (n1456) were picked based on the scravy phenotype and analyzed as newly hatched L1 larvae to overcome the L1 lethality of egl-15 null mutation. Some progeny was analyzed at later larval stages (L2 and older) and are thus expected to represent heterozygous n1456/+ genotype since they have escaped L1 lethality. Loss of function in let-756/FGF in s2887 allele similarly leads to lack of pklo-1::GFP expression. The parental strain contains let-756 rescuing array (see “Experimental Procedures”; Ref. 25); progeny that had lost the rescuing array were selected for analysis as newly hatched L1 to overcome the L1 lethality of s2887. Reduction of LET-756 function in the hypomorphic mutant carrying the s2613 allele causes no defect in pklo-1::GFP expression, * full genotype of let-756 mutants are let-756(s2887) unc-32(e1899); ptph-1::mCherry) and dpy-17(e164) let-756(s2887) unc-32(e1899); je109. Magnification was ×400 (A–D); scale bars, 20 μm.
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KLO-1 Associates with EGL-15/FGFR—To establish whether KLO-1 and EGL-15 interact, total protein lysates of wild type C. elegans were immunoprecipitated with monoclonal Klotho or polyclonal EGL-15 antibodies. Polyclonal EGL-15 antibody enriched both EGL-15 and KLO-1 from total C. elegans protein lysates, as detected by Western blotting using anti-Klotho and anti-EGL-15 (Fig. 4). Conversely, monoclonal anti-Klotho enriched both KLO-1 and EGL-15 from total C. elegans protein lysates. These results demonstrate that KLO-1 associates with EGL-15. KLO-1 migrated on SDS-PAGE corresponding to molecular mass of 110 kDa, which is greater than the theoretical mass of 50 kDa. A number of causes may lead to altered mobility of proteins on SDS-PAGE, which for KLO-1, include the possibility that its two potential N-glycosylation sites, which are conserved in KLO-2 (Fig. 1D), are glycosylated and the formation of SDS-resistant dimers.

Levels of EGL-15/FGFR Activity Critically Control Excretory Canal Development—Excess EGL-15 activity leads to accumulation of fluid within the animal body cavity (17, 18), suggesting defects in the excretory/secretory functions. Mutants with altered levels of EGL-15 activity were used to assess for morphological defects in excretory canal development. All strains were analyzed between larval stages L2 and L4 to ensure the canals should have been normally developed (by late L1) and to overcome the fact that the posterior ends of the excretory canals can sometimes “snap” in adult worms as they move, leading to shortened excretory canals. CLR-1 is a phosphatase that was originally identified as a negative regulator of EGL-15 activity, defects in which lead to the Clr phenotype (17). All current data point to CLR-1 being specific to EGL-15 (N401A,N407A,N433A), and EGL-15 (5B) isoforms from total worm protein lysates and from immunoprecipitates using either anti-Klotho or anti-EGL-15 antibodies. WB, Western blot.

FIGURE 4. KLO-1 associates with EGL-15/FGFR. Total protein lysates and immunoprecipitates of wild type C. elegans proteins with either anti-EGL-15 or anti-Klotho antibodies were blotted with either anti-Klotho or anti-EGL-15 antibodies. Monoclonal anti-Klotho antibodies recognize an ~110-kDa protein from total worm protein lysates or from immunoprecipitates with either anti-Klotho or anti-EGL-15 antibodies. Anti-EGL-15 recognizes two bands of ~90 and 80 kDa corresponding to EGL-15 (5A) and EGL-15 (5B) isoforms from total worm protein lysates and from immunoprecipitates using either anti-Klotho or anti-EGL-15 antibodies. WB, Western blot.

FIGURE 5. Abnormal levels of EGL-15/FGFR signaling lead to defects in excretory canal development. A, schematic drawing of the H-shaped excretory canals (green). B and C, in temperature-sensitive clr-1 mutants grown at nonpermissive temperature, the excretory canals fail to reach the length of the animal and display a short stop phenotype. clr-1 (e1745ts) grown at nonpermissive temperature displays excess EGL-15 activity. The developing gonad (gon) at midbody is indicated in C. D and E, reduced levels of EGL-15 activity in soc-2 (n1774) mutants also lead to short stop phenotype of the excretory canals. Additionally, large cysts can be seen especially at the ends of the canals (arrows, inset in D). F and G, EGL-15 N-glycosylation mutants with excess EGL-15/FGFR activity (18) have defects in fluid homeostasis and display clear phenotype. However, the excretory canals in these animals extend normally. H, numbers and percentages of excretory canals that fail to extend the entire length of the animal in mutants with altered levels of EGL-15 signaling. Transgenic overexpression of pklo::KLO-1 (jtEx168) in soc-2 (n1774) animals partially rescues the phenotype. Neither pklo::GFP marker nor pklo::KLO-1 transgene alone causes defects in excretory canal extension. Magnification, ×200 (F and G), ×400 (B–E), and ×630 (D, inset). Scale bars, 20 μm.

| strain                  | n  | wt | short stop | % defective |
|------------------------|----|----|------------|-------------|
| pklo-1::GFP (jtEx109)  | 49 | 46 | 3          | 6           |
| pklo-1::KLO-1 (jtEx168)| 26 | 24 | 2          | 8           |
| clr-1 (e1745) 16°C     | 79 | 52 | 27         | 34          |
| clr-1 (e1745) 21.5°C   | 47 | 22 | 25         | 53          |
| egl-15 (N401-440A)     | 27 | 27 | 0          | 0           |
| soc-2 (n1774)          | 31 | 15 | 16         | 52          |
| soc-2; pklo::KLO-1     | 45 | 36 | 9          | 20          |

Defects in excretory canal extension contributing to the fluid accumulation in these mutants.

Genetic removal of specific N-glycosylation sites from the extracellular domain of EGL-15 also leads to excess EGL-15 activity and the Clr phenotype, suggesting that N-glycans negatively regulate EGL-15 (18). egl-15 (N401A,N407A,N433A,N440A) mutants, with four putative N-glycosylation sites
abolished, develop the Clr phenotype. However, analysis of the excretory canals in these mutants showed that the excretory canals extended the entire length of the animal (Fig. 5, F and G). These results suggest that the excess EGL-15 activity due to lack of N-glycans does not affect the extension and cross-morphology of the canals. However, the fact that these animals display the Clr phenotype suggests that excretory function is disrupted in these mutants. N-glycans of the extra-cellular domain of EGL-15 may regulate EGL-15 interactions with molecules involved in osmoregulation such as ion channels, and removal of these glycans may hinder these interactions and thus ion channel function. Removal of N-glycans from IgD3 of EGL-15 does not, however, abolish association of EGL-15 with KLO-1 (see supplemental Fig. S2).

The morphology of the excretory canals was also analyzed in mutants with reduced levels of EGL-15/FGFR signaling. soc-2 is an EGL-15 downstream signaling component, hypomorphic mutations of which suppress the clear phenotype of hyperactive egl-15 mutants (18, 34). In soc-2 (n1774) mutants, the excretory canals fail to extend the entire length of 52% of the animals (n = 31) and stop short. Furthermore, in soc-2 (n1774) mutants, the canals frequently contain enlarged cysts (Fig. 5D, inset). The soc-2 short stop phenotype can be partially suppressed to 20% (n = 45) by transgenic overexpression of klo-1 (Fig. 5H), suggesting that increasing the level of KLO-1 can overcome reduction in EGL-15 downstream signaling. It is plausible that KLO-1 facilitates EGL-15 signaling complex formation. Reducing the levels of LET-756/FGF in the hypomorphic mutant s2613, did not lead to defects in excretory canal extension (n = 20), suggesting that the core domain of LET-756 (see above) is sufficient for excretory canal extension. Taken together, these results indicate that the excretory canal development is sensitive to levels of EGL-15/FGFR signaling. EGL-15 acts in concert with KLO-1 to regulate excretory canal development and disturbances in EGL-15/KLO-1 signaling lead to defects in fluid homeostasis.

klo-1 Gain-of-function Leads to Molting Defects and Accumulation of Fluid-filled Cysts—To obtain further insight into KLO-1 function, klo-1 gain-of-function (referred as klo-1(gf) from here on) animals were obtained by transgenic expression of the klo-1 promoter driving genomic klo-1 expression in wild type C. elegans. klo-1 (gf) animals molting defects (Fig. 6, A–B). klo-1 (gf) also lead to defects in gonad development (Fig. 6, A–B). In klo-1 (gf) animals, 19% of the anterior gonad leader cells or distal tip cells (n = 42) failed to execute ventral to dorsal reorientation (phase 2) resulting in overextension of the anterior gonad arms (Fig. 6B).

The most prominent phenotype of the klo-1 (gf) was accumulation of fluid-filled cysts under the hypodermis (Fig. 6, D and F). These cysts appeared along the entire length of the animal and varied in size. This phenotype is reminiscent of a weak Clr phenotype. Although the Clr phenotype of the klo-1 (gf) animals is weaker than that observed in strong clr-1 mutants or in hyperactive egl-15 mutants, the similarity in the phenotype suggest that consistent with vertebrate Klotho functionally interacting with FGF receptors, KLO-1 interacts with EGL-15/FGFR to regulate excretory canal development and function.

Abnormal Levels of EGL-15/FGFR and KLO-1/Klotho Signaling Sensitize to Physiological Stress—In mice, overexpression of Klotho extends life span and disruption of Klotho leads to shorter life span with multiple disorders resembling human premature aging syndromes (6, 7). Analysis of klo-1 (gf) animals in standard laboratory conditions showed significant differences in survival curves, as compared with wild type controls (Fig. 7A; p < 0.05 (*) significance; Log-rank test) with a median survival of 21 days for klo-1 (gf) (n = 56) and 17 days for wild type controls (n = 48). These results suggest that in addition to the evolutionary conservation of KLO-1 function in ion homeostasis, the life span-enhancing effects of KLO-1/Klotho are also evolutionarily conserved.

Mutants with abnormal levels of either EGL-15/FGFR or KLO-1/KLO-2 signaling were subjected to physiological stress by altering the ion strength of their environment. Wild type C. elegans tolerated changes in their microenvironment well and did not show a significant delay in reaching adulthood when grown in limited sources of Ca2+ or Mg2+ ions. In contrast, egl-15 (N401A,N407A,N433A,N440A) N-glycosylation mutants showed delay in development when subjected to physiological stress. egl-15 (N401A,N407A,N433A,N440A) mutants show ~55% embryonic and larval lethality (data not shown) and delay in development to adulthood even when grown under normal laboratory conditions. As some of the egl-15 (N401A,N407A,N433A,N440A) mutants become very severely clear, it is difficult to accurately determine their developmental stage. However, those egl-15 (N401A,N407A,N433A,N440A) mutants that do not develop a severe Clr phenotype have delayed development, as compared with wild type worms even under normal culture conditions (Fig. 7B). This delay becomes more accentuated when the mutants are subjected to physiological stress (Fig.
ok1862 is a Deletion Allele of klo-2—We have sequenced the deletion breakpoints (see "Experimental Procedures") and established that the ok1862 deletion leads to a premature stop codon resulting in truncation of the KLO-2 protein after Ile259 (Fig. 1). The ok1862 is thus considered as a null allele of klo-2. ok1862 mutants have superficially wild type morphology and normal fertility (data not shown). However, similarly to egl-15 N-glycosylation and klo-1 (gf) mutants, the ok1862 mutants show delayed development under physiological stress as compared with wild type (Fig. 7, B and C).

DISCUSSION

We have shown that the role of FGFs as endocrine regulators of physiological functions is evolutionarily conserved from nematodes to mammals. The C. elegans FGF signaling system with two FGF ligands, LET-756 and EGL-17, and one FGF receptor, EGL-15, has both morphogen and endocrine functions. The role of Klotho/beta-Klotho in endocrine functions of FGF is evolutionarily conserved.

Given the abundant expression of FGF receptors in vertebrates, Klotho and beta-Klotho expression is more restricted and is thought to specify the tissues of endocrine FGF action. We found C. elegans klo-1 and klo-2 expression predominantly in the intestine, the excretory canal, and the hypodermis, tissues that are responsible for regulation of metabolic homeostasis in the worm. We show that klo-1 expression in the excretory canal is responsive to LET-756/EGL-15 signaling. We show that KLO-1 and EGL-15 associate biochemically in pulldown assays. Given the previously demonstrated role of EGL-15 signaling in control of fluid homeostasis (17, 18), our results strongly suggest that KLO-1 and EGL-15 associate in vivo and that the role of Klotho/beta-Klotho in defining the endocrine actions of FGFs is thus evolutionarily conserved.

The C. elegans excretory canal is formed from a single, large, H-shaped excretory cell that sends processes anteriorly and posteriorly from the cell body. The excretory cell together with the duct cell and the pore cell confine the excretory organ functionally and structurally paralogous to the mammalian kidney (22). Abolishing any of these cells by laser ablation causes the animal to swell with fluid and die (23). The lack of klo-1 expression in the excretory canals of egl-15 (lf) or let-756 (lf) animals and the aberrant morphology of the canals in clr-1 mutants, which have hyperactive EGL-15 signaling, reveal a role for FGF signaling in the development of the excretory canal. The morphogenesis of the excretory canals is completed by the end of the first larval stage, after which the canals grow in size as the worms grow. C. elegans thus survives without functional excretory canals until late L1 stage. Both egl-15 (lf) and let-756 (lf) mutants die at late L1 stage for reasons that have so far been unknown. Our results strongly suggest that the lack of functional excretory canals in these animals is the underlying cause for lethality.

In vertebrates, FGF21 selectively requires beta-Klotho for signaling as a major metabolic regulator of glucose and lipid metabolism and obesity. Fgf23 and Klotho loss-of-function mice display identical aging like phenotypes with defects in phosphate, Ca^{2+} and vitamin D homeostasis (6, 35). In C. el-
egans, overexpression of klo-1 also led to statistically significant enhancement of life span as compared with wild type siblings. The effect on life span was prominent throughout adulthood, and klo-1 (gf) animals lived longer than wild type siblings. This was somewhat surprising, considering the molting defect and accumulation of fluid filled cysts within the klo-1 (gf) animals. Given the current lack of a klo-1 loss-of-function allele as a genetic tool, we cannot at this stage comprehensively address the role of KLO-1 in life span extension.

Removal of negative regulation of EGL-15 signaling by inactivating mutations in CLR-1 phosphatase leads to a failure of the excretory canals to extend fully and accumulation of fluid inside the animals. Similarly, mutations in soc-2, an intracellular downstream regulator of EGL-15, lead to shortened excretory canals and enlarged cysts within the canal ends. These results suggest that the levels of intracellular EGL-15 signaling are important for normal canal development and extension. Both too much and too little of Egl-15 signaling lead to abnormal excretory canal extension. We have previously shown that N-glycosylation of the extracellular domain of EGL-15 negatively regulates receptor activity and removal of specific N-glycans of EGL-15 leads to accumulation of fluid and the clear phenotype (18). Intriguingly, we could not detect any cross morphological defects in the excretory canals of EGL-15 N-glycosylation mutants, despite defects in osmoregulation. These results suggest a different mechanism by which N-glycans regulate EGL-15 signaling in fluid homeostasis as compared with negative regulation of intracellular phosphorylation of EGL-15 by CLR-1 phosphatase. N-glycosylation of EGL-15 may mediate extracellular/membrane interactions of EGL-15/FGFR with other membrane proteins, such as ion channels, involved in osmoregulation. Consistent with this suggestion, the EGL-15 N-glycosylation mutants are sensitive to physiological stress, as shown by delayed development under ion depletion in their environment. Similarly, klo-1 (gf) mutants show slightly delayed development under physiological stress. Klotho has been shown to stimulate transient receptor potential ion channel (TRPV5) by increasing the channel retention time on plasma membrane in murine and human cells in vitro (36, 37). TRPV5 is a Ca\(^2+\) channel involved in renal Ca\(^2+\) handling, and mutations in TRPV5 cause disturbances in Ca\(^2+\) homeostasis similar to those seen in Klotho-deficient in mice. It is thus plausible that EGL-15/FGFR forms a complex with an ion channel to regulate fluid balance, and KLO-1/KLO-2 may facilitate this complex formation. Klotho has also been suggested to possess glycosidase activity (6, 38) and to modulate glycans on TRPV5 (36, 37). This raises the possibility that C. elegans KLO-1 may modulate N-glycans of EGL-15 or alternatively that N-glycans of EGL-15 mediate interaction with KLO-1. Unlike the vertebrate Klotho proteins, KLO-1 and KLO-2 lack a transmembrane domain, suggesting they may exist as soluble or membrane-associated proteins. KLO-1/KLO-2 may thus function either as co-factors to facilitate ligand binding to EGL-15/FGFR or as hormone-like ligands for EGL-15. Vertebrate Klothos retain the soluble forms because they are detected in serum and cerebrospinal fluid (39) and in the cell supernatant in vitro (36), presumably due to shedding following cleavage by matrix metalloproteinases (40, 41) or translation of alternatively spliced mRNA in which the transmembrane and intracellular domains are lost (29, 30). Based on current data, the membrane-anchored and the shed forms of the vertebrate Klothos mediate different physiological responses (42).

In conclusion, we have shown that compared with mammals, in which the morphogen and endocrine functions of FGFs have diverged, in C. elegans, with reduced complexity of the FGF signaling system, the morphogen and endocrine functions of FGF signaling are retained in parallel. EGL-15/FGFR signaling regulates both the development and the function of the excretory system. We have identified the C. elegans Klotho/beta-Klotho homologues, klo-1 and klo-2, and show that klo-1 and klo-2 are specifically expressed in organs involved in osmoregulation in C. elegans. EGL-15 regulates klo-1 expression and associates biochemically with KLO-1. Abnormal levels of EGL-15 or KLO-1 lead to defects in osmoregulation and sensitivity to physiological stress. Finally, our results thus demonstrate that the role of Klotho/beta-Klotho in endocrine functions of FGF is evolutionarily conserved nematodes to mammals.

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