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

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Loss of αklotho causes reduced motor ability and short lifespan in zebrafish

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Abstract

The *klotho* gene encodes a transmembrane protein αKlotho 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, αKlotho-deficient mice show accelerated aging and shortened life span in addition to dysregulation of serum phosphorus. However, physiological basis of aging-related function of αklotho and its generality in animals remain unclear. The αklotho-deficient vertebrate animals other than mice have been awaited as an alternative premature aging model. We here employed zebrafish in our αklotho study and revealed that αklotho 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 αklotho mutant zebrafish displayed reduced swimming performance before their survival declined. A recent study also reported a similar finding that αklotho-deficient zebrafish exhibited short life span and reduced spontaneous movements. Taken together, these results suggest that αKlotho 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 [1]. 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 [1]. 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 [2-5]. Zebrafish (*Danio rerio*), which is an emerging alternative vertebrate model, has also been used to study progressive deterioration of biological function in aging [6,7].

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

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

Results

Zebrafish *klotho* gene and *klotho* mutation

To study αklotho in zebrafish, we first retrieved gene information of zebrafish αklotho from NCBI database. An amino acid alignment of αklotho protein showed that αklotho 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 αklotho 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 αklotho is overall conserved among vertebrates.

To investigate physiological function of αklotho in zebrafish, we next obtained an αklotho mutant allele $kl^{sa18644}$, which was identified by a targeting induced local lesions in genomes (TILLING) project in zebrafish [17]. 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 ($kl^{+/-}$), we obtained homozygous *kl* mutants ($kl^{-/-}$). The $kl^{-/-}$ mutant embryos showed no apparent defects and grew up to become adults. The ratio of $kl^{-/-}$ 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 [16]. Although the skin of the previously reported *kl*-deficient zebrafish appeared to be pale at 5 mph [16], we did not see apparent reduction of skin tone in $kl^{-/-}$ compared to $kl^{+/+}$ or $kl^{+/-}$ 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 [16]. We also recapitulated that our $kl^{-/-}$ fish become thinner after 5 months of age and die within 9 months (Fig. 2g-i). To detail the short life span of $kl^{-/-}$ zebrafish, we monitored the survival of our $kl^{+/+}$, $kl^{+/-}$ and $kl^{-/-}$ fish from 2 mpf (Fig. 2j). We found that all of the $kl^{-/-}$ fish (n = 28) died between 4 and 9 mpf, whereas all $kl^{+/+}$ (n = 32) and most $kl^{+/-}$ (n = 86) fish kept living after 9 mpf. It has been reported that the mean life span of zebrafish is 42 months [18]. These results indicate that $kl^{-/-}$ zebrafish undergo premature aging and death, just like αklotho-deficient mice exhibit progeroid phenotypes [8].

Zebrafish *klotho* mutants show reduced motor ability

Since aging affects the integrity of motor system [19], we addressed whether motor ability is impaired in $kl^{-/-}$ zebrafish before they initiate to show premature aging. As swimming ability of adult zebrafish is governed by body size especially by caudal fin length [20], 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 [21]. Both standard length and caudal fin length at 4 mpf were comparable between $kl^{+/+}$ (standard length 2.86 ± 0.04 cm, caudal fin length 0.67 ± 0.02 cm; n = 19; Fig. 3a), $kl^{+/-}$ (standard length 2.71 ± 0.06 cm, caudal fin length 0.69 ± 0.07 cm; n = 21) and $kl^{-/-}$ (standard length 2.74 ± 0.06 cm; $P = 0.13$ for $kl^{+/+}$, $P = 0.79$ for $kl^{+/-}$; caudal fin length 0.63 ± 0.06 cm; $P = 0.53$ for $kl^{+/+}$, $P = 0.44$ for $kl^{+/-}$; n = 12). These data confirm that $kl^{-/-}$ fish are indistinguishable from $kl^{+/+}$ and $kl^{+/-}$ 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 [20]. 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 [22]. The U_{crit} of $kl^{-/-}$ zebrafish (17.6 ± 0.9 cm/s; n = 12; Fig. 3b) was significantly lower than that of $kl^{+/+}$ (26.7 ± 0.6 cm/s; $P < 0.001$; n = 19) and $kl^{+/-}$ fish (28.6 ± 1.3 cm/s; $P < 0.001$; n = 21). These results demonstrate that $kl^{-/-}$ 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 αklotho in zebrafish and found that $kl^{-/-}$ 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 [16]. Although age-related frailty and premature death in our $kl^{-/-}$ fish were recapitulations of a recent report, we uniquely examined swimming performance of $kl^{-/-}$ fish using a swimmill and demonstrated that $kl^{-/-}$ fish exhibit a compromised motor ability before they become thinner and undergo

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

Conservation of αklotho gene

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

Decline of motor integrity in αklotho-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 [29]. The $kl^{-/-}$ 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 [16]. On the other hand, our swimmill assay, which assesses motor performance in forced swimming, revealed that $kl^{-/-}$ fish exhibited lower U_{crit}, which is the maximum speed of fish swimming, compared to $kl^{+/+}$ and $kl^{+/-}$ fish at 4 mpf. Reduction of U_{crit} has been reported in aged zebrafish [30], implying that motor deterioration in $kl^{-/-}$

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 αklotho

Kuro-o and his colleagues have demonstrated that αklotho mutant mice show several age-related disorders such as increase of serum phosphorus, ectopic calcification and hypokinesis [8]. 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 αklotho is implicated in short life span [31]. In addition, a 13 years old girl who carries a homozygous H193R missense mutation in αklotho showed premature tumoral calcinosis with dural and carotid artery calcifications [32]. Thus, physiological symptoms linked to αklotho mutations appear to be common in mammals. Although pathological studies of αklotho deficiency has only been done in mice, human and *C. elegans* until recently, the latest publication of αklotho mutant zebrafish [16] and our current study demonstrated pathologically-relevant progressive motor deterioration and premature death in zebrafish $kl^{-/-}$ mutants. Collectively, zebrafish $kl^{-/-}$ 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 [33]. 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 αklotho mutant line ($kl^{sa18644}$) 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 ºC 30 sec, 35 cycles; 72 ºC 1 min; 4 ºC 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 [34]. 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 $kl^{+/-}$ 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 [20]. 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 [20].

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.

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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 αklotho.

a. The αklotho 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 αklotho mutants show protruding eyes and short life span.

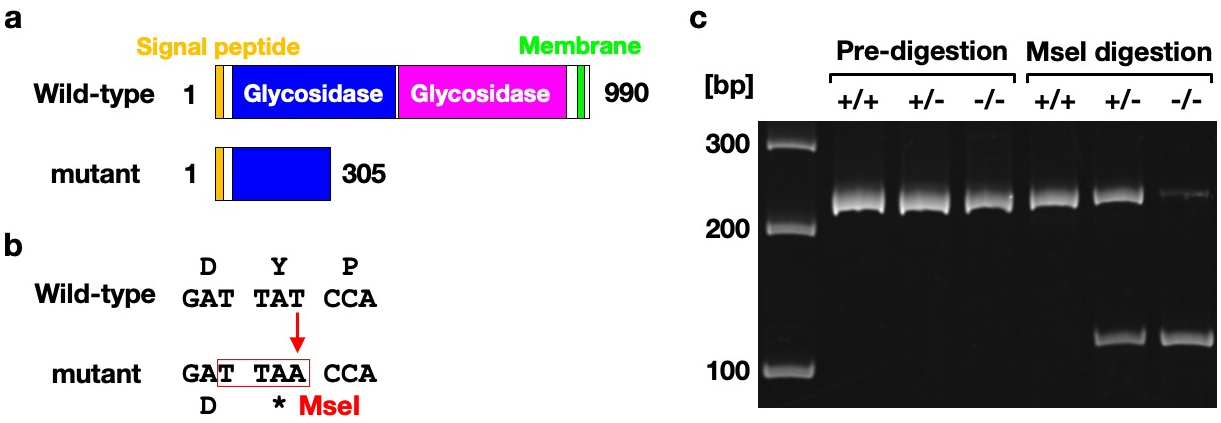
a-c. The $kl^{+/+}$ (a), $kl^{+/-}$ (b) and $kl^{-/-}$ (c) zebrafish at 4 mpf. $kl^{-/-}$ fish showed no morphological defects at this stage. **d-f.** Lateral and dorsal views of $kl^{+/+}$ (d), $kl^{+/-}$ (e) and $kl^{-/-}$ (f) head at 5 mpf. Note that the eyeball is protruding to the outside in $kl^{-/-}$ 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.

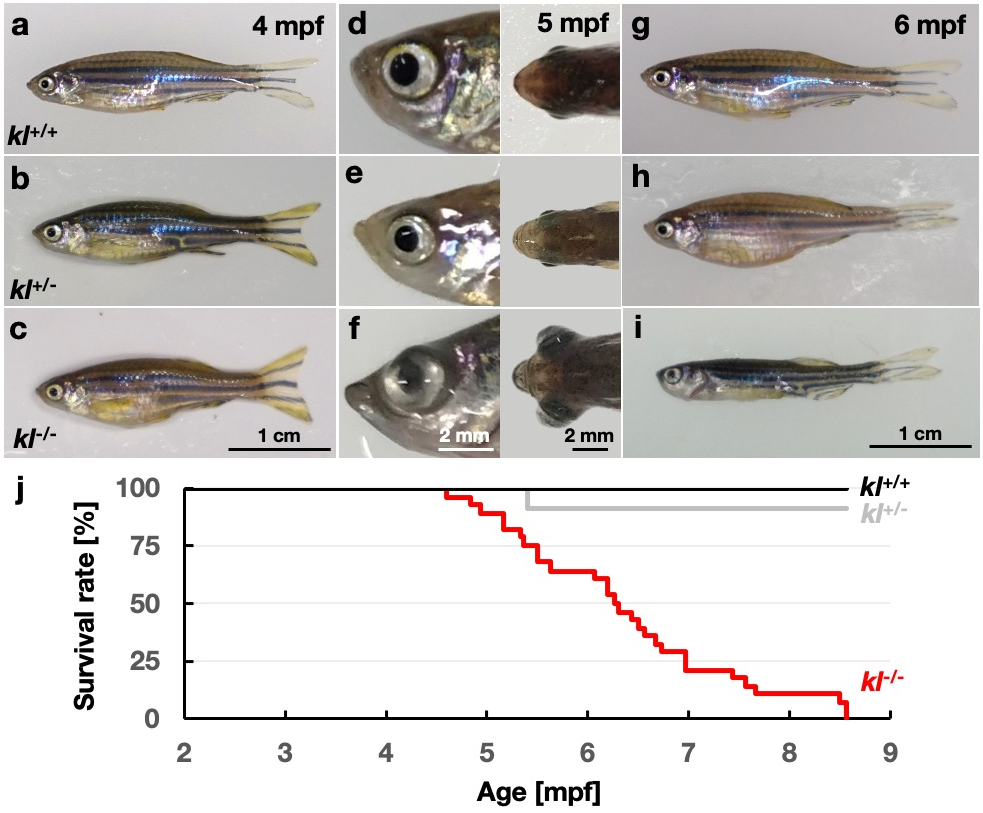
a. The standard length and caudal fin length of individual fish at 5 mpf. Note that both standard length and caudal fin length were comparable among $kl^{+/+}$ (n = 19), $kl^{+/-}$ (n = 21) and $kl^{-/-}$ (n = 12). **b.** The U_{crit} of $kl^{+/+}$ (n = 19), $kl^{+/-}$ (n = 21) and $kl^{-/-}$ (n = 12)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

Figure S1. The αKlotho is conserved in vertebrates.

Multiple amino acid sequence alignment of αKlotho 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 $kl^{sa18644}$ allele. Following NCBI data were used: human (*H. sapines*) 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



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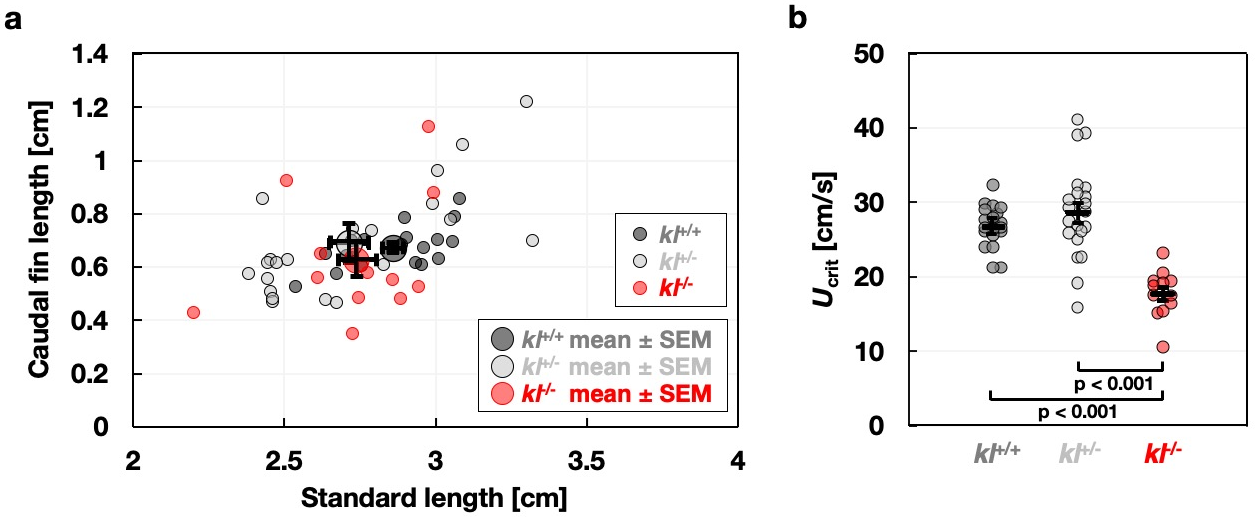

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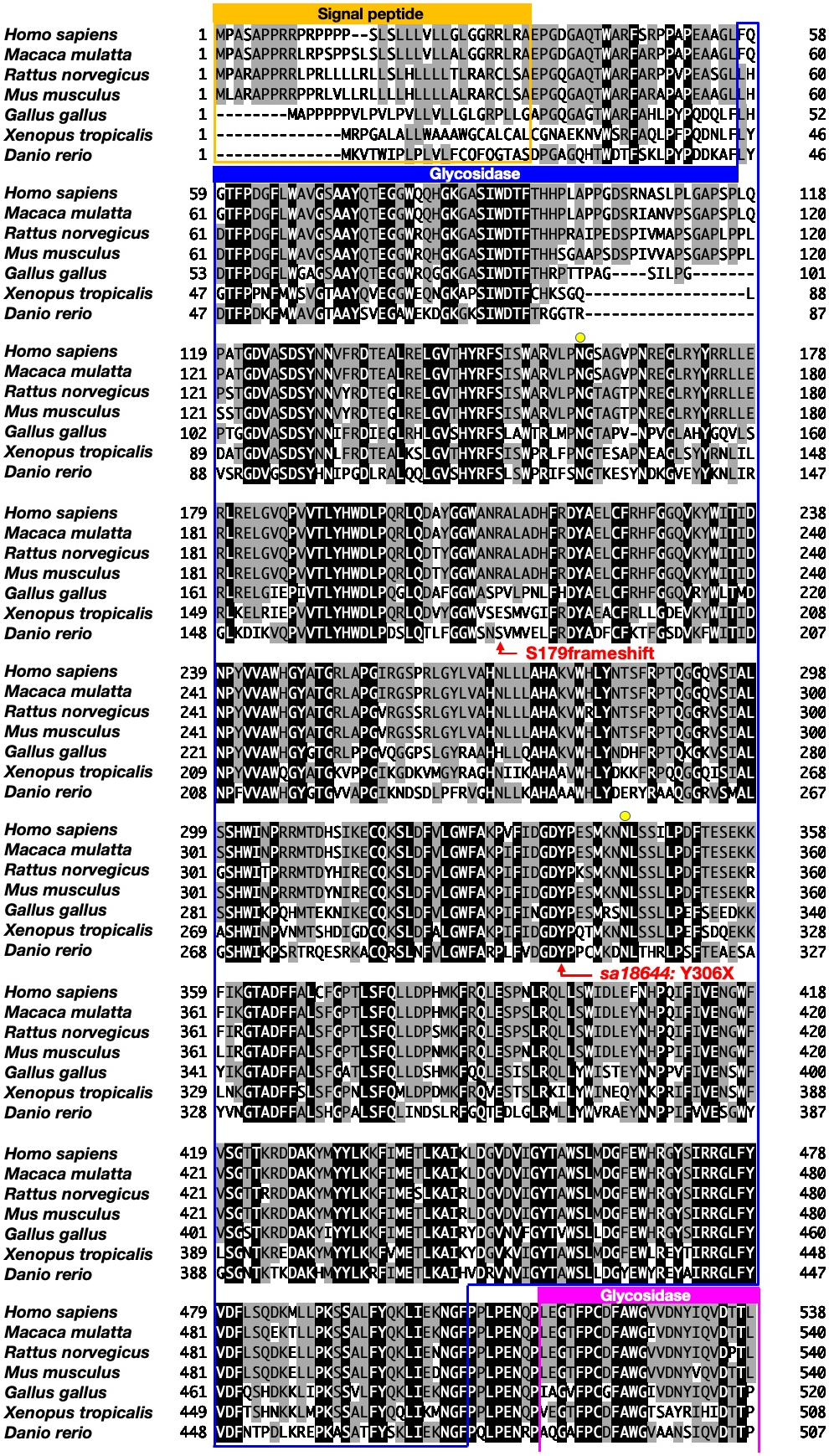
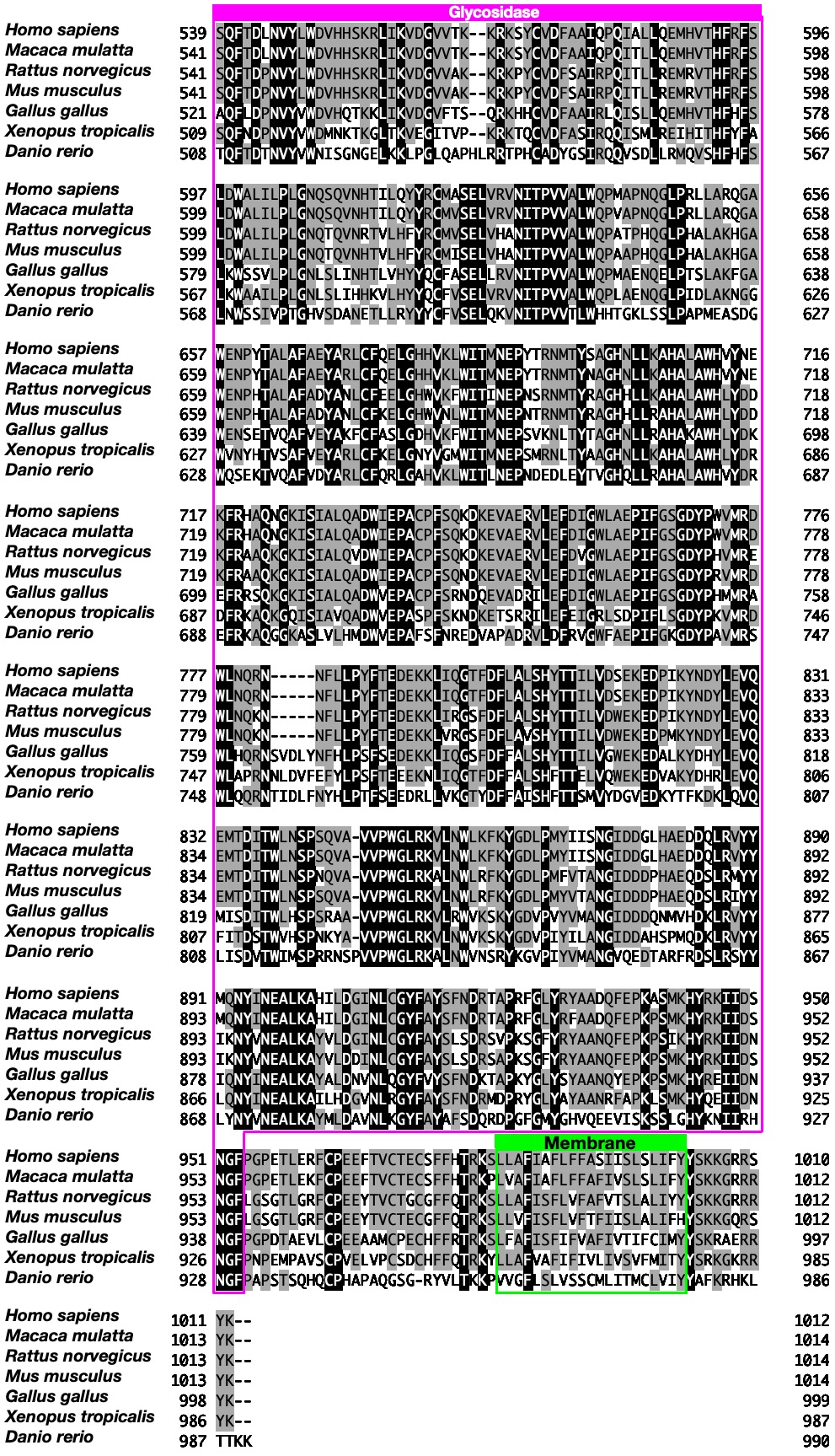


Ogura Y et al. Supplementary Fig. S1



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Figures

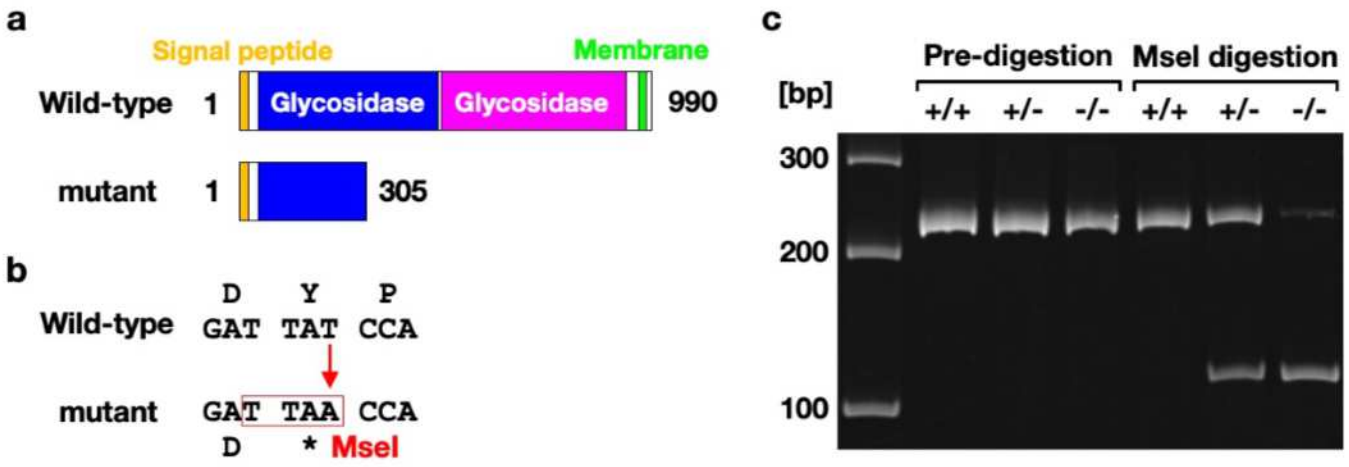

Figure 1

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.

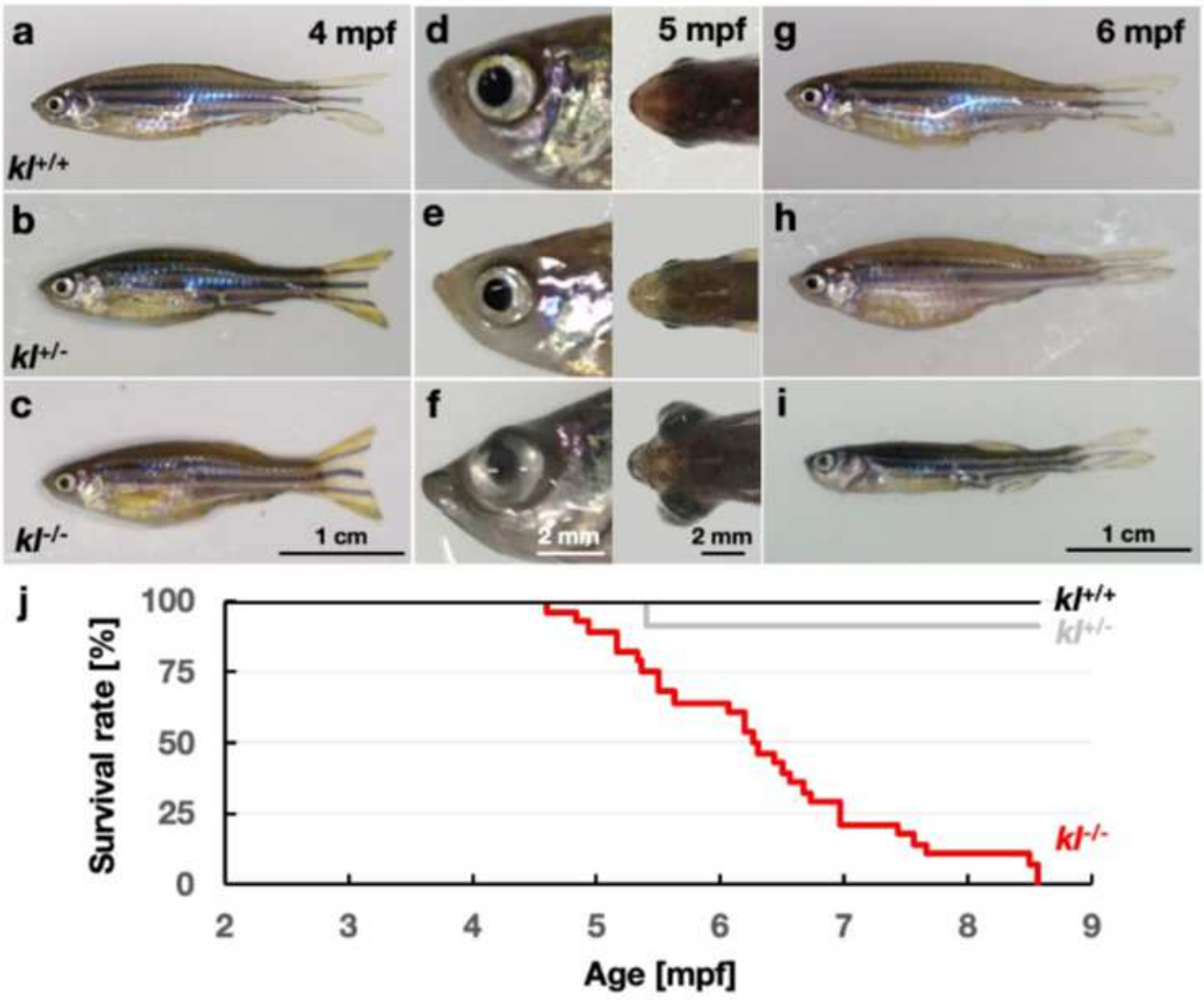

Figure 2

Zebrafish aklotho mutants show protruding eyes and short life span. a-c. The kl+/+ (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.

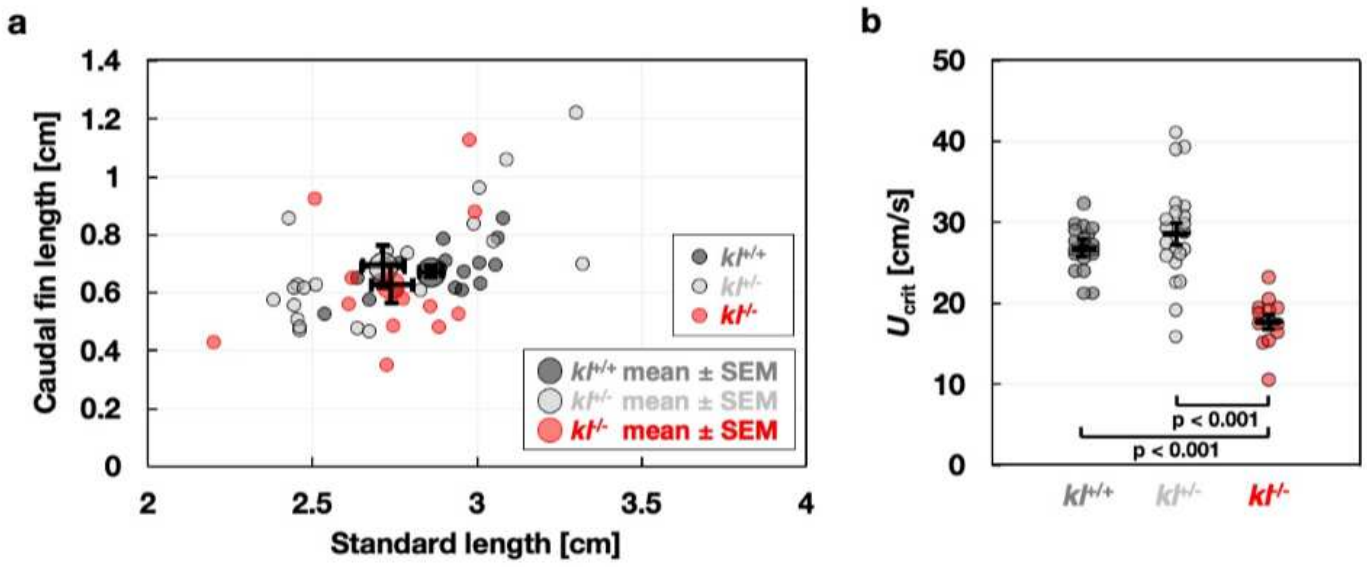

Figure 3

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 Ucrit 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