

# Loss of Aklotho Causes Reduced Motor Ability and Short Lifespan in Zebrafish

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## Research Article

**Keywords:** aging, development, mutation, swimming, zebrafish

**Posted Date:** December 11th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-123446/v1>

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**Version of Record:** A version of this preprint was published at Scientific Reports on July 23rd, 2021. See the published version at <https://doi.org/10.1038/s41598-021-93909-y>.

1 **Loss of  $\alpha$ klotho causes reduced motor ability and short lifespan in zebrafish**

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9  
10 Number of words in Title: 12/20 words

11 Number of words in Abstract: 200/200 words

12 Number of words in Main text: 1,975/4,500 words

13 Number of words in Materials and Methods: 493/1,500 words

14 Number of figures: 3

15 Number of supplementary figure: 1

16  
17 **Keywords:** aging, development, mutation, swimming, zebrafish

## Abstract

The *klotho* gene encodes a transmembrane protein  $\alpha$ Klotho that interacts with a fibroblast growth factor receptor in renal tubular epithelial cells and functions as a co-receptor for FGF23, which is an osteocytes-derived hormone. It is known that this bone-to-kidney signal promotes urinary phosphate excretion. Interestingly,  $\alpha$ Klotho-deficient mice show accelerated aging and shortened life span in addition to dysregulation of serum phosphorus. However, physiological basis of aging-related function of  $\alpha$ klotho and its generality in animals remain unclear. The  $\alpha$ klotho-deficient vertebrate animals other than mice have been awaited as an alternative premature aging model. We here employed zebrafish in our  $\alpha$ klotho study and revealed that  $\alpha$ klotho mutant zebrafish appear to be normal at 3 months postfertilization (mpf) in young adults but eventually undergo premature death by 9 mpf, while normal zebrafish is known to survive for 42 months. We also assessed motor ability of zebrafish in a forced swimming assay and found that  $\alpha$ klotho mutant zebrafish displayed reduced swimming performance before their survival declined. A recent study also reported a similar finding that  $\alpha$ klotho-deficient zebrafish exhibited short life span and reduced spontaneous movements. Taken together, these results suggest that  $\alpha$ Klotho mutant zebrafish show premature aging and are useful to investigate aging in vertebrates.

## Introduction

Aging is a process of becoming older. In human, a number of physiological decline of biological functions such as skin wrinkling, soft tissue calcification, neural degeneration, muscle weakness and motor deterioration occur over time<sup>1</sup>. Numerous aging-related disorders and premature aging diseases have been pathologically identified in human. Although it has been suggested that aging is triggered by an accumulation of DNA/cell damage or by a genetic limitation of cell proliferation, the physiological basis and causes of aging are still largely unknown<sup>1</sup>. To investigate aging-related genes and to fight against aging, several animal models such as mouse, fruit fly and *C. elegans* have been used to assay longevity and premature death<sup>2-5</sup>. Zebrafish (*Danio rerio*), which is an emerging alternative vertebrate model, has also been used to study progressive deterioration of biological function in aging<sup>6,7</sup>.

The *klotho* (*kl*) gene was originally identified as an aging-suppressor in mutant mice that show accelerated aging and short lifespan<sup>8</sup>. The *kl* gene encodes a single-pass transmembrane protein  $\alpha$ klotho and is predominantly expressed in the distal convoluted tubules. The  $\alpha$ klotho protein binds to fibroblast growth factor (FGF) receptors and functions as a co-receptor for FGF23, which is secreted from osteocytes in bone<sup>9</sup>. This bone-to-kidney signal is activated by an increase of serum phosphorus, promoting phosphate discharge from blood to urine<sup>10</sup>. The  $\alpha$ klotho-deficient mice as well as FGF23 knockout mice exhibited increased blood phosphate that results in arteriosclerosis and vascular calcification<sup>11,12</sup>. In addition to these blood vessel disorders, multiple aging phenotypes such as skin atrophy, auditory disturbance, osteopenia, sarcopenia and premature death has been reported in these mutant mice<sup>13</sup>. Conversely, transgenic mice that overexpress  $\alpha$ klotho displayed longevity compared to normal mice<sup>14</sup>. Thus, one important contributing factor to  $\alpha$ klotho-mediated suppression of aging appears to be the regulation of phosphate levels. But how phosphate homeostasis determines the life span remains unclear<sup>15</sup>. The  $\alpha$ klotho-deficient vertebrate animals other than mice would contribute toward understanding aging.

In this study, we employed zebrafish for an  $\alpha$ klotho study and found that  $\alpha$ klotho mutant zebrafish (Y306X) showed shortened lifespan. A recent study reported the same finding that  $\alpha$ klotho-deficient zebrafish (S179frameshift) undergo premature death<sup>16</sup>. We also assessed swimming performance of zebrafish in forced swimming assay using a swimmill and demonstrated that  $\alpha$ klotho mutant zebrafish display reduced swimming ability before their survival declined.

## Results

## **Zebrafish *klotho* gene and *klotho* mutation**

To study  $\alpha$ klotho in zebrafish, we first retrieved gene information of zebrafish  $\alpha$ klotho from NCBI database. An amino acid alignment of  $\alpha$ klotho protein showed that  $\alpha$ klotho is conserved among vertebrates from zebrafish to human, especially in two glycosidase domains (Supplemental Fig. 1). Although amino acid residues of the transmembrane domain at the C-terminus of  $\alpha$ klotho appeared to be less conserved in chicken, frog and zebrafish, online prediction tools of the protein secondary structure such as PredictProtein and SOSUI suggested that this region is a putative membrane-spanning domain in these non-mammalian animals, verifying that  $\alpha$ klotho is overall conserved among vertebrates.

To investigate physiological function of  $\alpha$ klotho in zebrafish, we next obtained an  $\alpha$ klotho mutant allele *kl<sup>sal18644</sup>*, which was identified by a targeting induced local lesions in genomes (TILLING) project in zebrafish<sup>17</sup>. This allele harbors a T to A base substitution that generates a premature nonsense codon (Y306X) in the middle of the first glycosidase domain (Fig. 1a). This mutation also generated an MseI restriction site that enabled genotyping by a restriction enzyme digestion of the genomic PCR products (Fig. 1b, c).

## **Zebrafish *klotho* mutants exhibit short life span**

By crossing heterozygous *kl* mutant carrier fish (*kl<sup>+/-</sup>*), we obtained homozygous *kl* mutants (*kl<sup>-/-</sup>*). The *kl<sup>-/-</sup>* mutant embryos showed no apparent defects and grew up to become adults. The ratio of *kl<sup>-/-</sup>* mutants in a progeny of a heterozygous carrier cross was about one quarter (23%, 23/100) at 4 months postfertilization (mpf), indicating that the loss of *kl* does not affect development or survival of zebrafish until they become young adults (Fig. 2a-c) as reported recently<sup>16</sup>. Although the skin of the previously reported *kl*-deficient zebrafish appeared to be pale at 5 mpf<sup>16</sup>, we did not see apparent reduction of skin tone in *kl<sup>-/-</sup>* compared to *kl<sup>+/+</sup>* or *kl<sup>+/-</sup>* fish at any age. Intriguingly, we noticed that some mutant female displayed protruding eye (30%, 6/20) just like telescope goldfish (Fig. 2d-f). This malformation of the eye was not seen in mutant male fish (n = 30). The same eye phenotype in female fish was also reported in the other *kl* mutant allele<sup>16</sup>. We also recapitulated that our *kl<sup>-/-</sup>* fish become thinner after 5 months of age and die within 9 months (Fig. 2g-i). To detail the short life span of *kl<sup>-/-</sup>* zebrafish, we monitored the survival of our *kl<sup>+/+</sup>*, *kl<sup>+/-</sup>* and *kl<sup>-/-</sup>* fish from 2 mpf (Fig. 2j). We found that all of the *kl<sup>-/-</sup>* fish (n = 28) died between 4 and 9 mpf, whereas all *kl<sup>+/+</sup>* (n = 32) and most *kl<sup>+/-</sup>* (n = 86) fish kept living after 9 mpf. It has been reported that the mean life span of zebrafish is 42 months<sup>18</sup>. These results indicate that *kl<sup>-/-</sup>* zebrafish undergo premature aging and death, just like  $\alpha$ klotho-deficient mice exhibit progeroid phenotypes<sup>8</sup>.

### **Zebrafish *klotho* mutants show reduced motor ability**

Since aging affects the integrity of motor system<sup>19</sup>, we addressed whether motor ability is impaired in *kl*<sup>-/-</sup> zebrafish before they initiate to show premature aging. As swimming ability of adult zebrafish is governed by body size especially by caudal fin length<sup>20</sup>, we began with a physical measurement. We measured standard length and caudal fin length defined as the length from the head to the root of the caudal fin and from the root of the caudal fin to the edge of the fin, respectively<sup>21</sup>. Both standard length and caudal fin length at 4 mpf were comparable between *kl*<sup>+/+</sup> (standard length  $2.86 \pm 0.04$  cm, caudal fin length  $0.67 \pm 0.02$  cm;  $n = 19$ ; Fig. 3a), *kl*<sup>+/-</sup> (standard length  $2.71 \pm 0.06$  cm, caudal fin length  $0.69 \pm 0.07$  cm;  $n = 21$ ) and *kl*<sup>-/-</sup> (standard length  $2.74 \pm 0.06$  cm;  $P = 0.13$  for *kl*<sup>+/+</sup>,  $P = 0.79$  for *kl*<sup>+/-</sup>; caudal fin length  $0.63 \pm 0.06$  cm;  $P = 0.53$  for *kl*<sup>+/+</sup>,  $P = 0.44$  for *kl*<sup>+/-</sup>;  $n = 12$ ). These data confirm that *kl*<sup>-/-</sup> fish are indistinguishable from *kl*<sup>+/+</sup> and *kl*<sup>+/-</sup> fish by body size at 4 mpf.

To quantify swimming performance of adult zebrafish, we then employed a swimmill, which is a treadmill for aquatic animals<sup>20</sup>. A zebrafish was put in a swimming chamber, where water flow is generated by a voltage-controlled spinning propeller. Since zebrafish instinctively swim against water flow to maintain the position, fish is compelled to swim at the water velocity. The water velocity was initially set to 10 cm/s for 1 min successively followed by 15 cm/s for 1 min. Eventually, the water velocity increased 1 cm/s every 1 min. Zebrafish kept swimming until the water velocity went above the swimming ability of the fish. The water velocity at when zebrafish can no longer keep swimming was defined as critical swimming speed ( $U_{crit}$ ) as described previously<sup>22</sup>. The  $U_{crit}$  of *kl*<sup>-/-</sup> zebrafish ( $17.6 \pm 0.9$  cm/s;  $n = 12$ ; Fig. 3b) was significantly lower than that of *kl*<sup>+/+</sup> ( $26.7 \pm 0.6$  cm/s;  $P < 0.001$ ;  $n = 19$ ) and *kl*<sup>+/-</sup> fish ( $28.6 \pm 1.3$  cm/s;  $P < 0.001$ ;  $n = 21$ ). These results demonstrate that *kl*<sup>-/-</sup> fish were less capable of swimming at 4 mpf when they do not exhibit premature death.

### **Discussion**

In this study, we investigated a nonsense mutant allele of  $\alpha$ klotho in zebrafish and found that *kl*<sup>-/-</sup> fish show shortened life span. Recently, Singh and his colleagues have reported the other *kl* mutant zebrafish allele, which harbors a CRISPR-mediated 5-bp deletion and thus carries a frameshift in the first glycosidase domain<sup>16</sup>. Although age-related frailty and premature death in our *kl*<sup>-/-</sup> fish were recapitulations of a recent report, we uniquely examined swimming performance of *kl*<sup>-/-</sup> fish using a swimmill and demonstrated that *kl*<sup>-/-</sup> fish exhibit a compromised motor ability before they become thinner and undergo

premature death. Taken together, we conclude that  $\alpha$ klotho mutant zebrafish show premature aging phenotypes and that  $\alpha$ klotho-deficient zebrafish can be an alternative animal model of aging.

### Conservation of $\alpha$ klotho gene

While invertebrate animals use calcium carbonate for exoskeleton, vertebrate animals utilize calcium phosphate for endoskeleton but with a risk of phosphate toxicity<sup>23</sup>. Since  $\alpha$ klotho plays an essential role in discharging excess phosphate from blood to prevent unwanted ectopic calcification, it is reasonable that  $\alpha$ klotho function is conserved among vertebrates<sup>24</sup>. Our amino acid alignment confirmed that  $\alpha$ klotho proteins have two putative glycosidase domains that are highly conserved among vertebrates. These domains share the highest homology with lactase, which hydrolyses lactose to produce galactose and glucose<sup>25,26</sup>. Interestingly however, the catalytic glutamate residues in both glycosidase domains were substituted to the other residues; i.e. N239 and S872 in human  $\alpha$ klotho, N241 and A874 in mouse  $\alpha$ klotho and N208 and A849 in zebrafish  $\alpha$ klotho. Instead of losing glycosidase activities, these domains might have acquired an affinity to a calciotropic hormone FGF23, enabling  $\alpha$ klotho to function as a coreceptor of FGF receptors that in turn promotes phosphate excretion from renal tubules<sup>9</sup>. These amino acid substitution might have been critical for  $\alpha$ klotho function in vertebrates. An  $\alpha$ klotho homologue gene (*klo1*) was also found in *C. elegans*, and *klo1*-deficient worms showed short life spans<sup>27,28</sup>. The  $\alpha$ klotho protein in *C. elegans* has only one glycosidase domain with its catalytic residue intriguingly conserved as glutamate, potentially possessing the glycosidase activity. But how  $\alpha$ klotho-mediated suppression of aging takes place in *C. elegans* also remains unsolved.

### Decline of motor integrity in $\alpha$ klotho-deficient zebrafish

Previous *in situ* hybridization analyses revealed that *kl* gene is expressed by brain, liver, pancreas and pronephros in embryonic and larval zebrafish and by mesonephric kidney tubules in adult zebrafish<sup>29</sup>. The *kl*<sup>-/-</sup> zebrafish did not show any apparent defects during development and in young adults around 3~4 mpf. But they eventually became thinner and showed premature death at 5~8 mpf. Singh et al. reported that *kl*-deficient zebrafish showed progressive reduction of motor speed during spontaneous free swimming at the onset of 2 mpf<sup>16</sup>. On the other hand, our swimmill assay, which assesses motor performance in forced swimming, revealed that *kl*<sup>-/-</sup> fish exhibited lower  $U_{crit}$ , which is the maximum speed of fish swimming, compared to *kl*<sup>+/+</sup> and *kl*<sup>+/-</sup> fish at 4 mpf. Reduction of  $U_{crit}$  has been reported in aged zebrafish<sup>30</sup>, implying that motor deterioration in *kl*<sup>-/-</sup>

fish is a premature aging phenotype. Taken together, the decline of motor integrity is likely the earliest physiological phenotype of premature aging in *kl* mutant zebrafish. But whether this was caused by neuronal dysfunction, muscle weakness or cardiovascular defects remains obscured.

## **Pathology of $\alpha$ klotho**

Kuro-o and his colleagues have demonstrated that  $\alpha$ klotho mutant mice show several age-related disorders such as increase of serum phosphorus, ectopic calcification and hypokinesia<sup>8</sup>. The life span of the mutant mice was approximately 60 days with no mice surviving over 100 days. In agreement with this short life span in mutant mice, a population-based association study suggested that a SNP that generates an F352V missense variant of human  $\alpha$ klotho is implicated in short life span<sup>31</sup>. In addition, a 13 years old girl who carries a homozygous H193R missense mutation in  $\alpha$ klotho showed premature tumoral calcinosis with dural and carotid artery calcifications<sup>32</sup>. Thus, physiological symptoms linked to  $\alpha$ klotho mutations appear to be common in mammals. Although pathological studies of  $\alpha$ klotho deficiency has only been done in mice, human and *C. elegans* until recently, the latest publication of  $\alpha$ klotho mutant zebrafish<sup>16</sup> and our current study demonstrated pathologically-relevant progressive motor deterioration and premature death in zebrafish *kl*<sup>-/-</sup> mutants. Collectively, zebrafish *kl*<sup>-/-</sup> mutants provide an alternative animal model to study aging and progeria in vertebrates.

## **Materials and Methods**

### **Animals**

Zebrafish (*Danio rerio*) were reared and maintained in 1.5 L tanks in a recirculating Meito System (Meito System) under a 14 h light and 10 h dark photoperiod according to the standard protocol<sup>33</sup>. Larvae were fed paramecia and Gemma Micro ZF 75 (Funakoshi) twice a day from 5 dpf to 20 dpf. Juvenile fish were fed brine shrimp (Tokai Guppy) and Gemma Micro ZF 75 twice a day. Adults fish were fed brine shrimp and Otohime B2 (Marubeni Nissin Feed) twice a day. Zebrafish  $\alpha$ klotho mutant line (*kl*<sup>sal8644</sup>) was purchased from Zebrafish International Resource Center (<https://zebrafish.org/home/guide.php>).

### **Genotyping**

The missense region of *kl* gene was amplified by genomic PCR using KAPA Taq Extra PCR Kit (Kapa Biosystems) in ProFlex PCR System (Thermo Fisher Scientific). Following program was used for amplification: 94 °C 2 min; 94 °C 10 sec, 63 °C 20 sec,



72 °C 30 sec, 35 cycles; 72 °C 1 min; 4 °C forever. Following primers were used; *kl* genotyping forward: CTCTGGGATCTCACTGGATC; *kl* genotyping reverse: AACTAAGAGCAGGTCCATGAGAC. PCR products were digested with MseI restriction enzyme (Takara) and separated by 15% polyacrylamide gel electrophoresis at 300 V for 90 min as described previously <sup>34</sup>. The gel images were captured using the Printgraph AE-6933FXCF (Atto).

### **Image capture**

Zebrafish were anesthetized in 0.004% Tricaine (MS-222, Sigma-Aldrich) for 1 min. Images of zebrafish were captured using a digital camera SONY α5000.

### **Survival**

Adult zebrafish obtained by a cross of *kl*<sup>+/-</sup> fish were maintained in the regular care. Genotyping was done at 2 mpf. Zebrafish that keep floating at water surface or sinking at the bottom either without swimming were judged as reaching the end point and subjected to euthanasia. Number of zebrafish reaching the end point was counted.

### **Swimmill analysis**

Motor ability of zebrafish was quantified using a swimmill system Swim tunnel respirometer 170 ml (Loligo System) as described previously <sup>20</sup>. In brief, an adult zebrafish was put under a propeller-driven water flow in a chamber, and fish was compelled to swim in the water flow. The water velocity increased 1 cm/s every 1 min after initial warming up of 10 cm/s flow for 1 min and successive 15 cm/s flow for 1 min. Zebrafish swam at the speed of water flow until the water velocity reach the maximum swimming capability of the fish. The water velocity at when zebrafish can no longer keep swimming was defined as critical swimming speed  $U_{crit}$ . The standard and caudal fin lengths were measured by analyzing frames of swimming movies as described previously <sup>20</sup>.

### **Statistics**

Quantitative data were given as mean ± SEM. All error bars in graphs represent the SEM values. The sample numbers are indicated in Figure legends. Quantitative data were tested for normality by Shapiro-Wilk test ( $P < 0.05$ ). Statistical significance was determined using the two-tailed Student's t test.

### **Ethics statement**

This study was approved by Animal Care and Use Committee of Aoyama Gakuin University (A9/2020) and carried out according to the Aoyama Gakuin University Animal Care and Use Guideline.

### **Acknowledgments**

We thank Hirata Lab members for fish care. This work was supported by KAKENHI (Grant-in-Aid for Scientific Research B from the MEXT, Japan: 19H03329), the Takeda Science Foundation, the Naito Foundation.

### **Author contributions**

H.H. designed research; Y.O., K.U. and Y.W. performed research and analyzed data; Y.O. and H.H. wrote the manuscript.

### **Competing interests**

The authors declare no competing interests.

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**Figure legends**

**Figure 1.** A nonsense mutation of zebrafish  $\alpha$ klotho.

**a.** The  $\alpha$ klotho protein has a signal peptide, two glycosidase domains and a transmembrane domain. A nonsense mutation caused a truncation in the first glycosidase domain. **b.** The T-to-A mutation generated an MseI restriction enzyme site. **c.** The size of genomic PCR products was 240 bp. The PCR products amplified from mutant alleles were digested by MseI to generate two 120-bp fragments.

**Figure 2.** Zebrafish  $\alpha$ klotho mutants show protruding eyes and short life span.

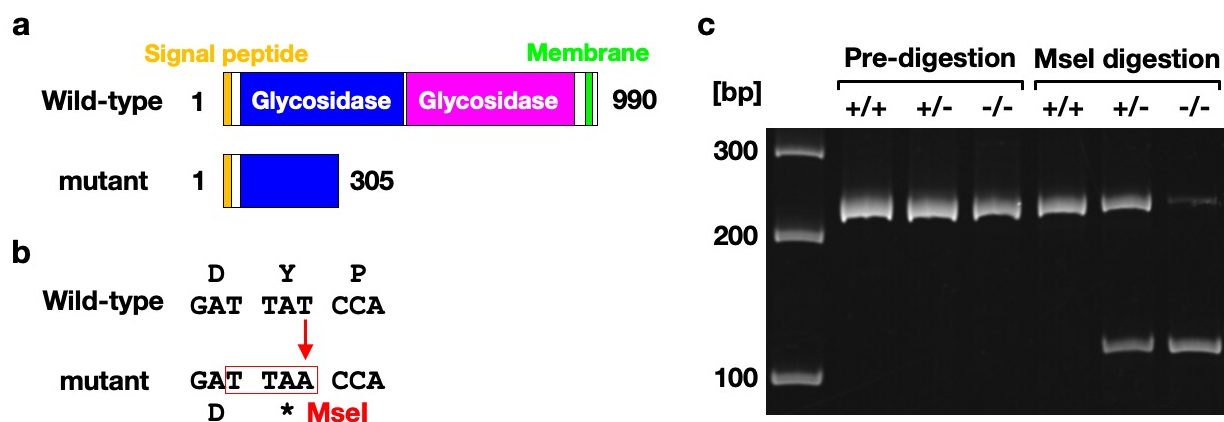
**a-c.** The  $kl^{+/+}$  (a),  $kl^{+/-}$  (b) and  $kl^{-/-}$  (c) zebrafish at 4 mpf.  $kl^{-/-}$  fish showed no morphological defects at this stage. **d-f.** Lateral and dorsal views of  $kl^{+/+}$  (d),  $kl^{+/-}$  (e) and  $kl^{-/-}$  (f) head at 5 mpf. Note that the eyeball is protruding to the outside in  $kl^{-/-}$  fish. **g-i.** The  $kl^{+/+}$  (g),  $kl^{+/-}$  (h) and  $kl^{-/-}$  (i) zebrafish at 6 mpf.  $kl^{-/-}$  fish became skinny. **g.** The  $kl^{+/+}$  (n = 32) and  $kl^{+/-}$  fish (n = 86) kept surviving after 9 mpf. Most of the  $kl^{-/-}$  fish (n = 28) underwent premature death at 5 ~ 8 mpf.

**Figure 3.**

**a.** The standard length and caudal fin length of individual fish at 5 mpf. Note that both standard length and caudal fin length were comparable among  $kl^{+/+}$  (n = 19),  $kl^{+/-}$  (n = 21) and  $kl^{-/-}$  (n = 12). **b.** The  $U_{crit}$  of  $kl^{+/+}$  (n = 19),  $kl^{+/-}$  (n = 21) and  $kl^{-/-}$  (n = 12) zebrafish were measured by a swimmill.

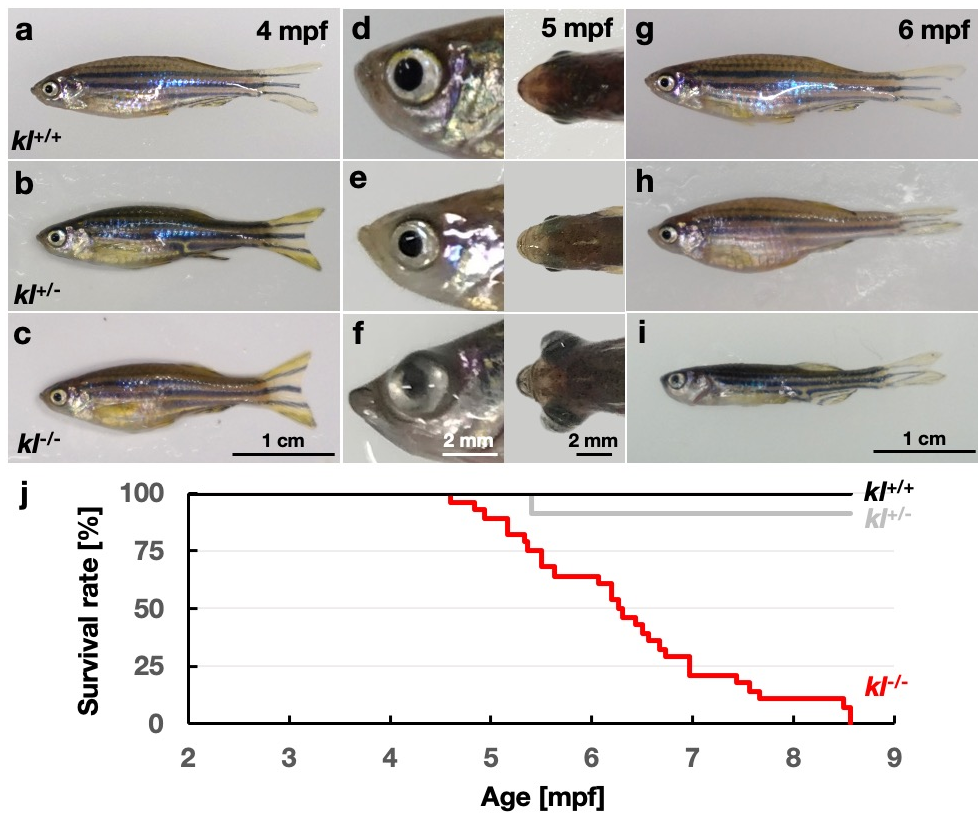
**Figure S1.** The  $\alpha$ Klotho is conserved in vertebrates.

Multiple amino acid sequence alignment of  $\alpha$ Klotho protein. Black and grey colors respectively indicate completely and highly conserved amino acid residues among vertebrates. The N-terminal signal peptide is highlighted by an orange box. The first and the second putative glycosidase domains are indicated by blue and magenta boxes, respectively. The C-terminal transmembrane domain is highlighted by a green box. Yellow circles indicate putative N-linked glycosylation sites. Two red arrows indicate the position of frameshift mutation (S179frameshift) in the previous *kl* mutant study and the position of protein truncation (Y306X) in our *kl<sup>sa18644</sup>* allele. Following NCBI data were used: human (*H. sapiens*) NP\_004786; rhesus monkey (*Macaca mulatta*) XP\_001101127; mouse (*Mus musculus*) NP\_038851; chicken (*Gallus gallus*) XP\_417105; tropical clawed frog (*Xenopus tropicalis*) XP\_002934067; zebrafish (*Danio rerio*) XP\_021335093.

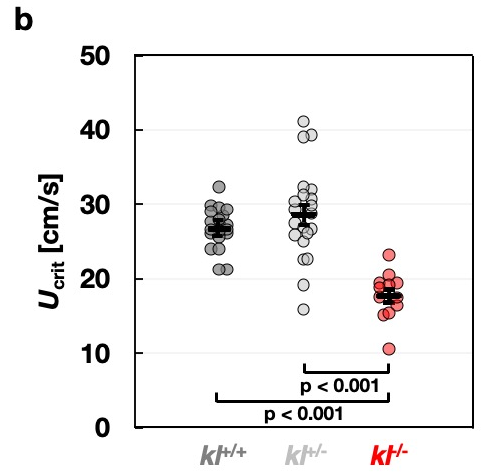
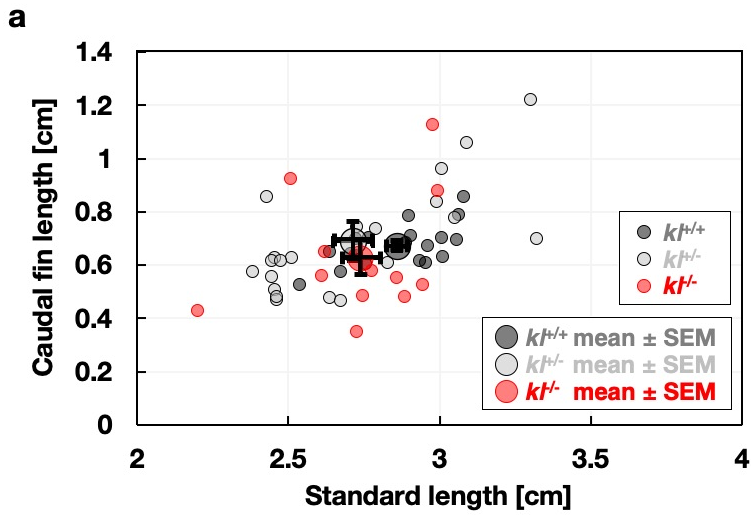


Ogura Y et al. Fig. 1





Ogura Y et al. Fig. 2



Ogura Y et al. Fig. 3

	Signal peptide	
<i>Homo sapiens</i>	1 MPASAPRRRPRPPPP--SLSLLLVLLGLGGRRLRAEPGDGAQTWARFSRPPAPEAAGLFQ	58
<i>Macaca mulatta</i>	1 MPASAPRRRLRSPSPSLSLSLLLVLLALGGRRLRAEPGDGAQTWARFARPPAPEAAGLFQ	60
<i>Rattus norvegicus</i>	1 MPARAPRRRLRLLLLLRLSLHLLLTLRARCLSAEPGQGAQTWARFARPPVPEASGLLH	60
<i>Mus musculus</i>	1 MLARAPRRRPPRLVLLRLLLHLLLLARCLSAEPGQGAQTWARFARAPAPEAAGLLH	60
<i>Gallus gallus</i>	1 -----MAPPPPPVLPVLPVLLVLLGLGRPLLGAPGQGAQTWARFAHLPPYQDQLFH	52
<i>Xenopus tropicalis</i>	1 -----MRPGALALLWAAWGCALCALCGNAEKVMSRFAQLPFPQDNLFY	46
<i>Danio rerio</i>	1 -----MKVTWIPLPLVLFQCOFGTASDPGAGQHTWDTESKLPYDDKAFY	46
	Glycosidase	
<i>Homo sapiens</i>	59 GTFPDGLWAVGSAAYQTEGGVQHGKGASINDTFTHHPLAPPGDSRNASLPLGAPSPLQ	118
<i>Macaca mulatta</i>	61 GTFPDGLWAVGSAAYQTEGGVQHGKGASINDTFTHHPLAPPGDSRIANVPSGAPSPLQ	120
<i>Rattus norvegicus</i>	61 DTFPDGLWAVGSAAYQTEGGVQHGKGASINDTFTHHPRAPEDSPIVMAPSGAPLPPL	120
<i>Mus musculus</i>	61 DTFPDGLWAVGSAAYQTEGGVQHGKGASINDTFTHSGAAPSDDSPIVVAPSGAPSPLQ	120
<i>Gallus gallus</i>	53 DTFPDGLWAGSAAYQTEGGVQHGKGASINDTFTHRPTTPAG-----SILPG-----	101
<i>Xenopus tropicalis</i>	47 GTFPDGLWAVGSAAYQTEGGVQHGKGASINDTFTCHKSGQ-----L	88
<i>Danio rerio</i>	47 DTFPDGLWAVGSAAYQTEGGVQHGKGASINDTFTTRGGTR-----	87
<i>Homo sapiens</i>	119 PATGDVADSNNVFRDTEALRELGVTHYRFSISWARVLPNGSAGVFNREGLRYRRLLE	178
<i>Macaca mulatta</i>	121 PATGDVADSNNVFRDTEALRELGVTHYRFSISWARVLPNGSAGVFNREGLRYRRLLE	180
<i>Rattus norvegicus</i>	121 PSTGDVADSNNVFRDTEALRELGVTHYRFSISWARVLPNGTAGTPNREGLRYRRLLE	180
<i>Mus musculus</i>	121 SSTGDVADSNNVFRDTEALRELGVTHYRFSISWARVLPNGTAGTPNREGLRYRRLLE	180
<i>Gallus gallus</i>	102 PTGDVADSNNVFRDTEALRELGVTHYRFSISWARVLPNGTAGTPNREGLRYRRLLE	160
<i>Xenopus tropicalis</i>	89 DATGDVADSNNVFRDTEALRELGVTHYRFSISWARVLPNGTESAFNEAGLSYRNLTL	148
<i>Danio rerio</i>	88 VSRGDVGSNNVFRDTEALRELGVTHYRFSISWARVLPNGTESAFNEAGLSYRNLTL	147
<i>Homo sapiens</i>	179 RLRELGVQPVVTLYHWDLPQRLQDAYGGWANRALADHFRDYAELCFRHFGGQVYWTITD	238
<i>Macaca mulatta</i>	181 RLRELGVQPVVTLYHWDLPQRLQDAYGGWANRALADHFRDYAELCFRHFGGQVYWTITD	240
<i>Rattus norvegicus</i>	181 RLRELGVQPVVTLYHWDLPQRLQDAYGGWANRALADHFRDYAELCFRHFGGQVYWTITD	240
<i>Mus musculus</i>	181 RLRELGVQPVVTLYHWDLPQRLQDAYGGWANRALADHFRDYAELCFRHFGGQVYWTITD	240
<i>Gallus gallus</i>	161 RLRELGVQPVVTLYHWDLPQRLQDAYGGWANRALADHFRDYAELCFRHFGGQVYWTITD	220
<i>Xenopus tropicalis</i>	149 RLRELGVQPVVTLYHWDLPQRLQDAYGGWANRALADHFRDYAELCFRHFGGQVYWTITD	208
<i>Danio rerio</i>	148 GLKDIKQPVVTLYHWDLPQRLQDAYGGWANRALADHFRDYAELCFRHFGGQVYWTITD	207
← S179frameshift		
<i>Homo sapiens</i>	239 NPYVVAWHGYATGRLAPGIRGSPRLGYLVAHNLAAHAKVHLYNTSFRPTQGGVSTIAL	298
<i>Macaca mulatta</i>	241 NPYVVAWHGYATGRLAPGIRGSPRLGYLVAHNLAAHAKVHLYNTSFRPTQGGVSTIAL	300
<i>Rattus norvegicus</i>	241 NPYVVAWHGYATGRLAPGIRGSPRLGYLVAHNLAAHAKVHLYNTSFRPTQGGVSTIAL	300
<i>Mus musculus</i>	241 NPYVVAWHGYATGRLAPGIRGSPRLGYLVAHNLAAHAKVHLYNTSFRPTQGGVSTIAL	300
<i>Gallus gallus</i>	221 NPYVVAWHGYATGRLAPGIRGSPRLGYLVAHNLAAHAKVHLYNTSFRPTQGGVSTIAL	280
<i>Xenopus tropicalis</i>	209 NPYVVAWHGYATGRLAPGIRGSPRLGYLVAHNLAAHAKVHLYNTSFRPTQGGVSTIAL	268
<i>Danio rerio</i>	208 NPYVVAWHGYATGRLAPGIRGSPRLGYLVAHNLAAHAKVHLYNTSFRPTQGGVSTIAL	267
<i>Homo sapiens</i>	299 SSHWINPRMTDHSIKECQKSLDFVLGWFAKPIFIDGDYPESMKNNLSLLPDTFSEKK	358
<i>Macaca mulatta</i>	301 SSHWINPRMTDHSIKECQKSLDFVLGWFAKPIFIDGDYPESMKNNLSLLPDTFSEKK	360
<i>Rattus norvegicus</i>	301 GSHWITPRMTDHSIKECQKSLDFVLGWFAKPIFIDGDYPESMKNNLSLLPDTFSEKK	360
<i>Mus musculus</i>	301 SSHWINPRMTDHSIKECQKSLDFVLGWFAKPIFIDGDYPESMKNNLSLLPDTFSEKK	360
<i>Gallus gallus</i>	281 SSHWIKPQMTDKNIKECQKSLDFVLGWFAKPIFIDGDYPESMKNNLSLLPDTFSEKK	340
<i>Xenopus tropicalis</i>	269 ASHWINPVMTSDHIGDQKSLDFVLGWFAKPIFIDGDYPQTMKNNLSLLPDTFSEKK	328
<i>Danio rerio</i>	268 GSHWIKPQMTDKNIKECQKSLDFVLGWFAKPIFIDGDYPQTMKNNLSLLPDTFSEKK	327
← sa18644: Y306X		
<i>Homo sapiens</i>	359 FIKGTADFFALSFGPTLSFQLLDPMKFRQLESPLRQLLSWIDLEYNPPTFIVENGWF	418
<i>Macaca mulatta</i>	361 FIKGTADFFALSFGPTLSFQLLDPMKFRQLESPLRQLLSWIDLEYNPPTFIVENGWF	420
<i>Rattus norvegicus</i>	361 FIKGTADFFALSFGPTLSFQLLDPMKFRQLESPLRQLLSWIDLEYNPPTFIVENGWF	420
<i>Mus musculus</i>	361 LIRGTADFFALSFGPTLSFQLLDPMKFRQLESPLRQLLSWIDLEYNPPTFIVENGWF	420
<i>Gallus gallus</i>	341 YIKGTADFFALSFGPTLSFQLLDPMKFRQLESPLRQLLSWIDLEYNPPTFIVENGWF	400
<i>Xenopus tropicalis</i>	329 LNKGTADFFALSFGPTLSFQLLDPMKFRQLESPLRQLLSWIDLEYNPPTFIVENGWF	388
<i>Danio rerio</i>	328 YVNGTADFFALSFGPTLSFQLLDPMKFRQLESPLRQLLSWIDLEYNPPTFIVENGWF	387
<i>Homo sapiens</i>	419 VSGITKRDDAKYIYLLKFFIMETLKATKLDGVDVIIGYTAWSLDGFIEWRCYSIRRGFLY	478
<i>Macaca mulatta</i>	421 VSGITKRDDAKYIYLLKFFIMETLKATKLDGVDVIIGYTAWSLDGFIEWRCYSIRRGFLY	480
<i>Rattus norvegicus</i>	421 VSGITKRDDAKYIYLLKFFIMETLKATKLDGVDVIIGYTAWSLDGFIEWRCYSIRRGFLY	480
<i>Mus musculus</i>	421 VSGITKRDDAKYIYLLKFFIMETLKATKLDGVDVIIGYTAWSLDGFIEWRCYSIRRGFLY	480
<i>Gallus gallus</i>	401 VSGITKRDDAKYIYLLKFFIMETLKATKLDGVDVIIGYTAWSLDGFIEWRCYSIRRGFLY	460
<i>Xenopus tropicalis</i>	389 LSGITKRDDAKYIYLLKFFIMETLKATKLDGVDVIIGYTAWSLDGFIEWRCYSIRRGFLY	448
<i>Danio rerio</i>	388 GSGITKRDDAKYIYLLKFFIMETLKATKLDGVDVIIGYTAWSLDGFIEWRCYSIRRGFLY	447
	Glycosidase	
<i>Homo sapiens</i>	479 VDFLSQDKMLLPKSSALFYQKLIENGFPPLENQPLEGTFPCDFAWGVVDNYIQVDITL	538
<i>Macaca mulatta</i>	481 VDFLSQDKMLLPKSSALFYQKLIENGFPPLENQPLEGTFPCDFAWGVVDNYIQVDITL	540
<i>Rattus norvegicus</i>	481 VDFLSQDKMLLPKSSALFYQKLIENGFPPLENQPLEGTFPCDFAWGVVDNYIQVDITL	540
<i>Mus musculus</i>	481 VDFLSQDKMLLPKSSALFYQKLIENGFPPLENQPLEGTFPCDFAWGVVDNYIQVDITL	540
<i>Gallus gallus</i>	461 VDFLSQDKMLLPKSSALFYQKLIENGFPPLENQPLEGTFPCDFAWGVVDNYIQVDITL	520
<i>Xenopus tropicalis</i>	449 VDFLSQDKMLLPKSSALFYQKLIENGFPPLENQPLEGTFPCDFAWGVVDNYIQVDITL	508
<i>Danio rerio</i>	448 VDFLSQDKMLLPKSSALFYQKLIENGFPPLENQPLEGTFPCDFAWGVVDNYIQVDITL	507



Glycosidase			
Homo sapiens	539	SQFTIDLNVYLWDVHHSKRLLIKVDGVVTK--KRKSYCVDFAAITQPOITLLQEMHVTHERFS	596
Macaca mulatta	541	SQFTIDLNVYLWDVHHSKRLLIKVDGVVTK--KRKSYCVDFAAITQPOITLLQEMHVTHERFS	598
Rattus norvegicus	541	SQFTIDPNVYLWDVHHSKRLLIKVDGVVAK--KRKPYCVDFAIRPOITLLREMRVTHFRFS	598
Mus musculus	541	SQFTIDPNVYLWDVHHSKRLLIKVDGVVAK--KRKPYCVDFAIRPOITLLREMRVTHFRFS	598
Gallus gallus	521	AQFIDPNVYVWDVHQTKLLIKVDGVFTS--QRKHCVDFAAIRLQISLLQEMHVTHERFS	578
Xenopus tropicalis	509	SQFVDPNVYVWDMNKTGLTIKVEGITVP--KRKTQCVDFASIRQQLISMREIHITHFYFA	566
Danio rerio	508	TQFTIDPNVYWNISGNGLKKLPGLQAPHLRRTPHCADYGSIRQQVSDLLRMQVSHFHF	567
Homo sapiens	597	LDWALILPLGNQSQVNHTILQYYRCMASELVRVNITPPVVALWQPMAPNQGLPRLLARQGA	656
Macaca mulatta	599	LDWALILPLGNQSQVNHTILQYYRCMASELVRVNITPPVVALWQPMAPNQGLPRLLARQGA	658
Rattus norvegicus	599	LDWALILPLGNQSQVNHTILQYYRCMASELVRVNITPPVVALWQPMAPNQGLPRLLARQGA	658
Mus musculus	599	LDWALILPLGNQSQVNHTILQYYRCMASELVRVNITPPVVALWQPMAPNQGLPRLLARQGA	658
Gallus gallus	579	LKWSVSLPLGNLSLNIHTLVHYQCFASELLVRVNITPPVVALWQPMAPNQGLPRLLARQGA	638
Xenopus tropicalis	567	LKWAATLPLGNLSLIHHKVLHYQCFASELLVRVNITPPVVALWQPMAPNQGLPRLLARQGA	626
Danio rerio	568	LNWSSIVPTGHSVSDANETLLRYYCYFSELQKVNITPPVVALWQPMAPNQGLPRLLARQGA	627
Homo sapiens	657	WENPYTALAFAYEARLCFQELGHHYKLWITWNEPYTRNMTYSAGHLLKAHALAWHLYNE	716
Macaca mulatta	659	WENPYTALAFAYEARLCFQELGHHYKLWITWNEPYTRNMTYSAGHLLKAHALAWHLYNE	718
Rattus norvegicus	659	WENPHYALAFADYANLCFELGHHYKLWITWNEPTNRNMTYRAGHLLKAHALAWHLYDD	718
Mus musculus	659	WENPHYALAFADYANLCFELGHHYKLWITWNEPTNRNMTYRAGHLLKAHALAWHLYDD	718
Gallus gallus	639	WENSETVQAFVEYAKFCFASLGDHKKFWITWNEPSVKNLTYTAGHLLKAHALAWHLYDK	698
Xenopus tropicalis	627	WVNYHTVSAFVEYARLCFQELGHHYKLWITWNEPSNRNMTYAGHLLKAHALAWHLYDR	686
Danio rerio	628	WQSEKTVQAFVDYARLCFQELGHHYKLWITWNEPDEDELEYTVGHLLKAHALAWHLYDR	687
Homo sapiens	717	KFRHAQKGKISIALQADWIEPACPFQSKQKEVAERVLEFDIGWLAEPFIFGSGDYPVWVRD	776
Macaca mulatta	719	KFRHAQKGKISIALQADWIEPACPFQSKQKEVAERVLEFDIGWLAEPFIFGSGDYPVWVRD	778
Rattus norvegicus	719	KFRAAQKGKISIALQADWIEPACPFQSKQKEVAERVLEFDIGWLAEPFIFGSGDYPVWVRD	778
Mus musculus	719	KFRAAQKGKISIALQADWIEPACPFQSKQKEVAERVLEFDIGWLAEPFIFGSGDYPVWVRD	778
Gallus gallus	699	EFRRSQKGKISIALQADWIEPACPFQSKQKEVAERVLEFDIGWLAEPFIFGSGDYPVWVRD	758
Xenopus tropicalis	687	DFRKAQKGKISIALQADWIEPACPFQSKQKEVAERVLEFDIGWLAEPFIFGSGDYPVWVRD	746
Danio rerio	688	EFRKAQGGKASLVLHMDWIEPAFSEFNREDVAPADRVLDERGVGFAEPFIFGSGDYPVWVRD	747
Homo sapiens	777	WLNQRN-----NFLLPYFTEDEKKLIQGTDFLALSHYTTILVDSEKEDIPIKYNDYLEVQ	831
Macaca mulatta	779	WLNQRN-----NFLLPYFTEDEKKLIQGTDFLALSHYTTILVDSEKEDIPIKYNDYLEVQ	833
Rattus norvegicus	779	WLNQRN-----NFLLPYFTEDEKKLIQGTDFLALSHYTTILVDSEKEDIPIKYNDYLEVQ	833
Mus musculus	779	WLNQRN-----NFLLPYFTEDEKKLIQGTDFLALSHYTTILVDSEKEDIPIKYNDYLEVQ	833
Gallus gallus	759	WLHQRNVDLNFHLPSPFSEDEKKLIQGTDFLALSHYTTILVDSEKEDIPIKYNDYLEVQ	818
Xenopus tropicalis	747	WLAPRNLDVFEFLPSPFSEDEKKLIQGTDFLALSHYTTILVDSEKEDIPIKYNDYLEVQ	806
Danio rerio	748	WLQRNTIDLNFHLPSPFSEDEKKLIQGTDFLALSHYTTILVDSEKEDIPIKYNDYLEVQ	807
Homo sapiens	832	EMTDITWLN SPSQVA--VVPWGLRKVLNWLKFKYGDLP MYIISNGIDDGLHAEDDQLRVYY	890
Macaca mulatta	834	EMTDITWLN SPSQVA--VVPWGLRKVLNWLKFKYGDLP MYIISNGIDDGLHAEDDQLRVYY	892
Rattus norvegicus	834	EMTDITWLN SPSQVA--VVPWGLRKVLNWLKFKYGDLP MYIISNGIDDGLHAEDDQLRVYY	892
Mus musculus	834	EMTDITWLN SPSQVA--VVPWGLRKVLNWLKFKYGDLP MYIISNGIDDGLHAEDDQLRVYY	892
Gallus gallus	819	MISDITWLN SPSRAA--VVPWGLRKVLNWLKFKYGDLP MYIISNGIDDGLHAEDDQLRVYY	877
Xenopus tropicalis	807	FITDITWLN SPSKVA--VVPWGLRKVLNWLKFKYGDLP MYIISNGIDDGLHAEDDQLRVYY	865
Danio rerio	808	LISDITWLN SPSRNPVVPWGLRKVLNWLKFKYGDLP MYIISNGIDDGLHAEDDQLRVYY	867
Homo sapiens	891	MONYINEALKAHILDGINLCGYFAYSFNDRTPARFGLYRYAADQFEPKPSMKHYRKITDS	950
Macaca mulatta	893	MONYINEALKAHILDGINLCGYFAYSFNDRTPARFGLYRYAADQFEPKPSMKHYRKITDS	952
Rattus norvegicus	893	IKNYINEALKAYVLDGINLCGYFAYSFNDRTPARFGLYRYAADQFEPKPSMKHYRKITDS	952
Mus musculus	893	IKNYINEALKAYVLDGINLCGYFAYSFNDRTPARFGLYRYAADQFEPKPSMKHYRKITDS	952
Gallus gallus	878	IQNYINEALKAYALDNVNLGYFYYSFNDRTPARFGLYRYAADQFEPKPSMKHYRKITDS	937
Xenopus tropicalis	866	LQNYINEALKAILHDGINLCGYFAYSFNDRTPARFGLYRYAADQFEPKPSMKHYRKITDS	925
Danio rerio	868	LYNYINEALKAYMLDAVNLGYFAYSFNDRTPARFGLYRYAADQFEPKPSMKHYRKITDS	927
Membrane			
Homo sapiens	951	NGFPGPETLERFCPEEFTVCTECSFFHTRKSLAFIAFLFFASISLSLIFYYSKKGRRR	1010
Macaca mulatta	953	NGFPGPETLEKFCPEEFTVCTECSFFHTRKSLAFIAFLFFAFIVLSLIFYYSKKGRRR	1012
Rattus norvegicus	953	NGFLGSGTLGRFCPEEFTVCTCGGFFQTRKSLAFISFLVFAFVTSLSLIFYYSKKGRRR	1012
Mus musculus	953	NGFLGSGTLGRFCPEEFTVCTCGGFFQTRKSLAFISFLVFAFVTSLSLIFYYSKKGRRR	1012
Gallus gallus	938	NGFPGPDTAEVLCPEEAMCECHFFRTRKSLAFISFLVFAFIVTIFCIMIYSKRAERR	997
Xenopus tropicalis	926	NGFPNPEMPAVSCPVELVPCSDCHFFQTRKSLAFIAFLFFAFIVLSLIFYYSKKGRRR	985
Danio rerio	928	NGFPAPSTSQHQCPHAPAGSGG-RYVLTCKPVGFLSLVSSQMLITMCLVIYAFKRHL	986
Homo sapiens	1011	YK--	1012
Macaca mulatta	1013	YK--	1014
Rattus norvegicus	1013	YK--	1014
Mus musculus	1013	YK--	1014
Gallus gallus	998	YK--	999
Xenopus tropicalis	986	YK--	987
Danio rerio	987	TTKK	990

# Figures

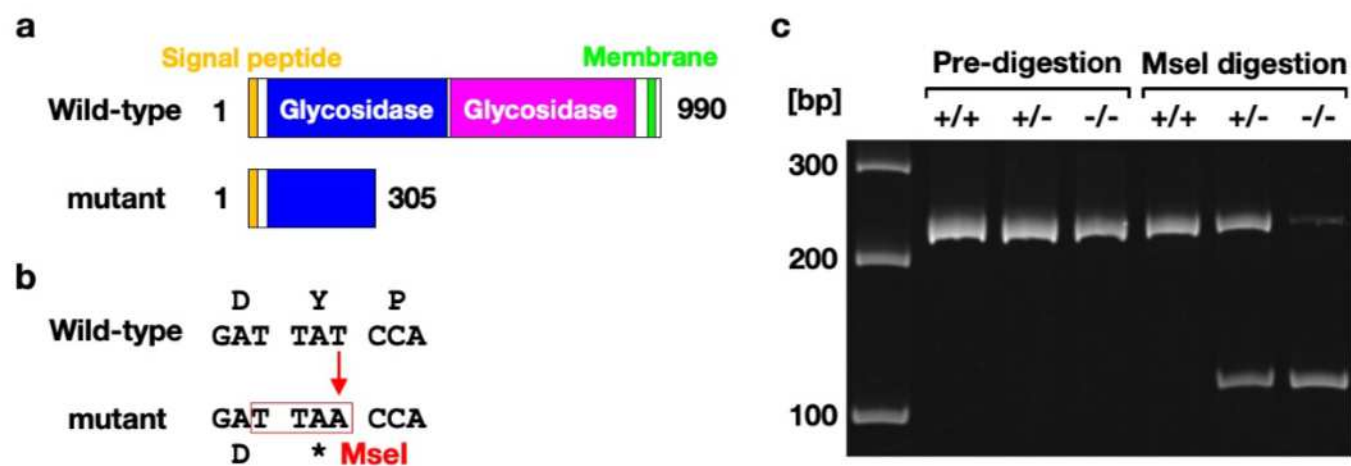
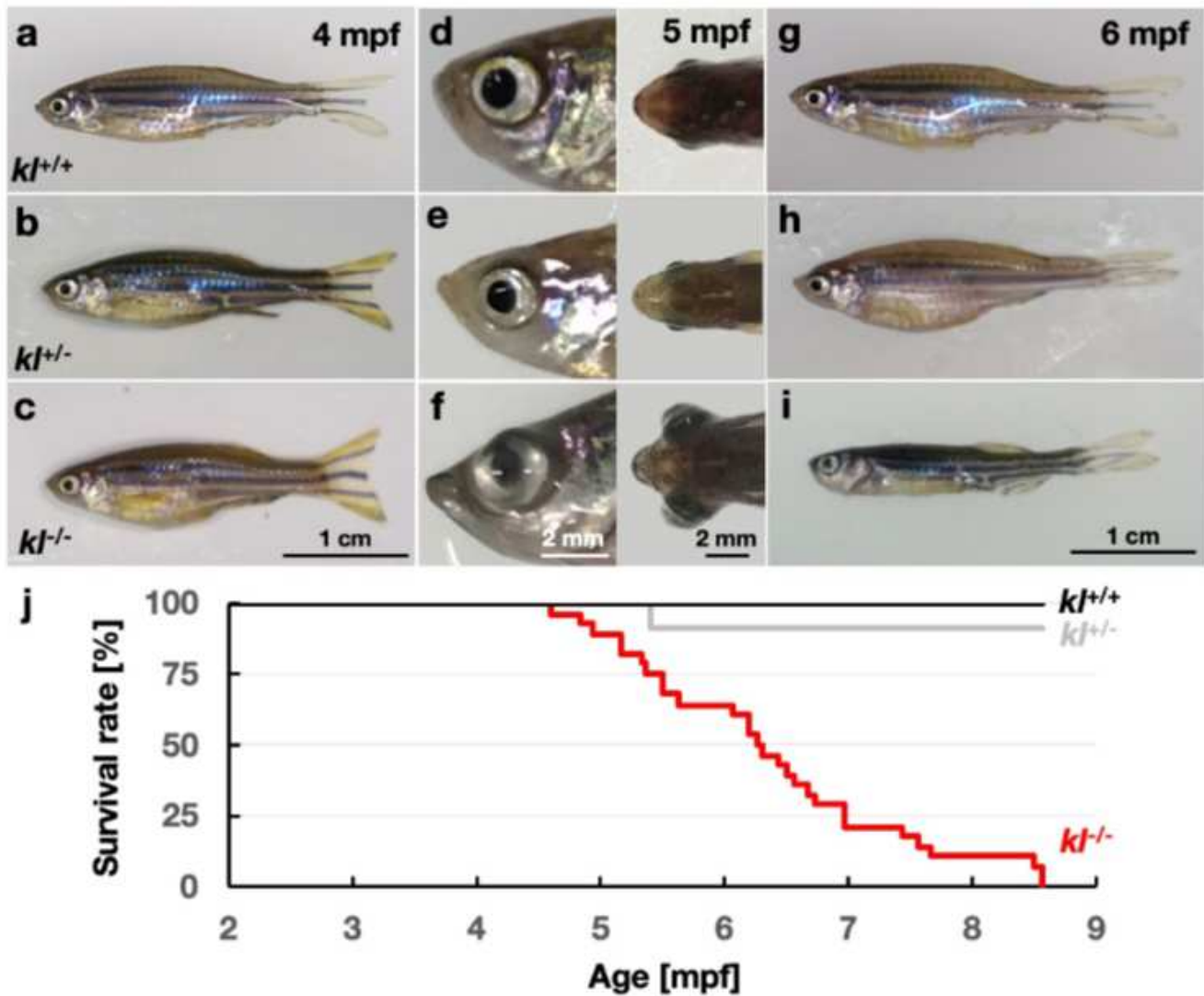


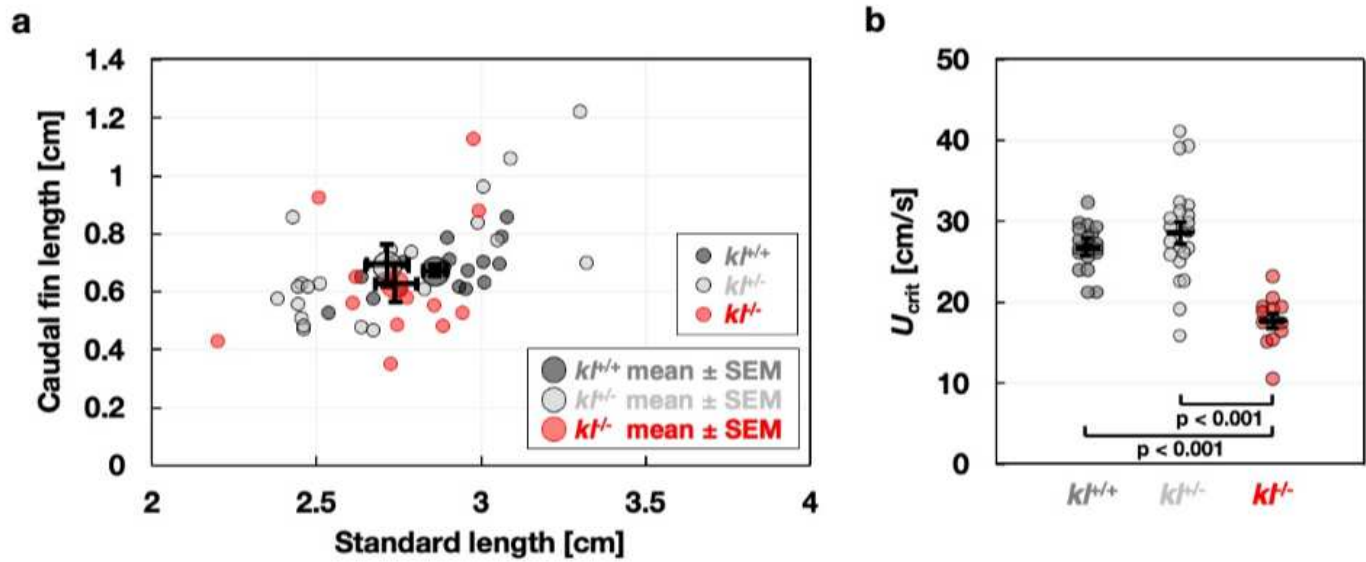
Figure 1

A nonsense mutation of zebrafish aklotho. a. The aklotho protein has a signal peptide, two glycosidase domains and a transmembrane domain. A nonsense mutation caused a truncation in the first glycosidase domain. b. The T-to-A mutation generated an MseI restriction enzyme site. c. The size of genomic PCR products was 240 bp. The PCR products amplified from mutant alleles were digested by MseI to generate two 120-bp fragments.



**Figure 2**

Zebrafish *aklotho* mutants show protruding eyes and short life span. a-c. The *kl*<sup>+/+</sup> (a), *kl*<sup>+/-</sup> (b) and *kl*<sup>-/-</sup> (c) zebrafish at 4 mpf. *kl*<sup>-/-</sup> fish showed no morphological defects at this stage. d-f. Lateral and dorsal views of *kl*<sup>+/+</sup> (d), *kl*<sup>+/-</sup> (e) and *kl*<sup>-/-</sup> (f) head at 5 mpf. Note that the eyeball is protruding to the outside in *kl*<sup>-/-</sup> fish. g-i. The *kl*<sup>+/+</sup> (g), *kl*<sup>+/-</sup> (h) and *kl*<sup>-/-</sup> (i) zebrafish at 6 mpf. *kl*<sup>-/-</sup> fish became skinny. g. The *kl*<sup>+/+</sup> (n = 32) and *kl*<sup>+/-</sup> fish (n = 86) kept surviving after 9 mpf. Most of the *kl*<sup>-/-</sup> fish (n = 28) underwent premature death at 5 ~ 8 mpf.



**Figure 3**

a. The standard length and caudal fin length of individual fish at 5 mpf. Note that both standard length and caudal fin length were comparable among  $kl^{+/+}$  ( $n = 19$ ),  $kl^{+/-}$  ( $n = 21$ ) and  $kl^{-/-}$  ( $n = 12$ ). b. The  $U_{crit}$  of  $kl^{+/+}$  ( $n = 19$ ),  $kl^{+/-}$  ( $n = 21$ ) and  $kl^{-/-}$  ( $n = 12$ ) zebrafish were measured by a swimmill.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [figS1.png](#)