Identification of kit-ligand a as the Gene Responsible for the Medaka Pigment Cell Mutant few melanophore

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ABSTRACT The body coloration of animals is due to pigment cells derived from neural crest cells, which are multipotent and differentiate into diverse cell types. Medaka (Oryzias latipes) possesses four distinct types of pigment cells known as melanophores, xanthophores, iridophores, and leucophores. The few melanophore (fm) mutant of medaka is characterized by reduced numbers of melanophores and leucophores. We here identify kit-ligand a (kit-lga) as the gene whose mutation gives rise to the fm phenotype. This identification was confirmed by generation of kit-lga knockout medaka and the findings that these fish also manifest reduced numbers of melanophores and leucophores and fail to rescue the fm mutant phenotype. We also found that expression of sox5, pax7a, pax3a, and mitfa genes is down-regulated in both fm and kit-lga knockout medaka, implicating c-Kit signaling in regulation of the expression of these genes as well as the encoded transcription factors in pigment cell specification. Our results may provide insight into the pathogenesis of c-Kit-related pigmentation disorders such as piebaldism in humans, and our kit-lga knockout medaka may prove useful as a tool for drug screening.

The body coloration of animals is attributable to pigment cells in the skin that are derived from neural crest cells and which provide protection from ultraviolet light as well as play a role in sexual selection and mimesis. Whereas mammals and birds possess a single type of pigment cell known as a melanocyte, six types of pigment cells known as chromatophores—melanophores (black), xanthophores (yellow), iridophores (iridescent), erythrophores (red), cyanophores (blue), and leucophores (white)—have been identified in fish (Fujii 1993). Given that these pigment cells are all derived from neural crest cells and can be readily distinguished on the basis of their color, fish have been studied as model organisms for characterization of the mechanisms underlying regulation of cell fate determination in multipotent cells. Medaka (Oryzias latipes) possesses four of these chromatophore types: melanophores, xanthophores, iridophores, and leucophores (Takeuchi 1976; Kelsh et al. 1996; Kelsh et al. 2004).

Intermediate progenitors and key transcription factors required for fate specification in neural crest cells have been identified (Bhatt et al. 2013). Although the molecular mechanisms of melanophore differentiation in fish have been relatively well characterized, those underpinning the differentiation of other pigment cell types have remained largely unknown. Characterization of the molecular mechanisms responsible for abnormal body coloration is expected to provide insight into the development of chromatophores, with such mutants also being applicable to the screening of drugs and studies of regenerative medicine related to skin pigmentation disorders in humans. In fact, Fukamachi et al. reported that the solute carrier family 45 member 2 (slc45a2) gene, which is also called antigen isolated from immuno-selected melanoma 1 (aim-1), is mutated in b-locus mutants of medaka (Fukamachi et al. 2001).
This gene was later found to be mutated in human patients with oculocutaneous albinism type 4 (OCA4) and related to population differences in human skin color (Fukamachi et al. 2008).

Various medaka mutants with abnormal body coloration have been described (Tomita 1992), and causal genes for such mutants have been identified. Such medaka mutant collections provide an important resource for studies of the genetic basis of fate determination in neural crest cells. One such recessive mutant, few melanophore (fm), is characterized by reduced numbers of melanophores and leucophores (Kelsh et al. 2004). The causal gene for this mutant has remained unknown, but its identification would be expected to provide insight into the differentiation of these two pigment cell types.

The pax7a gene has been implicated in fate specification of a shared, partially restricted progenitor of the xanthophore and leucophore lineages in medaka (Kimura et al. 2014), and sox5 functions in a cell-autonomous manner to control the specification of xanthophores from the shared xanthophore-leucophore progenitor (Nagao et al. 2014). Therefore, we have here adopted genetic approaches to identify the relationship with causal gene and molecular mechanisms underlying the phenotype of fm medaka. We found that the fm locus includes a mutated version of the kit-ligand gene (kitlga, DK099743) and that expression of pax7a, sox5, pax3a, and miifga is down-regulated in both fm and kitlga knockout (KO) medaka. Our results thus suggest that the abnormal coloration of fm medaka is caused by disruption of the kitlga gene.

### MATERIALS AND METHODS

#### Medaka strains and maintenance

The fm strain (ID: MT48) of medaka has been described previously (Kelsh et al. 2004). The Sakyo strain (ID: WS1164) is normal with regard to the production of all four pigment cell types and was thus studied as the wild type (WT). The Kaga strain (ID: IB833) was used for crossing in genetic mapping. All medaka strains were obtained from the Laboratory of Fish Biology, Keio University, Japan. The strains were maintained in a recirculating system with a 14-h-light, 10-h-dark cycle at 28°C. Larvae and adult fish were anesthetized with tricaine mesylate, and embryos were also evaluated at stages 26, 30, and 36. The area of leucophores of each embryo at stage 30 and the area of individual melanophores at stage 36 were measured with Image J software (Schneider et al. 2012). Xanthophores were counted in larvae and on the scales of adult fish observed under ultraviolet light after treatment with 10µM melatonin for 10min and fixation with 10% paraformaldehyde-hyde. Iridophores were evaluated on the basis of iris luminosity and with the use of Image J software (Schneider et al. 2012).

#### Observation of pigment cells

Larvae and adult fish were anesthetized with tricaine mesylate, and dorsal body images were obtained at a constant magnification and resolution (1280 × 968 pixels) with a Leica MZ12.5 stereomicroscope or a Nikon SMZ225 microscope. For counting the number of melanophores, scales of adults were treated with epinephrine (2 mg/ml) to induce melanin aggregation. Melanophores and leucophores on the dorsal side of each larva were counted at 3 days posthatching (dph), and embryos were also evaluated at stages 26, 30 and 36. The total area of leucophores of each embryo at stage 30 and the area of individual melanophores at stage 36 were measured with Image J software.

#### Positional cloning of the gene mutated in fm medaka

Crossing of the F1 generation obtained by breeding the fm mutant with the Kaga strain yielded 156 F2 offspring with the fm phenotype and 30 siblings with the WT phenotype, which were collected and subjected to bulk segregation analysis with the M-marker 2009 system as described previously (Kimura and Naruse 2010). For further recombination analysis, polymorphic markers were isolated with reference to the medaka genome database (https://shigen.nig.ac.jp/medaka/). Detailed information on the markers, including primers for polymerase chain reaction (PCR) amplification and restriction enzymes for genotyping, is provided in Table 1.

#### 5'-RACE analysis

Body tissue of medaka at 3 dph was minced and then processed with a RNasey Minikit (Qiagen) for extraction of total RNA. The RNA was subjected to reverse transcription (RT) for 15 min at 37°C with the use of a PrimeScript RT Reagent Kit with gDNA Eraser (Takara), after which the reaction was terminated by incubation at 85°C for 5 s. The resulting cDNA was subjected to 5' rapid amplification of cDNA ends (5'-RACE) with the use of a GeneRACE Kit (Invitrogen) and with region-specific primers.

#### Generation of kitlga KO medaka

Gene targeting with the CRISPR/Cas9 system was performed as described previously (Ansai and Kinoshita 2014). The single guide RNAs (sgRNAs) were designed to target exons 2 and 4 of kitlga (see Figure 5A), and microinjection was performed with the Sakyo strain. Genomic DNA was purified from fin clips. For sequencing, exons 2 to 4 of kitlga were amplified with the use of the Ampdirect reagent and BIOTAQ HS DNA polymerase (Shimadzu). The PCR incubation protocol included an initial incubation at 95°C for 10 min; 35 cycles at 95°C for 10 s, 68°C for 30 s, and 72°C for 90 s; and a final incubation at 72°C for 10 min. The PCR products were separated by electrophoresis, purified with the use of a QIAquick Gel Extraction Kit (Qiagen), and sequenced. The PCR primers for amplification and sequencing were KLG ex2-F (5'-TGATCTTAGTCATGTTTTT-3') and Cas9 (5'-AGCAGCACAATGGAATTGCC-3').

#### Genotyping of fm medaka

Genomic DNA was extracted from fin clips that were prepared from anesthetized fish and fixed in 100% methanol. The samples were suspended in 100 µl of lysis buffer [20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 400 mM NaCl, 0.3% SDS, and proteinase K (10 mg/ml)],
incubated at 56°C for at least 2 h, and then stored at −80°C until analysis. They were subsequently applied directly to a PCR reaction mixture containing Ampdirect (Shimadzu). The PCR conditions for fm medaka included an initial incubation at 95°C for 10 min; 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s; and a final incubation at 72°C for 10 min. The primers were KLG ex2-F (5′- TGATCTTAGT- CATGTTTTT -3′), fm ex2-R (5′-TGTTGTCATTTACACGCACA- TCT-3′), KLG ex5-F (5′-GTGTTGTAACAGGCACGTG-3′), and KLG ex5-R (5′-ACTGTTGTGAGTGACTGTTGC-3′).

**RT and real-time PCR analysis**

Total RNA was extracted and subjected to RT as described for 5′-RACE. The resulting cDNA was subjected to real-time PCR analysis with the use of a Thermal Cycler Dice Real Time System (TAKARA BIO INC.). The PCR primers for medaka kitlga, pax7a, sox5, pax3a, and mitfa are listed in Table 2. The abundance of each target mRNA was normalized by that of elongation factor (EF)-1α mRNA as an invariant control.

**Phylogenetic analysis of kitlga genes in teleosts**

A phylogenetic tree for kitlga genes was generated by the maximum likelihood method on the basis of amino acid sequences listed in Supplemental Material, Table S1 and with the use of MEGA-X software (Kumar et al. 2018).

**Statistical analysis**

Quantitative data are presented as means ± 95% confidence interval and were compared among groups by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. A p value of <0.05 was considered statistically significant.

**Data availability**

fm strains are available from NBRP Medaka (https://shigen.nig.ac.jp/medaka/top/top.jsp). Sakyo strains are available from NBRP Medaka (https://shigen.nig.ac.jp/medaka/top/top.jsp). Kitlga KO strains are available from Keio University. Sequence data are available at GenBank; the accession numbers are listed in Table S1 and S2. Supplemental material available at figshare: https://doi.org/10.25387/g3.9906305.

**RESULTS**

**The fm medaka mutant has reduced numbers of melanophores and leucophores**

fm medaka is a spontaneous mutant discovered by Takahashi in 1965 and established by Tomita in 1971 (Tomita 1975). It was first described as having a reduced number of melanophores, but was later shown by Kelsh et al. (2004) to also be characterized by a reduced number of leucophores and an abnormal shape of melanophores (Kelsh et al. 2004). We first examined the numbers of melanophores, leucophores, and iridophores (determined by iris luminosity measurement) in embryos, larvae, and adults of WT, fm heterozygous (fm het), and fm medaka. Xanthophores were also examined in the scales of adult fish as well as on the lateral side of larvae examined under ultraviolet light.

Melanophores were apparent on the dorsal side and yolk sphere and leucophores were detected on the dorsal side of the head in WT embryos at stage 26 (Figure 1A). Iridophores could not be examined because eyes were not silver at this stage. In contrast to WT and fm het medaka at this stage, melanophores and leucophores were not found in the center of the head in fm embryos (Figure 1A–C). At stage 30, melanophores had spread throughout the lateral side of the back and their number had increased in WT and fm het medaka, whereas their number remained low in fm embryos (Figure 1D–F). The area of leucophore pigmentation was also narrower in fm medaka than in WT and fm het embryos (Figure 1D–F). At stage 36, many melanophores were visible throughout the entire body, with leucophores coexisting with melanophores along the back, of WT and fm het embryos (Figure 1G, H). The numbers of melanophores and leucophores in fm had remain lower than those in WT or fm het medaka (Figure 1G–I). Quantitative analysis revealed that the numbers of both melanophores and leucophores were significantly lower in fm embryos than in WT or fm het embryos from stages 26 to 36 (Figure 1J, K). It was difficult to count the number of leucophores at stage 30, but the total area of these cells was significantly lower in fm embryos than in WT or fm het embryos at this stage (Figure 1K).

Moreover, melanophores appeared smaller in fm medaka than in WT and fm het embryos at stage 36 (Figure 1L). The luminosity value of iridophores at stage 30 or 36 did not differ among the three genotypes (Figure 1M).

Examination of WT and fm het larvae at 3 dph revealed that most melanophores colocalized with leucophores on the dorsal side and in the head region (Figure 2A). In fm medaka, although the differentiation of all chromatophores was apparent, and melanophores and leucophores were also positioned at the dorsal midline, the melanophores appeared smaller than in WT and fm het larvae (Figure 2A). Furthermore, whereas the numbers of melanophores and leucophores had increased to ~25 in the dorsal midline of the trunk in WT and fm het larvae at 3 dph, those in the fm mutant remained significantly smaller (Figure 2B). There was no apparent difference in the number of xanthophores on the lateral side of larvae examined under ultraviolet light (Figure 2C). The luminosity value of iridophores in the iris of larvae at 3 dph also did not differ significantly among the three genotypes (Figure 2D, E).

The back of adult WT and fm het medaka appeared black as a result of the large number of melanophores, whereas the fm mutant appeared paler because of the continued reduction in melanophore number (Figure 3A). The numbers of melanophores and leucophores on scales were also larger for WT and fm het adults than for fm adults (Figure 3B, C). The number of xanthophores on scales did not differ significantly among adults of the three genotypes (Figure 3B, D). The luminosity value of iridophores in the iris was also similar for adults of all three genotypes (Figure 3E, F). As with embryos and larvae, there were no apparent differences in chromatophores between WT and fm het adults, consistent with the

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**Table 2 Primer sequences for quantitative RT-PCR**

| Gene     | Genebank accession No. | Primer sequence (5′-3′) forward | Primer sequence (5′-3′) reverse |
|----------|------------------------|---------------------------------|---------------------------------|
| kit-ligand | GFI001037170          | TGCCCATAGTGAATCTGGAACC          | ATTGCTCTGTGGCCACACTGC          |
| pax7a    | AB827303              | CGTITTTGAGGGCCCCGATTGTG         | TAGTTGCTCAGCTGGAAGG            |
| sox5     | EF577484              | TGGAGAGTAAACATGGCCACAC          | GGGCTTTCAGATTTCGATTAG          |
| pax3a    | GFI00100990           | CCACACCTGACTCTGACCTGTT          | TACGCGCCACACCTTTACAG           |
| mitfa    | JF489982              | CAATGTCAGTGTCGGACCA             | AGCTTCCCAGGATGTCGTCT           |
The fm locus contains the kitlga gene

To identify the fm locus, we adopted a positional cloning approach. Bulk segregation analysis with M-marker 2009 (Kimura and Naruse 2010) suggested that the fm locus was present in linkage group 6. Analysis of linkage among the fm locus and DNA markers in linkage group 6—including MID0602, MID0621, OLe1804f, MF01SSA036H12, and MF01SSA105H04 (Naruse et al. 2004)—confirmed that fm maps to chromosome 6 (Figure 4A).

We next focused on the c-Kit signaling pathway given that c-Kit receptor mutants of zebrafish and guppy (Kelsh et al. 2004; Kottler et al. 2013) show marked similarity to the fm medaka mutant. In particular,
the embryonic phenotype of the fm mutant, characterized by a reduced number and smaller size of melanophores, was found to be highly reminiscent of that of the zebrafish sparse/kit mutant (Kelsch et al. 2004). We searched the genomic regions of kita (kit receptor a) and kitb (kit receptor b) in the Ensembl database and found that kita is located on chromosome 4 and kitb on chromosome 1 of the medaka genome. An Ensembl-based search for the map position of the gene encoding Kit ligand (kitlg) revealed that this gene is located in scaffold121, which had not been mapped to a chromosome in MEDAKA1 (Ensembl release 93). The kitlg gene was subsequently mapped to the region spanning 2469132 to 2512640 bp of chromosome 6 (Ensembl genome assembly ASM223467v1). A second kitlg gene was also identified on chromosome 23, however. To examine the relation between these two kitlg genes, we constructed a phylogenetic tree of teleost kitlg genes based on amino acid sequences shown in Table S1. The kitlg gene on chromosome 6 of medaka was thus found to belong to the kitlg clade and that on chromosome 23 to the kitlgb clade (Figure S1). We therefore designated these two medaka kitlg genes as kitlg1 and kitlg2, respectively. We designed a kitlg1 gene marker and found that the gene maps to chromosome 6 between MID0602 and MID0621 and that there was no recombination between the kitlg1 gene marker and the fm phenotype (Figure 4A).

To determine whether the fm mutant harbors a mutation in kitlg1, we performed RT-PCR analysis. Such analysis revealed deletion of a portion of kitlg1 cDNA in the mutant (Figure 4B). Analysis by 5’-RACE identified a 475-bp deletion corresponding to skipping of exons 2 to 5 (Figure 4C). Sequencing of this genomic region of fm medaka revealed substitution of the 3.6-kb region encompassing exons 2 to 5 of the WT gene with a 3.9-kb sequence of unknown origin (Figure 4D). The medaka kitlg1 gene comprises 10 exons with a 756-bp open reading frame that encodes a 252–amino acid protein. The fm mutation results in the generation of an open reading frame for a truncated protein that lacks the stem cell factor (SCF) domain and would therefore be expected to be nonfunctional (Figure 4E). A BLASTX analysis of the genomic sequence of the mutated kitlg1 gene in fm medaka revealed that the insertion shows marked sequence similarity to the transposase encoded by the transposon Caenorhabditis briggsae 1 (Tcb1), which has been identified in the genomes of other fish species such as zebrafish and rainbow trout. Moreover, we found that this transposon-like sequence is also present at >100 additional regions of the current Ensembl genome assembly (for medaka ASM223467v1). These results suggested that the phenotype of fm medaka is attributable to insertion of a Tcb1-like transposon at the kitlg1 locus.

CRISPR/Cas9–mediated knockout of the kitlg1 gene induces an fm-like phenotype

To confirm kitlg1 as the causal gene of the fm mutant, we generated kitlg1 KO medaka with the use of the CRISPR/Cas9 system and sgRNAs targeted to the splice donor sites of exons 2 and 4 (Ansai and Kinoshiita 2014). Microinjection of one cell–stage WT embryos resulted in the generation of some larvae with reduced numbers of melanophores and leucophores at 3 dph, a phenotype similar to that of the fm mutant. Control embryos injected with only sgRNA or Cas9 mRNA failed to give rise to larvae that mimicked the fm phenotype. We outcrossed the kitlg1 G0 medaka with WT fish to obtain F1 medaka, sequence analysis of which revealed that the CRISPR/Cas9 system
induced a 9-bp deletion in exon 4 of Kitlga that altered the amino acid sequence of the encoded protein (Figure 5A, B). We then generated homozygous Kitlga KO medaka, which again manifested a phenotype indistinguishable from that of the fm mutant (Figure 5C, D). Given that the Kitlga KO medaka were viable and fertile, we performed a complementation test to further verify that Kitlga is the causal gene of the fm mutant. We obtained a total of 30 embryos from a cross between Kitlga KO medaka and the fm mutant, with all larvae showing the same

with a 3.9-kb sequence (green line) in the fm genome. (E) The WT Kitlga gene encodes a 252-amino acid protein containing an SCF domain (red box). The fm mutation is predicted to result in the generation of a truncated protein, with the gray box representing the altered frame.
phenotype as the fm mutant (Figure 5C) characterized by reduced numbers of melanophores and leucophores (Figure 5D). Together, these results thus indicated that mutation of the kitlga gene is responsible for the fm phenotype of medaka.

Expression of pax3a, pax7a, sox5, and mitfa is down-regulated in fm medaka

The pax7a gene is expressed in neural crest cells of medaka and functions as a molecular switch for the differentiation of multipotent progenitor cells into either xanthophores and leucophores or iridophores and melanophores, whereas the sox5 gene is expressed in differentiating xanthophores and functions as a molecular switch in the specification of xanthophores vs. leucophores (Kimura et al. 2014) (Nagao et al. 2014). Sox5 belongs to the SOXD group of proteins and also plays a role in formation of the cephalic neural crest (Perez-Alcala et al. 2004). Pax3 and Pax7 are closely related transcription factors of the Pax family that manifest similar DNA binding activity in vitro, and Pax3 regulates the promoter of the Mitf (mouse microphthalmia-associated transcription factor) gene (Schafer et al. 1994; Lacosta et al. 2005). To examine the molecular mechanisms underlying the reduction in the numbers of melanophores and leucophores in fm and kitlga KO medaka, we therefore determined the expression levels of pax3a, sox5, pax3a, and mitfa. RT and real-time PCR analysis revealed that the expression of each of these four genes was down-regulated in fm and kitlga KO larvae relative to WT larvae (Figure S2), suggesting that such down-regulation may contribute to the mutant phenotype.

DISCUSSION

We have here identified kitlga as the gene responsible for the fm mutant of medaka, which is characterized by reduced numbers of melanophores and leucophores in embryos, larvae, and adult fish. Moreover, genomic PCR, RT-PCR, and 5’-RACE analyses revealed that the fm mutation is a deletion of exons 2 to 5 of kitlga and their replacement with a transposon-like sequence, which likely gives rise to a null allele of kitlga. Larvae of kitlga KO medaka established with the CRISPR/Cas9 system also manifested reduced numbers of melanophores and leucophores, and the progeny of a cross between fm and kitlga KO fish is similar to that of the fm mutant, indicating that the kitlga mutant could not rescue the fm phenotype.

Kit ligand, also known as stem cell factor (SCF), plays a key role in melanogenesis, gametogenesis, and hematogenesis in mammals (Copeland et al. 1990; Geissler et al. 1991). Homozygous mutation of the mouse Kitl results in embryonic death due to severe macrocytic anemia, whereas heterozygous mutant animals are viable but manifest a wide spectrum of abnormalities including a variable extent of macrocytic anemia, a reduced number of mast cells, and reduced pigmentation including white spotting or a gray color of fur (Sarvella and Russell 1956; Broudy 1997). In mouse melanogenesis, melanoblasts
are specified from neural crest cells, with Mitf and Kit being the earliest known markers for melanoblasts. After their differentiation, melanoblasts migrate dorsolaterally through the dermis between the somites and the developing epidermis from embryonic day 10.5 (Mort et al. 2015). Both fm mutant and our homozygous kitlga KO medaka are viable and manifest reduced numbers of melanophores and leucophores. This phenotype is thus similar to that of the heterozygous Kitl mutant mice, which have a reduced number of melanocytes.

Zebrafish has two kitlga genes, kitlga and kitlgb, with the former, but not the latter, playing a key role in the survival and migration of melanophores (Hultman et al. 2007). A zebrafish kitlga null mutant is viable and manifests a reduced number of melanophores, similar to our kitlga KO medaka. We found that medaka also harbors kitlga and kitlgb genes and that kitlga is the causal gene of the fm mutant, indicating that medaka kitlga is likely equivalent to zebrafish kitlga.

Tcb1 belongs to the Tc family of transposons and has been identified in nematodes and fruit flies. Transposons of the Tc family are ~1.6 kb in size, are associated with a TA repeat sequence, and contain a DDE motif in the open reading frame encoding the transposase (Harris et al. 1990; Hoekstra et al. 1999). Their inverted terminal repeats (ITRs) comprise 20 to 400 bp and contain CAGT at the 5′ end (Rosenzweig et al. 1983; Harris et al. 1988). The inserted sequence found in kitlga of fm medaka is similar to a Tcb1-like sequence found in other fish species. However, no ITR was associated with the Tcb1-like sequences detected in fm or WT medaka. Furthermore, this transposon-like sequence of medaka contains stop codons, indicating that the kitlga product in the fm mutant does not function like that in WT medaka. With the use of the dot plot program “dotmatcher” (http://www.bionformatics.nl/cgi-bin/emboss/dotmatcher), we also did not find any other inverted repeats on either side of the inserted sequence in fm medaka, suggesting that the transposase is not active and that the insertion arose as the result of a “cut and paste” type mechanism.

c-Kit signaling activates the expression of Mitf via the Ras-Raf-Mek-Mapk and mechanistic target of rapamycin (mTOR) pathways in mammals, and Mitf promotes transcription of the gene for tyrosinase, which is the rate-limiting enzyme of melanin production (Ronnstrand 2004; Dahl et al. 2016; Franceschi et al. 2017). Mice with white spotting also harbor heterozygous loss-of-function mutations in the c-Kit gene (Geissler et al. 1991). We did not detect tumors or organ abnormalities in either fm or kitlga KO medaka. Although the mechanistic insights gained by our results are also limited, the present study allows us to propose mechanistic hypothesis which will be verified in the future studies. Moreover, given that, as in the present study, changes in body coloration induced by drugs or genetic manipulation are readily detected in embryos or larvae of medaka within a couple of days, our kitlga KO medaka may prove useful as a tool for screening of drugs for conditions related to loss of c-Kit signaling.

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