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Generalized neuromuscular hypoplasia, reduced smooth muscle myosin and altered gut motility in the *klotho* model of premature aging

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Abstract

Background—Gastrointestinal symptoms, particularly constipation, increase with aging, but their underlying mechanisms are poorly understood due to a lack of experimental models. Previously we established the progeric *klotho* mouse as a model of aging-associated anorexia and gastric dysmotility. We also detected reduced fecal output in these animals; therefore, the aim of this study was to investigate in-vivo function and cellular make-up of the small intestinal and colonic neuromuscular apparatus.

Methods—Klotho expression was studied by RT-PCR and immunohistochemistry. Motility was assessed by dye transit and bead expulsion. Smooth muscle and neuron-specific gene expression was studied by Western immunoblotting. Interstitial cells of Cajal (ICC) and precursors were analyzed by flow cytometry, confocal microscopy and 3-dimensional reconstruction. HuC/D⁺ myenteric neurons were enumerated by fluorescent microscopy.

Key Results—Klotho protein was detected in neurons, smooth muscle cells and some ICC classes. Small intestinal transit was slower but whole-gut transit of *klotho* mice was accelerated due to faster colonic transit and shorter intestinal lengths, apparent only after weaning. Fecal water content remained normal despite reduced output. Smooth muscle myosin expression was reduced. ICC, ICC precursors, as well as nitrergic and cholinergic neurons maintained their normal proportions in the shorter intestines.

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DISCLOSURES

Author contributions: DTA performed experiments, analyzed data, and drafted the manuscript; YH, FI, LNP, DLY, MRB, and AL contributed to the experiments; MK contributed the mutant mice for the study and provided advice; DRL contributed to the in vivo studies; GF provided key advice on the interpretation of the results; TO conceived the study, analyzed data, and contributed to the writing of the manuscript. All authors revised the manuscript and approved the final version.

Competing Interests: the authors have no competing interests.

SUPPORTING INFORMATION

Supporting information including 4 Supplemental Tables and 4 Supplemental Figures are available in the file entitled “Gut dysfunction in *klotho* mice_Supporting Info.pdf”.

Conclusions & Inferences—Progeric *klotho* mice express less contractile proteins and develop generalized intestinal neuromuscular hypoplasia mainly arising from stunted post-weaning growth. Since reduced fecal output in these mice occurs in the presence of accelerated colonic and whole-gut transit, it likely reflects reduced food intake rather than intestinal dysmotility.

Keywords

Aging; gastrointestinal motility; smooth muscle; interstitial cells of Cajal; enteric neurons

In most “developed” countries, life expectancies have increased to more than 80 years, bringing about a major demographic restructuring.¹ The proportion of individuals older than 65 years is predicted to increase to more than 20% in the United States during the next 15 years, and this change is expected to raise the cost of treating older patients with gastrointestinal complaints by 2- to 3-fold.¹ Many of the older patients will require treatment for disorders involving neuromuscular dysfunction such as constipation, diarrhea, diverticulosis, irritable bowel syndrome, fecal incontinence, rectal prolapse and gastroesophageal reflux.^{1–3} Furthermore, the prevalence of constipation, chronic diarrhea, and fecal incontinence is disproportionately higher in older patients, reflecting several factors including natural, aging-associated decline in organ function and consequent increased vulnerability to pathogenetic factors.^{1,2}

Mechanisms underlying aging of the gastrointestinal neuromuscular apparatus are incompletely understood. While studying aged humans and animals remains indispensable,⁴ these systems are not amenable to experimental manipulations required for molecular-level, mechanistic understanding of aging-associated pathologies. In contrast, strains of mutant mice with progeric syndromes are relatively easy to maintain and manipulate experimentally. These models have provided important clues about genes critical for regulating lifespan and tissue aging. Prematurely aged *klotho* mice are hypomorphic for the anti-aging peptide Klotho (α -Klotho; Kl) due to a recessive insertional mutation at the 5' flanking region of the *Kl* gene. Reduced Klotho levels in these animals lead to a wide array of aging-associated phenotypes after 3 weeks of age and premature death at about 60–70 days.^{5,6} Conversely, mice overexpressing Klotho live 20–30% longer than their wild-type (WT) littermates.⁷ *Kl* gene variations have been reported to affect human lifespan,⁸ and Klotho expression declines naturally with age in mice, rats and monkeys,⁹ highlighting its role as a key regulator of lifespan and aging. Klotho exerts its anti-aging functions both as a membrane-anchored and soluble protein. In the brain and kidney primarily, membrane-associated Klotho regulates phosphate and vitamin D metabolism as co-receptor for fibroblast growth factor 23.¹⁰ Klotho is also cleaved in these tissues by membrane-anchored proteases to form a circulating peptide,⁶ which regulates cell surface glycoproteins through its putative sialidase activity and suppresses oxidative stress and cancer by inhibiting the insulin-like growth factor 1 (Igf1), Wnt, and transforming growth factor beta 1 signaling pathways.^{6,11–13}

We have recently established the *klotho* mouse as a model of aging-associated decline of ICC, ICC stem cells (ICC-SC), inhibitory neuromuscular neurotransmission and electrical pacemaker activity in the stomach.¹⁴ Because fecal output is also reduced in these animals, we hypothesized they may also be useful as a model of age-related intestinal pathologies. In small intestinal and colonic tissues of older patients and aged animals, reduced numbers of cholinergic^{15–17} or nitrergic¹⁸ myenteric neurons have been described. There is also evidence of impaired smooth muscle function¹⁹ and reduced number and function of interstitial cells of Cajal (ICC).^{20–22} Therefore, here we investigated the effect of reduced

Klotho expression on intestinal and colonic transit, smooth muscle cells, enteric neurons, as well as ICC and their precursors.

MATERIALS AND METHODS

Animals and tissue preparation

Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. Homozygous *klotho* mice⁵ and age-matched WT and heterozygous (Het) littermates were obtained from heterozygous breeders and their genotype verified by PCR.⁵ Mice were housed in the same conventional mouse room and fed ad libitum. Animals were inspected daily and killed only after they had displayed all key aging-related signs characteristic of *klotho* mice including cataracts, kyphosis, ataxia and reduced stride lengths,⁵ which occurred at a median age of 57 days (range: 38–90 days; n=50). Data from WT and Het mice were pooled when results indicated no significant differences. Experimental groups were balanced for sex in all physiological studies and adequate balancing was verified by statistical analysis. Mice were killed by decapitation under deep isoflurane (Baxter Healthcare, Deerfield, IL, USA) anesthesia. Abdominal viscera were removed in toto and dissected into jejunum and ileum (defined as the orad and aborad half, respectively, of the small intestines spanning from the duodenojejunal flexure to the ileocecal junction) and proximal and distal colon (defined as the haustrated region orad to the insertion of the mesocolon and the region lacking haustration and spanning from the insertion of the mesocolon to the pelvic diaphragm, respectively; see ref.²³). Intact tunica muscularis tissues were prepared as described^{24–26} and used immediately or flash-frozen in liquid nitrogen and stored at –80°C.

In vivo physiological studies

Physiological studies were started between 0900 and 1000 h before feeding as described.¹⁴ *Whole-gut transit* was estimated by gavaging 0.3 ml carmine red (6% w/v in 0.5% w/v methylcellulose in water; Sigma-Aldrich, St. Louis, MO, USA) and measuring time until excretion of first colored fecal pellet. *Gastrocecal transit* was measured by gavaging 0.3 ml carmine red as above, killing the mice after 20 minutes by decapitation, and measuring distance of dye front travel along the small intestine. *Fecal output and water content* were quantified by collecting pellets for 3 h and weighing before and after drying to weight constancy. *Colonic transit* was measured by inserting a single glass bead (diameter: 3 mm) 2 cm orad to the anus and measuring time till bead expulsion.²⁷ To estimate *food consumption*, mice were placed singly into standard cages lined with sheets of white absorbent paper. Pre-weighed mouse chow was placed onto the sheets and all cage contents were recovered after 72 h and dried under a heat lamp until weight constancy (~48 h). Food scraps were separated from small pieces of paper and feces and their weight subtracted from the weight of the chow offered.

Western immunoblotting

Tissues were homogenized in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with Protease Inhibitor Cocktail Set I (Calbiochem, Gibbstown, NJ, USA) for 30 s then boiled for 5 min at 95 °C. After centrifugation at 15,000 g, 15 µg protein from each supernatant was subjected to 8–10% SDS-PAGE (90 V for 100 min) in Laemli buffer containing β-mercaptoethanol and transferred to 0.45-µm Immobilon-FL PVDF blotting membranes (Millipore, Billerica, MA; 15 V for 30 min). After washing with Tris-buffered saline (TBS; Bio-Rad, Hercules, CA, USA) for 5 min and blocking with LI-COR Odyssey Blocking Buffer (LI-COR Bioscience, Lincoln, NE, USA) for 1 h at room temperature, protein bands were probed simultaneously with antibodies against

glyceraldehyde-3-phosphate dehydrogenase (Gapdh) used as loading control and a protein of interest (Supplemental Table S1 for detailed information on antibodies used). The antibodies in each pair were from different hosts and were applied in the above blocking buffers at 4 °C overnight. After washing with TBS containing 0.1% Tween 20 (Bio-Rad), bound antibodies were visualized simultaneously with the aid of appropriate secondary antibodies labeled with near-infrared and infrared dyes (IRDye 680 and IRDye 800CW; LI-COR) applied in Odyssey Blocking Buffer for 1 h at room temperature. Membranes were washed and scanned with the Odyssey Infrared Imaging System (LI-COR). The 16-bit, 2-channel image files were analyzed with Bio-Rad Quantity One 4.5.1 software. Bands of interest were expressed in densitometric units normalized to the loading control (Gapdh), detected simultaneously in the same sample. In each experiment, lysates from a WT and a *klotho* tissue were processed in parallel and results were expressed as fold change relative to control, represented by a horizontal line in the figures. *Total neuronal nitric oxide synthase* (Nos1) protein (including α , β and γ isoforms) was detected with antibodies raised against a peptide mapping near the C-terminus. *Nos1 dimerization* was estimated by low temperature preparation (4 °C; without boiling) and immunoblotting with antibodies raised against amino acids mapping at the N-terminus of Nos1 specific for the dimerization-capable, functionally active α isoform^{28,29} (Supplemental Figure S1).

Immunohistochemistry, confocal microscopy and image analysis

Whole-mounts of freshly dissected intact tunica muscularis tissues were processed using established techniques.^{24,25} Briefly, tissues were fixed with cold acetone (10 min) and blocked with 1% bovine serum albumin (Sigma-Aldrich). ICC were detected with rat monoclonal anti-murine Kit antibodies (ACK2; 48 h at 4°C; see Supplemental Table S2 for detailed antibody information). *Enteric neurons and nerve fibers* were detected with human serum containing type 1 antineuronal nuclear antibodies (ANNA-1) reacting with Hu antigens C and D (ELAV (embryonic lethal, abnormal vision, Drosophila)-like 3 and 4; a generous gift from Dr. Vanda Lennon, Mayo Clinic; 24 h at room temperature)^{30,31} and rabbit polyclonal anti-protein gene product 9.5 (PGP 9.5; Uchl1) antibodies¹⁴ applied in PBS containing 50 mg mL⁻¹ beef liver powder (a generous gift from Dr. Vanda Lennon, Mayo Clinic).

klotho protein was detected in acetone-fixed, 5–10 μ m cryosections³² using rat monoclonal anti- α -Klotho antibodies (4°C overnight). *Smooth muscle cells, neurons/nerve fibers and ICC* were labeled with rabbit polyclonal anti-smooth muscle myosin antibodies (recognizing smooth muscle myosin heavy polypeptide; Myh11), anti-PGP 9.5 and anti-Kit antibodies, respectively. *Nuclei* were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Wide-field fluorescence images of cryosections were captured either with a Nikon (Melville, NY, USA) Eclipse TS-100F microscope equipped with a Modulation Optics (Glen Cove, NY, USA) HMC ELWD Plan Fluor $\times 40$, 0.60 NA air objective and a Jenoptik (Brighton, MI, USA) MFCool CCD camera or an Olympus (Center Valley, PA, USA) Magnafire camera mounted on an Olympus BX51 microscope equipped with a UPlanFI $\times 40$, 0.75 NA air objective. Whole-mounts were imaged with an Olympus FV1000 confocal system equipped with a UPlan $\times 40$, 1.00 NA oil immersion objective. Confocal images (512 pixels $\times$ 512 pixels) were collected using optimal pinhole size and Z-axis step (0.91 μ m). *Kit*⁺ ICC network volumes were calculated by 3-dimensional (3-D) reconstruction using ANALYZE software (Mayo Foundation).¹⁵ Mast cells were excluded from this quantification. ANNA-1⁺ enteric neurons were visualized with an Olympus BX61 microscope equipped with a Zeiss $\times 25$, 0.80 NA water immersion objective and an Olympus Magnafire camera and counted frame-by-frame with the aid of a Prior (Rockland, MA, USA) automated stage along the circumference of the organs to minimize the effect of organ size on cell counts and to take into account the inhomogeneous distribution of myenteric

ganglia along the circumference of e.g. the proximal colon.²³ Specificity of immunolabeling was verified by omitting the primary antibodies, by examining the samples with filter sets not designed for the fluorochrome used, and by pre-incubating primary antibody overnight with 40× excess recombinant mouse Klotho protein (aa 35–982; carrier-free) (100 µg mL⁻¹; R&D Systems, Minneapolis, MN, USA). Post-acquisition modification of images was limited to maximum-transparency projection, noise reduction, assignment of pseudocolor, and adjustment of brightness and contrast, which were always applied to the entire image. Personnel performing quantitative analyses were blinded to the identity of the samples.

Flow cytometry

ICC and ICC stem/progenitor cells were quantified by flow cytometry in the hematopoietic marker-negative fraction of dissociated jejunum, ileum and proximal colon tunica muscularis as Kit⁺Cd44⁺Cd34⁻ and Kit^{low}Cd44⁺Cd34⁺ cells, respectively.^{25,26,33} A previously published protocol was used¹⁴ (see Supplemental Figure S2 for gating scheme and Supplemental Table S3 for detailed antibody information). Samples were analyzed using a Becton Dickinson (San Jose, CA, USA) LSR II flow cytometer (see Supplemental Table S4 for configuration). Data files were analyzed by FlowJo software (Treestar, Ashland, OR, USA).

Real-time (quantitative) reverse transcription–polymerase chain reaction (qRT-PCR)

Previously published methods were used;¹⁴ cDNA was amplified on a Bio-Rad MyiQ or CFX96 real-time PCR detector.

Statistical analyses

Data are expressed as means±S.E.M. or median [interquartile range (IQR)] and analyzed by Student's t test, Mann–Whitney rank sum test, or one-way ANOVA (on raw data or on ranks) and all-pairwise multiple comparisons. *P*<0.05 was considered significant.

RESULTS

Klotho mRNA and protein expression

Whereas Klotho's anti-aging effects may be partly due to the circulating peptide,⁶ direct or paracrine effects may complement these actions in several tissues including the stomach¹⁴ and the colon.⁵ Therefore, we first identified Klotho-expressing cells in the small and large intestines. By qRT-PCR we detected *Kl* mRNA in the tunica muscularis of WT mouse jejunum and ileum and, to a much lesser degree, in the proximal and distal colon (Figure 1A–B; Supplemental Figure S3A). This finding indicates that most of the *Kl* mRNA previously detected in the colon⁵ probably arose from the mucosa. *Kl* mRNA was much reduced in the small intestines of the hypomorphic *klotho* mice, but in the colon, expression was not significantly different from the low levels detected in the WT controls (Figure 1A–B). Despite the low mRNA levels, specific Klotho immunoreactivity was detectable in the tunica muscularis of the distal colon of both WT and *klotho* mice (Figure 1C–H; Supplemental Figure S3B–C). Similarly to the stomach,¹⁴ we found Klotho expression in myenteric and submucosal neurons of the small and large intestines in WT mice, as well as in smooth muscle cells of the circular and longitudinal muscle layers and the lamina muscularis mucosae but not in intraepithelial myofibroblasts or myenteric ICC (ICC-MY) (Figure 1C–H). However, in contrast to the stomach, and in agreement with the results of a previous microarray study,³⁴ we detected Klotho expression in some ICC associated with the deep muscular plexus (ICC-DMP) of the small intestines (Figure 1E) and also in ICC residing on the submucosal border of the circular muscle layer (ICC-SM) of the colon (Figure 1H). Unlike in the stomach, we also found Klotho protein in some intramural nerve

fibers of the colon (Figure 1G). Thus, our findings in the intestines indicate both similarities and differences in *Klotho* expression patterns in different organs of the alimentary canal.

In vivo physiological studies

Gastrocecal transit was studied by measuring the distance traveled by gavaged carmine red dye over a period of 20 min. In WT animals age-matched to the test cohort, this duration was sufficient for the dye front to reach ~60% of the length of the small intestines. In progeric *klotho* mice, dye transit was significantly slower (Figure 2A). As there was a small but significant reduction in small intestinal lengths in the mutant animals (Figure 2B), we normalized the results to intestinal length. Small intestinal transit was still markedly slower after normalization (Figure 2C). Gastric emptying is normal in the *klotho* mouse stomach,¹⁴ therefore the delayed dye transit found here reflects a slowing of small intestinal transit.

In contrast, *klotho* mice showed significantly accelerated colonic transit as measured by time till expulsion of a glass bead inserted 2 cm orad to the anus (Figure 2D). This technique has the advantage of providing motility data independent of colonic length.²⁷ However, we also found that *klotho* mice had significantly shorter colons than their age-matched, WT littermates (Figure 2E). This change did not reflect a developmental abnormality arising from reduced *Klotho* expression because we found no differences in colonic lengths between *klotho* and WT mice on postnatal day 15 (Figure 2F), i.e., before the segregation of their body weights,⁵ which begins around the introduction of solid food into the juvenile animals' diet. The reduced intestinal lengths did not match the mutants' small body size as weight-normalized lengths were significantly higher in the *klotho* mice (Figure 2G–H). The shorter colons in the adults likely accentuated the effects of increased colonic transit over the reduced small intestinal transit, leading to a significant reduction in whole-gut transit times ($54 \pm 3\%$ of WT; $n=15$ (*klotho*) and 36 (WT); $P<0.001$; see also ref.¹⁴). In spite of this acceleration, *klotho* mice produced well-formed fecal pellets with normal water content ($22 \pm 6\%$; $n=9$; WT: $27 \pm 4\%$; $n=10$; $P=0.31$).

Previously we reported reduced dry fecal output and low daily food intake in the progeric mutant mice¹⁴ and in the current cohort we verified low daily food consumption by direct measurement ($38 \pm 3\%$ of WT; $n=6$ /group; $P<0.001$). Together, these results indicate that reduced daily fecal output in the *klotho* mice is due to low food intake rather than intestinal dysmotility.

Smooth muscle gene expression

We next investigated whether any cellular defects might underlie the abnormal transit in *klotho* mice. Expression of the contractile protein smooth muscle myosin, assessed by Western immunoblotting, was significantly reduced throughout the small intestines and the colon (Figure 3). This change did not reflect an overall reduction in smooth muscle gene expression as we detected no significant changes in *Igf1* mRNA^{25,35} by qRT-PCR ($n=3$ /group/tissue; not shown) or in 31-kDa stem cell factor (Kitl) protein,²⁵ which includes both membrane-associated and secreted polypeptides (Figure 4). However, in the jejunum, there may have been an imbalance in the expression of the various *Kitl* isoforms, as we found significantly reduced mRNA encoding for the 248-amino-acid, rapidly cleavable isoform.

ICC and ICC precursors

Kitl is a key factor for the development and postnatal maintenance of ICC³⁶ and consistent with the unchanged normalized Kitl protein levels, the frequency of ICC and ICC-SC, analyzed by flow cytometry and expressed as the percentage of all cells, did not change significantly in any part of the intestines, although there was a general trend toward reduced ICC-SC frequencies (Figure 5A–B, Supplemental Figure S4). 3-dimensional reconstruction

of Kit⁺ structures from confocal stacks^{14,15} indicated that ICC network volumes throughout the thickness of the musculature also remained unchanged in the *klotho* small intestines and distal colon, and were only significantly reduced in the proximal colon, reflecting regional dystrophy of the ICC networks in the absence of selective ICC depletion (Figure 5C–F).

Together, these results indicate that, with the exception of a decline in contractile protein expression and regional decrease in ICC network density, mesodermally-derived components of the gut tunica muscularis are not disproportionately affected in the *klotho* gut. However, since all results obtained by Western immunoblotting, qRT-PCR and flow cytometry are normalized to the number of cells in the assays, and the ICC network volume data only reflect absolute values within small tissue volumes, these parameters could not reveal changes paralleling altered organ sizes. Due to the significantly reduced small intestinal and colonic lengths in these mice, the interpretation of the above results is that *klotho* mice display actual decreases in absolute terms (parameter/organ) as compared to their age-matched WT controls.

Enteric neurons

In order to minimize the influence of organ size and to avoid potential pitfalls arising from inhomogeneous distribution of myenteric ganglia along the circumference of the organs, particularly in the proximal colon,²³ we enumerated myenteric neurons along the circumference of the gut. With the exception of the jejunum, we found a significant decline in HuC/D-expressing neuronal perikarya (detected with the aid of ANNA-1 antibody-containing human serum) throughout the intestines of *klotho* mice (Figure 6). There was only minimal “drop-out” of perikarya and the lower cell counts rather reflected smaller ganglia with fewer total cell bodies. Ganglion numbers could not be reliably determined because many ganglia in mouse intestines do not have clearly defined boundaries (see e.g. Figure 6B). Intramural nerve fibers and trunks were not obviously affected. The expression of PGP 9.5, a marker for both perikarya and neuronal processes,³⁰ also did not change significantly by Western immunoblotting (fold changes vs. WT; n=6/group/tissue: ileum: 1.20[0.97;1.26]; proximal colon: 0.98[0.95;1.22]; distal colon: 1.17[0.99;1.82]; all $P>0.39$) indicating that the observed neuron depletion was likely also proportional to the reduced intestinal lengths in the *klotho* mice. Furthermore, Western immunoblotting revealed no significant changes in *klotho* tissues in the expression of 155-kDa Nos1 α protein, the enzyme responsible for the production of the key inhibitory enteric neurotransmitter nitric oxide²⁹ (fold changes vs. WT; n=6–7/group/tissue: jejunum: 1.17[0.65;1.40]; ileum: 0.75[0.53;0.89]; proximal colon: 1.14[1.02;1.58]; distal colon: 1.84[0.71;2.77]; all $P>0.06$) or in the expression of 75-kDa choline acetyltransferase (Chat), the enzyme responsible for the synthesis of acetylcholine, a key excitatory enteric neurotransmitter (fold changes vs. WT; n=6/group/tissue: jejunum: 1.05[0.84;1.28]; ileum: 0.98[0.77;1.14]; proximal colon: 1.02[1.01;1.34]; distal colon: 0.75[0.54;1.56]; all $P>0.06$). Since Nos1- and Chat-expressing neurons comprise about 80% of myenteric ganglia,³¹ these results also support the conclusion that the neuron loss was proportional to the shortening of the intestines. Predictably, Nos1/Chat ratios were also not different between *klotho* and WT animals (Figure 7A–D). Dimerization of the Nos1 α isoform, which is required for calmodulin binding and Nos1 catalytic activity,^{28,29} also remained unaffected both in the small intestines and colon (Figure 7E–H). These observations indicate nonselective, indiscriminate neuron deficits in absolute values but proportional to the reduction in organ size in the small and large intestines of *klotho* mice relative to their age-matched, WT littermates.

DISCUSSION

Here we show that in progeric *klotho* mice, reduced daily fecal output is associated with accelerated, rather than delayed, transit and is, therefore, due to the animals' reduced food

intake. *klotho* mice display several key changes in the gastrointestinal neuromuscular apparatus that have been previously described in naturally aging humans and animals including an anally predominant¹⁶ reduction in enteric neurons,^{3,4,15,16,37–40} ICC depletion²⁰ and smooth muscle involvement.¹⁹ However, unlike in natural aging, cell depletion in the *klotho* intestines is due to generalized neuromuscular hypoplasia manifesting in abnormally short small intestines and colon, and the relative proportion of each cell type is left unchanged. We also found disproportionately reduced expression of smooth muscle myosin throughout the gut. These findings show differences when compared to the *klotho* stomach,¹⁴ where ICC depletion due to premature senescence of ICC stem cells occurred in the absence of detectable changes in neurons, smooth muscle myosin mRNA or organ size. Also, unlike in the stomach,¹⁴ Klotho protein is expressed not only in enteric neurons and smooth muscle cells, but also in some ICC-DMP and ICC-SM of the small intestines and colon, respectively. The hypomorphic *klotho* mutation led to reduced *Kl* mRNA in the small intestines but did not significantly affect the already low *Kl* expression in the colon. The changes detected in the gastrointestinal tract of *klotho* mice including previously reported findings in the stomach¹⁴ are summarized in Table 1.

Constipation is the most prevalent gastrointestinal symptom specifically increased in the elderly.¹ A largely cholinergic-selective depletion of myenteric neurons, which has been consistently observed both in humans and animal models,^{3,4,15,16,37–40} is often cited as one of its major underlying factors.^{3,4} However, these changes, as well as a similarly dramatic reduction in ICC, do also occur without any gastrointestinal complaint or detectable abnormality in healthy aging humans^{15,20} or animals.³⁸ In a large community-based sample, delayed rectosigmoid transit was only detected in elderly with symptoms of functional constipation,⁴¹ and there is only little and conflicting evidence of slow intestinal transit in healthy older people,^{1,2,15,40} despite an apparently mandatory decrease in neuron and ICC numbers.^{15,20} Thus, assumptions of a linear relationship between age-related cell loss and intestinal dysfunction^{3,4,39} are not justified and age-related changes in the gastrointestinal neuromuscular apparatus are better viewed as reduced functional capacity limiting responsiveness to functional challenges.¹⁴ Our present results demonstrating intestinal neuromuscular hypoplasia without delayed transit also support this view.

The relationship between constipation and reduced intestinal motility is similarly tenuous in the literature.^{1,2,42} Although there is some evidence of slow colonic transit in aged people⁴³ and rats,^{44,45} according to other studies, most constipation may be owing to decreased nutrient and fluid intake and lack of exercise in older persons rather than frankly reduced transit.^{1,2,42} Our findings in the *klotho* mutants support the notion that aging-associated constipation can indeed occur from reduced food intake rather than overt delayed transit.

An interesting finding of this study was the shortening of the *klotho* small and large intestines, which is not a direct (i.e., not a progeric phenotype-independent) consequence of the *klotho* mutation. The degree of intestinal shortening in *klotho* mice was less than the decrease in the mice's body size, indicating that it is also not directly related to the hypofunction of the growth hormone–Igfl axis arising from a combination of pituitary dysfunction⁵ and low food intake.¹⁴ Proportions of ICC and ICC stem cells and normalized expression values for neuron- and smooth muscle-specific genes (PGP 9.5, Nos1, Chat, Igfl, and total Kitl) remained unchanged indicating a net loss of these cells in the shorter *klotho* intestines. We verified the decrease in myenteric neuron counts by enumerating HuC/D⁺ perikarya along the circumference of the gut, a method which is relatively insensitive to organ size. An increase in ICC numbers proportional to increased intestinal lengths has been reported in PRM/Alf mice.⁴⁶ However, whereas an increased slow wave frequency maintained intestinal transit times at normal levels in PRM/Alf mice, the gut hypoplasia (shortening) and cell deficit in the *klotho* mutants rather accentuated the effect of a length-

independent increase in colonic motility (discussed below) resulting in much accelerated whole-gut transit. Thus, cell deficits proportional to organ size can have functional consequences. The acceleration of colonic transit in the *klotho* mice may in fact allow the maintenance of normal fecal water content despite very low food intake and fecal output. Similarly, the slowing of small intestinal transit would also help maintain nutrient absorption in the presence of shorter intestinal length.

Our data indicate that in the *klotho* gut, hypoplasia (subnormal organ size with proportionally reduced cell numbers) dominates over tissue dystrophy. The most likely explanation is that, similarly to human progeric syndromes and unlike in naturally aged people and animals, the pro-aging effects of the *klotho* mutation such as reduced trophic signals, hyperphosphatemia and oxidative stress^{6,14} manifest during a period of intensive growth and thus lead primarily to stunted post-weaning development rather than loss of existing cells. For example, smooth muscle cells and ICC continue to develop and remain highly plastic during the postnatal period^{35,47,48} and reduced numbers of ICC stem cells¹⁴ may underlie the organ size-proportional decrease in ICC. Recent data indicate that neurogenesis may also continue into adulthood in mice⁴⁹ although at a much reduced rate.⁵⁰ The lack of an increased frequency of “drop-outs” in myenteric ganglia of *klotho* mice seems to support impaired development, rather than increased cell death, although ganglia in aged animals have also been shown to collapse and eliminate cavities caused by dead cells.¹⁶ The lack of cavities in the *klotho* ganglia also argues against undetectable HuC/D protein in existing neurons as an apparent cause of reduced neuron numbers.

Most studies found aging-related neuron loss to mainly or exclusively affect cholinergic neurons^{3,15,16,37} and this has been attributed to their increased sensitivity to reactive oxygen and nitrogen species.^{3,38} However, the same argument has also been made to explain a selective loss of nitrergic neurons in diabetes⁵¹ and reduced Nos1 mRNA, protein and catalytic activity have been reported in aged rats¹⁸ along with reduced nitrergic responses.⁵² Unlike in many other models and aging humans, in *klotho* mice, both cholinergic and nitrergic neurons appeared to be proportionally affected, but we failed to detect abnormal Nos1 dimerization, an indicator of Nos1 function.^{28,29} The mechanisms responsible for these differences remain unclear.

We also detected a specific decrease in the expression of the contractile protein smooth muscle myosin. Although overt smooth muscle dystrophy does not typically accompany aging,³ diminished contractility reflecting reduced K⁺ and Ca²⁺ currents, impaired activation of the protein kinase C and mitogen-activated kinase pathways, as well as reduced phosphorylation and association with other contractile proteins of heat shock protein 27 have been detected in the colon of aged rats.¹⁹ To our knowledge, this is the first report of reduced contractile protein expression in the gastrointestinal tract of an aging model. While the association of decreased contractile protein expression with accelerated colonic transit and bead expulsion may at first appear counterintuitive, it is in fact expected since the main function of the colon is retention of contents for water reclamation and formation of solid excrement. Indeed, ~90% of intestinal transit time is due to colonic transit.⁵³ Since this retention is not passive but reflects segmenting contractile activity, its decline due e.g. to diminished contractile protein expression should result in shorter transit times.

Previously we described Klotho protein expression in smooth muscle cells and enteric neurons of the stomach and noted lack of expression in ICC.¹⁴ Here we found similar distribution of Klotho in the intestines except that it was also found in some ICC-DMP and ICC-SM of the small and large intestines, respectively. This result is consistent with the expression of *Kl* mRNA previously detected in isolated ICC-DMP by oligonucleotide microarrays.³⁴ The selective expression of Klotho in these ICC classes is interesting because

the same subtypes have recently been identified as being resistant to oncogenic *Kit*-mediated transformation leading to gastrointestinal stromal tumors (GIST) due to the absence of endogenous *Etv1* expression.⁵⁴ Indeed, *Klotho* has been shown to possess anti-oncogenic properties¹³ and may also protect from GIST. We also found *Klotho* expression in the colon and small intestines of *klotho* mutant mice, albeit at reduced levels. However, the *klotho* mouse is not a knock-out model but rather a severe hypomorph.⁵ Therefore, while *Klotho* expression is greatly reduced in most tissues, it is still present at lower levels. The local expression of *Klotho* may be of little physiological importance however, since most of *Klotho*'s anti-aging effects appear to stem from reduced circulating levels originating mainly from the kidney and choroid plexus of the brain.^{5,6}

In summary, we provide the first evidence of gut dysmotility and underlying cellulopathies in the *klotho* model of aging. The rapidly aged *klotho* mouse provides a unique opportunity to study the process of aging and its underlying cellular and molecular changes. Studies from progeroid models highlight the possibility that aging is regulated by only a handful of overlapping signaling pathways. Understanding these pathways may lead to novel approaches to target the adverse effects of aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

<i>Actb</i>	actin, beta.
ANNA-1	type 1 antineuronal nuclear antibodies
Ano1	anoctamin 1
Chat	choline acetyltransferase
CM	circular muscle layer
DAPI	4',6-diamidino-2-phenylindole
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GE	gastric emptying
GIST	gastrointestinal stromal tumor(s)
Het	heterozygous
HuC/D	Hu antigens C and D (ELAV (embryonic lethal, abnormal vision, <i>Drosophila</i>)-like 3 and 4)
ICC	interstitial cell(s) of Cajal
ICC-DMP	ICC associated with the deep muscular plexus
ICC-MY	myenteric region ICC
ICC-SC	ICC stem/progenitor cells
ICC-SM	submucosal border ICC
Igf1	insulin-like growth factor 1
IJP	inhibitory junction potential
Kit	Kit oncogene

Kitl	stem cell factor
Kl	gene/mRNA encoding Klotho
LM	longitudinal muscle layer
LS	light scatter
MG	myenteric ganglion
Muc	tunica mucosa
MM	lamina muscularis mucosae
Myh11	smooth muscle myosin heavy polypeptide 11
NO	nitric oxide
Nos1	nitric oxide synthase 1, neuronal
PGP 9.5	protein gene product 9.5 (ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase; Uchl1)
RT	reverse transcriptase
SG	submucosal ganglion
SW	slow wave
TBS	Tris-buffered saline
WT	wild-type

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REFERENCES

1. Hall KE, Proctor DD, Fisher L, Rose S. American Gastroenterological Association Future Trends Committee Report: Effects of Aging of the Population on Gastroenterology Practice, Education, and Research. *Gastroenterology*. 2005; 129:1305–1338. [PubMed: 16230084]
2. Bhutto A, Morley JE. The clinical significance of gastrointestinal changes with aging. *Curr Opin Clin Nutr Metab Care*. 2008; 11:651–660. [PubMed: 18685464]
3. Camilleri M, Cowen T, Koch TR. Enteric neurodegeneration in ageing. *Neurogastroenterol Motil*. 2008; 20:418–429. [PubMed: 18371012]
4. Wiskur B, Greenwood-Van Meerveld B. The aging colon: the role of enteric neurodegeneration in constipation. *Curr Gastroenterol Rep*. 2010; 12:507–512. [PubMed: 20878508]
5. Kuro-o M, Matsumura Y, Aizawa H, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature*. 1997; 390:45–51. [PubMed: 9363890]
6. Kuro-o M. Klotho and aging. *Biochim Biophys Acta*. 2009; 1790:1049–1058. [PubMed: 19230844]
7. Kurosu H, Yamamoto M, Clark JD, et al. Suppression of aging in mice by the hormone Klotho. *Science*. 2005; 309:1829–1833. [PubMed: 16123266]
8. Arking DE, Krebsova A, Macek M Sr, et al. Association of human aging with a functional variant of klotho. *Proc Natl Acad Sci U S A*. 2002; 99:856–861. [PubMed: 11792841]

9. Duce JA, Podvin S, Hollander W, Kipling D, Rosene DL, Abraham CR. Gene profile analysis implicates Klotho as an important contributor to aging changes in brain white matter of the rhesus monkey. *Glia*. 2008; 56:106–117. [PubMed: 17963266]
10. Razzaque MS, Lanske B. The emerging role of the fibroblast growth factor-23-klotho axis in renal regulation of phosphate homeostasis. *J Endocrinol*. 2007; 194:1–10. [PubMed: 17592015]
11. Yamamoto M, Clark JD, Pastor JV, et al. Regulation of oxidative stress by the anti-aging hormone klotho. *J Biol Chem*. 2005; 280:38029–38034. [PubMed: 16186101]
12. Kuro-o M. Klotho as a regulator of oxidative stress and senescence. *Biol Chem*. 2008; 389:233–241. [PubMed: 18177265]
13. Doi S, Zou Y, Togao O, et al. Klotho inhibits transforming growth factor- β 1 (TGF- β 1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem*. 2011 (in press).
14. Izbeki F, Asuzu DT, Lorincz A, et al. Loss of Kitlow progenitors, reduced stem cell factor and high oxidative stress underlie gastric dysfunction in progeric mice. *J Physiol*. 2010; 588:3101–3117. [PubMed: 20581042]
15. Bernard CE, Gibbons SJ, Gomez-Pinilla PJ, et al. Effect of age on the enteric nervous system of the human colon. *Neurogastroenterol Motil*. 2009; 21 746-e46.
16. Phillips RJ, Powley TL. Innervation of the gastrointestinal tract: patterns of aging. *Auton Neurosci*. 2007; 136:1–19. [PubMed: 17537681]
17. Roberts D, Gelperin D, Wiley JW. Evidence for age-associated reduction in acetylcholine release and smooth muscle response in the rat colon. *Am J Physiol*. 1994; 267:G515–G522. [PubMed: 7943316]
18. Takahashi T, Qoubaitary A, Owyang C, Wiley JW. Decreased expression of nitric oxide synthase in the colonic myenteric plexus of aged rats. *Brain Res*. 2000; 883:15–21. [PubMed: 11063983]
19. Bitar KN, Patil SB. Aging and gastrointestinal smooth muscle. *Mech Ageing Dev*. 2004; 125:907–910. [PubMed: 15563937]
20. Gomez-Pinilla PJ, Gibbons SJ, Sarr MG, et al. Changes in interstitial cells of Cajal with age in the human stomach and colon. *Neurogastroenterol Motil*. 2011; 23:36–44. [PubMed: 20723073]
21. MacIntosh CG, Horowitz M, Verhagen MA, et al. Effect of small intestinal nutrient infusion on appetite, gastrointestinal hormone release, and gastric myoelectrical activity in young and older men. *Am J Gastroenterol*. 2001; 96:997–1007. [PubMed: 11316218]
22. Shimamoto C, Hirata I, Hiraike Y, Takeuchi N, Nomura T, Katsu K. Evaluation of gastric motor activity in the elderly by electrogastrography and the (13)C-acetate breath test. *Gerontology*. 2002; 48:381–386. [PubMed: 12393954]
23. Sibaev A, Franck H, Vanderwinden JM, Allescher HD, Storr M. Structural differences in the enteric neural network in murine colon: impact on electrophysiology. *Am J Physiol Gastrointest Liver Physiol*. 2003; 285:G1325–G1334. [PubMed: 12881230]
24. Horvath VJ, Vittal H, Ordog T. Reduced insulin and IGF-I signaling, not hyperglycemia, underlies the diabetes-associated depletion of interstitial cells of Cajal in the murine stomach. *Diabetes*. 2005; 54:1528–1533. [PubMed: 15855342]
25. Horvath VJ, Vittal H, Lorincz A, et al. Reduced stem cell factor links smooth myopathy and loss of interstitial cells of Cajal in murine diabetic gastroparesis. *Gastroenterology*. 2006; 130:759–770. [PubMed: 16530517]
26. Lorincz A, Bardsley M, Popko L, Izbeki F, Ördög T. Gastric slow wave dysrhythmias underlying gastroparesis in mouse models of eating disorders. *Gastroenterology*. 2008; 134(4 Suppl 1) A-534.
27. Raffa RB, Mathiasen JR, Jacoby HI. Colonic bead expulsion time in normal and mu-opioid receptor deficient (CXBK) mice following central (ICV) administration of mu- and delta-opioid agonists. *Life Sci*. 1987; 41:2229–2234. [PubMed: 2823042]
28. Gangula PR, Maner WL, Micci MA, Garfield RE, Pasricha PJ. Diabetes induces sex-dependent changes in neuronal nitric oxide synthase dimerization and function in the rat gastric antrum. *Am J Physiol Gastrointest Liver Physiol*. 2007; 292:G725–G733. [PubMed: 17347455]
29. Rao YM, Chaudhury A, Goyal RK. Active and inactive pools of nNOS in the nerve terminals in mouse gut: implications for nitrergic neurotransmission. *Am J Physiol Gastrointest Liver Physiol*. 2008; 294:G627–G634. [PubMed: 18096606]

30. Phillips RJ, Hargrave SL, Rhodes BS, Zopf DA, Powley TL. Quantification of neurons in the myenteric plexus: an evaluation of putative pan-neuronal markers. *J Neurosci Methods*. 2004; 133:99–107. [PubMed: 14757350]
31. Linden DR, Couvrette JM, Ciolino A, et al. Indiscriminate loss of myenteric neurones in the TNBS-inflamed guinea-pig distal colon. *Neurogastroenterol Motil*. 2005; 17:751–760. [PubMed: 16185315]
32. Gomez-Pinilla PJ, Gibbons SJ, Bardsley MR, et al. Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. *Am J Physiol Gastrointest Liver Physiol*. 2009; 296:G1370–G1381. [PubMed: 19372102]
33. Bardsley MR, Horvath VJ, Asuzu DT, et al. Kitlow stem cells cause resistance to Kit/platelet-derived growth factor alpha inhibitors in murine gastrointestinal stromal tumors. *Gastroenterology*. 2010; 139:942–952. [PubMed: 20621681]
34. Chen H, Ordog T, Chen J, et al. Differential gene expression in functional classes of interstitial cells of Cajal in murine small intestine. *Physiol Genomics*. 2007; 31:492–509. [PubMed: 17895395]
35. Flynn RS, Murthy KS, Grider JR, Kellum JM, Kuemmerle JF. Endogenous IGF-I and $\alpha V\beta 3$ integrin ligands regulate the increased smooth muscle hyperplasia of stricturing Crohn's disease. *Gastroenterology*. 2010; 138:285–293. [PubMed: 19751734]
36. Ordog T, Hayashi Y, Gibbons SJ. Cellular pathogenesis of diabetic gastroenteropathy. *Minerva Gastroenterol Dietol*. 2009; 55:315–342. [PubMed: 19829287]
37. Saffrey MJ. Ageing of the enteric nervous system. *Mech Ageing Dev*. 2004; 125:899–906. [PubMed: 15563936]
38. Thrasivoulou C, Soubeyre V, Ridha H, et al. Reactive oxygen species, dietary restriction and neurotrophic factors in age-related loss of myenteric neurons. *Aging Cell*. 2006; 5:247–257. [PubMed: 16842497]
39. Wade PR, Hornby PJ. Age-related neurodegenerative changes and how they affect the gut. *Sci Aging Knowledge Environ*. 2005; 2005:pe8. [PubMed: 15788792]
40. Wade PR, Cowen T. Neurodegeneration: a key factor in the ageing gut. *Neurogastroenterol Motil*. 2004; 16 Suppl 1:19–23. [PubMed: 15065999]
41. Evans JM, Fleming KC, Talley NJ, Schleck CD, Zinsmeister AR, Melton LJ 3rd. Relation of colonic transit to functional bowel disease in older people: a population-based study. *J Am Geriatr Soc*. 1998; 46:83–87. [PubMed: 9434670]
42. Morley JE. Constipation and irritable bowel syndrome in the elderly. *Clin Geriatr Med*. 2007; 23:823–832. vi–vii. [PubMed: 17923340]
43. Madsen JL, Graff J. Effects of ageing on gastrointestinal motor function. *Age Ageing*. 2004; 33:154–159. [PubMed: 14960431]
44. McDougal JN, Miller MS, Burks TF, Kreulen DL. Age-related changes in colonic function in rats. *Am J Physiol*. 1984; 247:G542–G546. [PubMed: 6149696]
45. Smits GJ, Lefebvre RA. Influence of aging on gastric emptying of liquids, small intestine transit, and fecal output in rats. *Exp Gerontol*. 1996; 31:589–596. [PubMed: 9415114]
46. Bellier S, Da Silva NR, Aubin-Houzelstein G, Elbaz C, Vanderwinden JM, Panthier JJ. Accelerated intestinal transit in inbred mice with an increased number of interstitial cells of Cajal. *Am J Physiol Gastrointest Liver Physiol*. 2005; 288:G151–G158. [PubMed: 15297259]
47. Lorincz A, Redelman D, Horvath VJ, Bardsley MR, Chen H, Ordog T. Progenitors of interstitial cells of Cajal in the postnatal murine stomach. *Gastroenterology*. 2008; 134:1083–1093. [PubMed: 18395089]
48. Mei F, Yu B, Ma H, Zhang HJ, Zhou DS. Interstitial cells of Cajal could regenerate and restore their normal distribution after disrupted by intestinal transection and anastomosis in the adult guinea pigs. *Virchows Arch*. 2006; 449:348–357. [PubMed: 16912883]
49. Liu MT, Kuan YH, Wang J, Hen R, Gershon MD. 5-HT₄ receptor-mediated neuroprotection and neurogenesis in the enteric nervous system of adult mice. *J Neurosci*. 2009; 29:9683–9699. [PubMed: 19657021]

50. Kruger GM, Mosher JT, Bixby S, Joseph N, Iwashita T, Morrison SJ. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. *Neuron*. 2002; 35:657–669. [PubMed: 12194866]
51. Celtek S, Qu W, Schmidt AM, Moncada S. Synergistic action of advanced glycation end products and endogenous nitric oxide leads to neuronal apoptosis in vitro: a new insight into selective nitroergic neuropathy in diabetes. *Diabetologia*. 2004; 47:331–339. [PubMed: 14676945]
52. Smits GJ, Lefebvre RA. Influence of age on cholinergic and inhibitory nonadrenergic noncholinergic responses in the rat ileum. *Eur J Pharmacol*. 1996; 303:79–86. [PubMed: 8804914]
53. Schuster, MM.; Crowell, MD.; Koch, KL., editors. *Schuster Atlas of Gastrointestinal Motility in Health and Disease*. 2nd edn.. Lewiston: B.C. Decker; 2002.
54. Chi P, Chen Y, Zhang L, et al. ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. *Nature*. 2010; 467:849–853. [PubMed: 20927104]
55. Majumdar MK, Feng L, Medlock E, Toksoz D, Williams DA. Identification and mutation of primary and secondary proteolytic cleavage sites in murine stem cell factor cDNA yields biologically active, cell-associated protein. *J Biol Chem*. 1994; 269:1237–1242. [PubMed: 7507105]
56. Hirano K, Shishido-Hara Y, Kitazawa A, et al. Expression of stem cell factor (SCF), a KIT ligand, in gastrointestinal stromal tumors (GISTs): a potential marker for tumor proliferation. *Pathol Res Pract*. 2008; 204:799–807. [PubMed: 18602222]

Figure 1. Klotho expression in the murine small and large intestines

A–B, Quantification of *Kl* transcript levels by qRT PCR in WT and *klotho* mice. Data from individual animals are shown ($n=3$ /group/tissue); error bars are from technical triplicates. *Actb*, β -actin used as reference gene. *Kl* expression in WT small intestines was variable but higher than in the colon or in *klotho* mice. There was no difference between WT and *klotho* animals in colonic *Kl* expression. **C–H**, Immunohistochemical localization of Klotho protein in the WT murine small intestine (**C–E**) and colon (**F–H**). Cryosections from at least 3 WT tissues were labeled with rat monoclonal anti- α Klotho antibodies (green pseudocolor; left panels) and a second primary antibody against a cell type-specific antigen (red pseudocolor; middle panels) as follows: Smooth muscle cells in **C** and **F** were identified with polyclonal

anti-smooth muscle myosin antibodies; enteric neurons and nerve fibers in **D** and **G** were detected with polyclonal anti-PGP 9.5 antibodies; and ICC in **E** and **H** were labeled with polyclonal anti-Kit and monoclonal anti-anoctamin 1 (Ano1) antibodies, respectively. Nuclei were counterstained with DAPI (blue pseudocolor). Right panels show overlaid images. Scale bars in **C–F** and **H**, 30 μm ; in **G**, 50 μm . In **E**, the bottom panels are enlargements of the areas identified by dotted lines in the upper panels. Klotho was detected in both the small and large intestines in the circular and longitudinal smooth muscles layers (CM and LM, respectively) and the lamina muscularis mucosae (MM); in myenteric and submucosal ganglion cells (MG and SG, respectively) and some intramural nerve fibers (in the colon only; arrowheads in **G**), but not in mucosal (Muc) intraepithelial myofibroblasts (arrows in **C**) or in myenteric ICC (ICC-MY). In the small intestines, Klotho expression was also found in some ICC associated with the deep muscular plexus (ICC-DMP; arrowheads in the bottom panels of **E**); and in the colon, in some submucosal border ICC (ICC-SM; arrowheads in **H**). Controls for the RT-PCR and immunohistochemistry experiments are shown in Supplemental Figure S3.

Figure 2. Accelerated whole-gut transit in *klotho* mice reflects increased colonic motility and shorter small intestinal and colonic lengths and occurs in the presence of reduced small intestinal motility

A, Gastrocecal dye transit is decreased in *klotho* mice relative to age-matched, WT/Het littermates (n=8 and 9, respectively). **B**, Small intestinal length is slightly but significantly reduced in *klotho* mice (n=11/group). **C**, Gastrocecal transit is still reduced in *klotho* mice when normalized to small intestinal length. **D**, Faster bead expulsion times in *klotho* mice signifying increased colonic motility independent of organ length (n=11/group). **E**, Shorter colons in progeric *klotho* mice (n=10 (*klotho*) and 11 (WT/Het)). **F**, Colon length is not different in *klotho* and WT mice at postnatal day 15, i.e., before the segregation of body weights (n=3/group). The relative sizes of the proximal and distal colon also remained unchanged (not shown). **G–H**, Lengths of the small intestines (**G**; n=11/group) and the colon (**H**; n=10–11/group) normalized to body weights. The significantly higher values in the *klotho* mice indicate that the reduced postnatal growth of the intestines of the mutants does not directly reflect their small body size arising from reduced activity of the growth hormone–Igfl axis.^{5,14}

Figure 3. Reduced expression of smooth muscle myosin in *klotho* mice

The smooth muscle-specific isoform of the contractile protein myosin was detected by Western immunoblotting using antibodies mainly recognizing its heavy polypeptide chain (Myh11). **A–B**, Representative Western immunoblots showing reduced Myh11 protein (~230 kDa) in the jejunum, ileum (**A**) or proximal and distal colon (**B**). Gapdh, glyceraldehyde-3-phosphate dehydrogenase used as reference (~36 kDa). Myh11 and Gapdh were detected simultaneously in different fluorescent channels. **C–D**, Quantification of Myh11 protein normalized to Gapdh; n=6–9/group. In each experiment, lysates from a WT and *klotho* tissue were processed simultaneously and results were expressed as fold change relative to control, which is represented by a horizontal line.

Figure 4. Stem cell factor (*Kitl*) expression is only minimally affected in the *klotho* small intestines and colon

A–B, Representative Western immunoblots showing no reduction in *Kitl* protein in the jejunum, ileum (**A**) or proximal and distal colon (**B**) when normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; ~36 kDa). *Kitl* protein was detected as a ~31-kDa band. The size of this band corresponds to the molecular weights reported for both cell-associated (uncleaved) *Kitl*²²⁰ (the so-called “membrane-bound” isoform, which is less likely to be cleaved by extracellular proteases) and secreted *Kitl* produced from the rapidly cleaved or “soluble” *Kitl*²⁴⁸ isoform.^{55,56} We also confirmed the mixed origin of this band by detecting it in lysates of *Kitl*-deficient *Sl/S^l* fibroblasts stably expressing either murine *Kitl*²⁴⁸ or *Kitl*²²⁰ (not shown). *Kitl* and *Gapdh* were detected simultaneously in different fluorescent channels. **C–D,** Quantification of *Kitl* protein normalized to *Gapdh*; n=6–9/group. In each experiment, lysates from a WT and *klotho* tissue were processed simultaneously and results were expressed as fold change relative to control, which is represented by a horizontal line. **E–F,** Detection and quantification of the “soluble” *Kitl* isoform (*Kitl*²⁴⁸), the main source of secreted *Kitl* protein,⁵⁵ by qRT-PCR. *Actb*, β -actin used as a reference gene. Soluble *Kitl* expression was reduced in *klotho* jejunum but no statistically significant changes were detected in the ileum or proximal and distal colon (n=3/group).

Figure 5. Unchanged frequency and regionally reduced network density of intestinal ICC in *klotho* mice

A–B, ICC and ICC-SC were identified in the hematopoietic marker-negative fraction of dissociated tunica muscularis cells by flow cytometry as Kit⁺Cd44⁺Cd34⁻ and Kit^{low}Cd44⁺Cd34⁺ cells, respectively. (See gating sequence and representative projections in Supplemental Figures S2 and S4, respectively). Neither mature ICC (**A**) nor ICC-SC frequencies (**B**) showed significant changes in any part of the gut (n=4/group/tissue) despite a general trend toward lower ICC-SC frequencies (**B**). **C–F**, Visualization of Kit⁺ structures by confocal microscopy (**C–D**, representative confocal stacks) and quantification by 3-dimensional volume reconstruction using ANALYZE software (**E–F**, n=3–4/group/tissue).

ICC network volumes remained unchanged in the *klotho* small intestines and distal colon but were significantly reduced in the proximal colon. Scale bars in **C** and **D**, 50 μm .

Figure 6. Myenteric neuron counts are reduced in the *klotho* intestines

A–B, Representative whole-mounts immunostained with ANNA-1 antibody-containing human serum to detect HuC/D⁺ perikarya (red) and anti-PGP 9.5 antibodies to reveal nerve fibers and cell bodies (green). Yellow color signifies colocalization. Only a very small number of HuC/D⁺ perikarya lacked PGP 9.5 (arrows in **B**). Note reduced ganglionic areas particularly in the *klotho* colon. Scale bars, 50 μ m. **C–D**, Myenteric neuron counts determined as HuC/D⁺ perikarya along the circumference of the gut ($n=3-5$ /group/tissue). Myenteric neuron numbers were reduced in the *klotho* ileum and proximal and distal colon but not in the jejunum.

Figure 7. Unchanged Nos1/Chat ratios and Nos1 dimerization in *klotho* mice

A–B, Representative Western immunoblots showing Nos1 α (~155 kDa) and Chat (~75 kDa) detected in the jejunum, ileum (**A**), and proximal and distal colon (**B**) of the same *klotho* and WT mice. Nos1 was detected with antibodies mapping to the C terminus and capable of recognizing both α , β and γ isoforms.^{28,29} Since the smaller β and γ isoforms were only barely detectable (not shown), only the 155-kDa α band was analyzed further. Gapdh, loading control (~36 kDa). **C–D**, Unchanged Nos1/Chat ratios in *klotho* jejunum, ileum (**C**), and proximal and distal colon (**D**) (n=6/group/tissue). **E–H**, Analysis of Nos1 α dimerization in *klotho* and WT mice using antibodies mapping to the N terminus of Nos1 specific for the dimerization-capable, catalytically active α isoform (see Supplemental

Figure S1 for verification of the technique used). **E–F**, Representative immunoblots and **G–H**, quantitative results showing no changes in Nos1 α dimer/monomer ratios in *klotho* jejunum, ileum (**E,G**) and proximal and distal colon (**F,H**) compared to age-matched, WT controls (n=6/group/tissue).

Table 1

Summary of key changes in the gastrointestinal neuromuscular apparatus in *klotho* mice^a

Organ	Size	Function	Smooth muscle	ICC & ICC-SC ^b	Neurons	Klotho expression
Stomach ^c	↔	SW amplitude↓ NO-ergic IIP↓ Solid GE↔	Myh11 mRNA↔ Igf1 mRNA↔ Kitl (total) mRNA↓ Kitl (soluble) mRNA↔	ICC %↓ ICC-SC %↔ Network volume↓ Kit mRNA↓ Kit protein↓	Number↔ Frequency↔ Nos1 mRNA↔ Nos1 protein↔	↓
Small intestine	Jejunum	Transit ↓	Myh11 protein↓ ^d Kitl protein↔ Kitl (soluble) mRNA↓	ICC %↔ ICC-SC %↔ Network volume↔	Number↔ Nos1 protein↔ Nos1 dimer/monomer↔ Chat protein↔ Nos1/Chat↔	(↓)
	Ileum		Myh11 protein↓ Kitl protein↔ Kitl (soluble) mRNA↔	ICC %↔ ICC-SC %↔ Network volume↔	Number↓ PGP 9.5 protein↔ Nos1 protein↔ Nos1 dimer/monomer↔ Chat protein↔ Nos1/Chat↔	↓
Colon	Proximal	Transit ↑	Myh11 protein↓ Kitl protein↔ Kitl (soluble) mRNA↔	ICC %↔ ICC-SC %↔ Network volume↓	Number↓ PGP 9.5 protein↔ Nos1 protein↔ Nos1 dimer/monomer↔ Chat protein↔ Nos1/Chat↔	↔
	Distal		Myh11 protein↓ Kitl protein↔ Kitl (soluble) mRNA↔	ICC %↔ ICC-SC %↔ Network volume↔	Number↓ PGP 9.5 protein↔ Nos1 protein↔ Nos1 dimer/monomer↔ Chat protein↔ Nos1/Chat↔	↔

^aThe organ-level changes listed in this table underlie the general functional abnormalities detected in *klotho* mice and reported herein and in reference¹⁴ including reduced food intake, low body weight and reduced fecal output without a change in fecal water content.

^b Abbreviations and symbols: ICC, interstitial cells of Cajal; ICC-SC, ICC stem/progenitor cells; SW, slow wave; ↓, reduced; (↓), strong trend toward reduction; ↑, increased; ↔, unchanged; PGP 9.5, protein gene product 9.5 (ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase; Uchl1); NO, nitric oxide; IIP, inhibitory junction potential; GE, gastric emptying; Myh11, smooth muscle myosin heavy polypeptide 11, the protein predominantly recognized by the BTI Anti-Smooth Muscle Myosin antibody; Igf1, insulin-like growth factor 1; Kitl, stem cell factor; Kit, Kit oncogene; %, percent of cells with light scatter properties characteristic of single live cells (LS⁺) defined by flow cytometry; Nos1, nitric oxide synthase 1, neuronal; Chat, choline acetyltransferase.

^c All gastric data are from reference¹⁴.

^d Note that with the exception of neuron numbers, Nos1 dimer/monomer and Nos1/Chat ratios, all parameters listed for the various cell types are either normalized to the number of cells in the assays or only reflect absolute values within small tissue volumes (ICC network volumes). Therefore, in organs whose size is reduced in *klotho* mice (small intestines, colon), unchanged (↔) values of these parameters signify actual decreases in absolute terms (parameter/organ) as compared to the age-matched WT controls.