Mouse hair significantly lightened through replacement of the cysteine residue in the N-terminal domain of Mc1r using the CRISPR/Cas9 system

Hitoshi Suzuki[1*], Gohta Kinoshita[2], Takeru Tsunoi[3], Mitsuki Noju[3], and Kimi Araki[4,]

[1]Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810, Japan

[2]Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

[3]Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

[4]Division of Developmental Genetics, Institute of Resource Development and Analysis, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan.

*Address correspondence to H. Suzuki at the address above, or e-mail: htsuzuki@ees.hokudai.ac.jp

Running head: Genome-edited mouse *Mc1r*

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Abstract

A loss-of-function mutation in the *melanocortin 1 receptor* gene (*MC1R*), which switches off the eumelanin production, causes yellowish coat color variants in mammals. In a wild population of sables (*Martes zibellina*) in Hokkaido, Japan, the mutation responsible for a bright yellow coat color variant was inferred to be a cysteine replacement at codon 35 of the N-terminal extracellular domain of the Mc1r receptor. In the present study, we validated these findings by applying genome editing on *Mc1r* in mouse strains C3H/HeJ and C57BL/6N, altering the codon for cysteine (Cys33Phe). The resulting single amino acid substitution (Cys33Phe) and unintentionally generated frameshift mutations yielded a color variant exhibiting substantially brighter body color, indicating that the Cys35 replacement produced sufficient MC1R loss of function to confirm that this mutation is responsible for producing the Hokkaido sable yellow color variant. Notably, the yellowish mutant mouse phenotype exhibited brown coloration in subapical hair on the dorsal side in both the C3H/HeJ and C57BL/6N strains, despite the inability of the latter to produce the agouti signaling protein (*Asip*). This darker hair and body coloration was not apparent in the Hokkaido sable variant, implying the presence of an additional genetic system shaping yellowish hair variability.

Introduction

Body color has significant ecological and evolutionary meaning in animals (Rosenblum et al. 2004). In vertebrates, a variety of loci are involved in pigmentation; among these, the melanocortin 1 receptor gene (*Mc1r*) and agouti signaling protein (*Asip*) contribute to the determination of pigmentation patterns (Västermark and Schiöth 2011) and play major roles in evolutionary body color changes (Miller et al. 1995; Hoekstra 2006; Cal et al. 2017). The products of these genes are the receptor and its antagonist, respectively, which regulate pigment production in melanocytes (Rees 2000; Abdel-Malek 2001). The *Mc1r–Asip* system functions to create banding patterns on a single hair, termed an agouti pattern. Eumelanin production is generally more intense on the dorsal side of an animal than on the ventral side (Caro and Mallarino 2020), resulting in a contrasting body pattern often called countershading (Ruxton et al. 2004). The molecular system responsible for these patterns lies in the *Asip* promoter regions (Vrieling et al. 1994; Linnen et al. 2013).

Mutations on *MC1R* are generally not lethal (Enshell-Seijffers et al. 2010). Gain-of-function *Mc1r* mutations are commonly found in the wild populations and domesticated animals and lead to melanistic phenotype through by single amino acid replacements (Våge et al. 1997; Theron et al. 2001; Kambe et al. 2011) and short amino acid segment deletions (Eizirik et al. 2003; Vidal et al. 2010). Loss-of-function *MC1R* mutations are known to cause yellowish color variants in wild and domestic populations of a number of species such as seals (Peters et al. 2016) and dogs (Everts et al. 2000; Newton et al. 2000; Schmutz et al. 2002). In a recent study, *MC1R* deletion mutant

rabbits created by the CRISPR/Cas9 system developed pale yellow coat color (Xiao et al. 2019).

The bright yellow variant of the sable *Martes zibellina* from Hokkaido, Japan is an example of a pale colored phenotype possibly caused by *MC1R* mutation in a wild population (Hosoda et al. 2005; Ishida et al. 2013; Suzuki 2013). While the yellow sable was found to have two substitutions (Cys35Phe and Asn68Ser) compared to the wild-type individuals (Hosoda et al. 2005), population analyses suggest that replacement of the cysteine residue at codon 35 of the first extracellular domain (Cys35Phe) of MC1R may be responsible for this variant (Ishida et al. 2013).

In this study, we applied genome editing techniques to investigate whether a single amino acid replacement of the cysteine residue in the N-terminal domain of Mc1r is sufficient to cause the yellow hair color variant. We also explored the possibility of contrasting color patterns in mice lacking functional Mc1r and Asip genes.

Materials and Methods

Animal experimental procedures

All animal experiments were approved by the Kumamoto University Ethics Committee for Animal Experiments and the Kumamoto University Recombinant DNA Advisory Committee for the generation of *Mc1r* knockout mice. C3H/HeJ and C57BL/6N mice were purchased from Crea-Japan, Tokyo, Japan and housed in a climate-controlled room under a 12-h light/dark cycle. We generated knockout mice by introducing Cas9 protein (317-08441; NIPPON GENE, Toyama, Japan), tracrRNA (GE-002; FASMAC,

Kanagawa, Japan), synthetic CRISPR RNA (FASMAC), and a single-strand oligodeoxy nucleotide (ssODN) into fertilized eggs by electroporation, as previously described (Takemoto et al. 2020). For replacement of the targeted cysteine residue at codon 33 of mouse *Mc1r*, we designed CRISPR RNA [ATGGACACATACAGGCACCA (AGG)] and ssODN (CTAGGCTGAGGAAGAGGCCATCTGGGATGGACACATACAGGAACCAAGGCT CTGACTGGTTGGTGGCCAGTCCAAGGTGAGAGG).

F_1 mice were generated by breeding G_0 mice with markedly lighter coat colors, and were subsequently used to generate homozygous F_2 mice.

Genotyping of Mc1r

We determined the nucleotide sequence, targeting the 5' half of the *Mc1r* coding region. Polymerase chain reaction (PCR) was performed using the AmpliTaq Gold 360 DNA Polymerase Kit (Invitrogen, Carlsbad, CA, USA) as previously described (Shimada et al. 2009). We used the primers 5'Mc1r-52 (5'-GCTCATACCACCTGGAGCTGCAGCC-3') and 3'Mc1r+504 (5'-AAGAGGGTGCTGGAGACGATGC TGACC-3').

Measurement of coat color variation

We quantified hair color variation using a spectrophotometer (CM-700d, Konica Minolta Co., Osaka, Japan) with a 3-mm diameter window and the Specular Component

Included option. We used F_2 mice for phenotype characterization to eliminate the mosaic phenotype, which is expected to occur in G_0 mice. For this purpose, we used the CIE L*a*b* colorimetry model, where L* is luminosity from black to white [0, 100], a* encodes the initial color from green to red [–60, 60], and b* encodes the initial color from blue to yellow [–60, 60]. Dorsal and ventral colors were quantified using 10 independent measurements in areas along the midline; we used average values after excluding outlier measurements in which at least one of the quantified values (L*, a*, or b*) exceeded 1.5 times the interquartile range.

The R software (R Development Core Team, 2008) was used to plot dorsal (x-axis) and ventral (y-axis) color values and to perform principal component analysis (PCA). The R 'prcomp' function and the 'ggbiplot' package were used to combine the dorsal and ventral measurement datasets.

Results

Variant Mc1r sequences in genome-edited mice

To test whether single amino acid replacement of Cys35 was sufficient for inducing total dysfunction of Mc1r via the CRIPR/Cas9 system, we targeted the cysteine residue at codon 33 (corresponding to codon 35 in other mammals) in the mouse strains used in this study (Figure 1a). G_0 mice (n = 53) generated in the C3H/HeJ genome-editing experiment showed variable coat color patterns among and within individuals, indicative of appearance of mosaicism (Supplementary Figure S1). Several G_0 mice showed markedly lighter coat color; this phenotype was designated as the yellowish

phenotype in contrast to wild-type (WT) mice, which had the original coat color.

We explored genotyping of *Mc1r* (Supplementary Table S1) and detected the c.98G>T mutation at the target site and a number of deletion and insertion mutations nearby the target site, including one or more nucleotide (1, 2, 3, and 6 bp) deletions (Figure 1c). Homozygous c.98G>T mice or a combination of c.98G>T and another mutation predicted to cause frameshift mutations had the yellowish phenotype. A non-frameshift mutation caused by the 6-bp deletion, which was expected to lose Cys33, was also found to induce the yellowish phenotype. By contrast, the 3-bp deletion, which resulted in the removal of Trp32 prior to the Cys33 target codon, exhibited the WT phenotype.

These results were reproduced in the C57BL/6N experiment, (Supplementary Table S2, Supplementary Figure S2). G_0 mice (n = 28) exhibited a variety of coat color patters ranging from the yellowish phenotype and a melanistic phenotype caused by the parental strain with the *Asip* null allele (Slominski et al. 2005).

We then conducted breeding experiments using representative G_0 c.98G>T, 1-bp deletion, and 2-bp deletion mice of the homozygous C3H/HeJ background. We genotyped 36 F_2 mice with the WT (n = 29) and yellowish (n = 7) phenotypes and found that their coat color phenotypes were those predicted based on the *Mc1r* genotypes. This trend was reproduced in c.98G>T F_2 mice of the B57BL/6N background, as observed among G_0 mice.

Hair color phenotypes among Mc1r mutant mice

We examined the hair color of the yellowish (Figure 2a) and WT phenotypes (Figure 2b) in Mc1r genotyped F_2 mice of the C3H/HeJ background. Hair on both the dorsal and ventral sides exhibited the typical agouti pattern, characterized by black hair color and subapical yellow bands in the WT (Figure 2c, d). In the yellowish phenotype, dorsal hair was creamy pale yellow with darkened tips (Figure 2e) and ventral hair was creamy pale yellow without any apparent banding pattern (Figure 2f).

We quantified hair from both coat color phenotypes in F_2 mice of the C3H/HeJ background (n = 14) using a spectrophotometer. The results showed that lightness (L*) differed substantially between the homozygous or heterozygous WT allele sequence (Cys33) and mice homozygous for c.98G>T (Cys33Phe), and 1-bp and 2-bp deletions in both dorsal and ventral body parts (Figure 3). The results indicated similar trends, particularly in L*, between mice with the homozygous c.98G>T (Phe33) genotype and frameshift mutations that altered the codon sequence including Cys33 (Supplementary Figure S3).

Discussion

The replacement or deletion of the cysteine residue (Cys35) in the extracellular N-terminus of MC1R has been suggested to be a significant driver of bright yellow coat color in sables (Hosoda et al. 2005; Ishida et al. 2013). In the present study, we replaced Cys33 of the first extracellular domain of the murine receptor Mc1r using the CRSPR-Cas9 system. Our results reveal that a single amino acid replacement is sufficient to cause a drastic change in coat color phenotype from WT to the yellowish

phenotype, and that this property is shared in mice with frameshift mutations (Figures 2, 3). These findings indicate that functional efficiency of the Cys33 replacement or deletion in mouse Mc1r is comparable to a null mutation. This cysteine residue is perfectly conserved in the N-terminus of all melanocortin receptors (Rana et al. 1999; Garcia-Borron et al. 2005) and plays an essential role in guiding MC1R to the plasma membrane, where Cys35 is thought to stabilize the MC1R receptor by mediating dimerization (Zanna et al. 2008). Cys35 substitution has also been reported in humans with red hair (Fargnoli et al. 2003).

Substitution or deletion of Cys33 of the Mc1r receptor markedly enhanced the lightness of coat color in mice, as also observed in the Hokkaido sables. However, an interesting difference between the yellowish-phenotype mice and sables is that the yellow hair of the mutant mice had dark brown tips, whereas the variant Hokkaido sables had bright yellow hair on both the dorsal and ventral sides (see photograph in Suzuki 2013). Such dark coloration, which was also reported in yellow-haired mice with a frameshift mutation and stop codon (Robbins et al. 1993), is known as “incomplete yellowing”, suggesting the ability to produce eumelanin in the absence of the MC1R signaling system (Bennett and Lamoreux 2003). Accordingly, several alternative signaling systems have been reported to cause adenylyl cyclase activity on the cell membrane of melanocytes to produce cAMP, possibly regulating melanogenesis independently of MC1R by acting directly on melanosomes (Schallreuter et al. 2008; Kondo and Hearing 2011; Enshell-Seijffers et al. 2010). Alternatively, this apparent difference in yellowish phenotype between mice and sables implies the presence of an

additional genetic system adding dark pigmentation. In dogs with the homozygous Mc1r null mutation, lighter hair varies from white to yellow, gold, orange and red (Everts et al. 2000; Newton et al. 2000; Schmutz et al. 2002; Oguro-Okano et al. 2011). The mutant mice created in the present study will facilitate our understanding of the genetic system underlying pigment production and variable yellowish hair color in the genetic background when the MC1R system is lost.

In conclusion, we demonstrated that a single amino replacement or deletion was sufficient to cause loss of function of MC1R producing effects comparable to those of a frameshift mutation. We therefore conclude that replacement of Cys35 is the mutation responsible for the bright yellow Hokkaido sable phenotype. Variability in the yellowish coat color between species with homozygous Mc1r null alleles should be examined in future studies.

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Data Availability

All relevant data are available in this paper.

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Figure Captions

Fig. 1 (**a**) Schematic representation of the mouse G-protein-coupled receptor Mc1r with the seven characteristic α-helical transmembrane segments, showing the target amino acid codon (Cys33) produced using the CRIPR/Cas9 genome editing system. (**b**) Direct sequencing traces (nucleotide positions 85–105 in the coding sequence) showing the mutation from TGC (Cys) to TTC (Phe) at target codon 33. (**c**) Six sequencing traces representing the (1) wild-type (WT) and (2–6) genome-editing experiment phenotypes, showing characteristic coding variants.

Fig. 2 Mice with (**a**) WT and (**b**) yellowish coat colors. Coat color patterns in dorsal and ventral views of F_2 mice with (**c, d**) WT and (**e, f**) yellowish coat color, showing banding patterns in small bundles of hair removed from the dorsal and ventral body parts. Scale bar, 5 mm.

Fig. 3 Plots of dorsal (X-axis) and ventral (Y-axis) body color measurements of color traits within CIE Lab color space: lightness (L*), red/green (a*), and yellow/blue (b*). We derived 14 F_2 mice from genome-edited mice with the wild type and altered coat colors. The representative types of the target site of the coat color-related melanocortin 1 receptor gene (*Mc1r*) were those having the cysteine residue at codon 33 (c.98G/c.98G; n = 2), a heterozygous target site (c.98G/c.98T; n = 5), replacement of the cysteine residue (c.98T/c.98T; n = 2), and 1-bp deletion (n = 3) and 2-bp deletion (n = 2) near the target site.

Supplementary Figure S1 (**a**) G_0 mice generated in the C3H/HeJ genome-editing experiment targeting the codon 33 (corresponding to codon 35 in other mammals) for the cysteine residue, resulting in variable coat color patterns among and within individuals, indicative of appearance of mosaicism. (**b**) Four G_0 mice showed markedly lighter coat color were subjected to breeding experiments to generate F_2 mice.

Supplementary Figure S2 (**a**) G_0 mice generated in the C57BL/6N genome-editing experiment targeting the codon 33 (corresponding to codon 35 in other mammals) for the cysteine residue, resulting in variable coat color patterns among and within individuals, indicative of appearance of mosaicism. (**b**) Four G_0 mice showed markedly lighter coat color were subjected to breeding experiments to generate F_2 mice.

Supplementary Figure S3 Principal component analysis results for the combined dorsal (d) and ventral (v) L*, a*, and b* measurement datasets.

Supplementary Table S1 Supplementary Table S1. Genotyping of Mc1r in G_0 mice in the C3H/HeJ background.

200 fertilized embryos from 10 female mice were subjected to electroporation. After cell culture, 198 injected eggsof the 2-cell stage were transferred to the oviducts of 10 pseudopregnant females, resulting in 53 offsprings.

*Coat color was temporarily categorized into three, yellowish (Y), wild (WT), and intermediate (I) types. See Supplementary Figure S1 for detail.

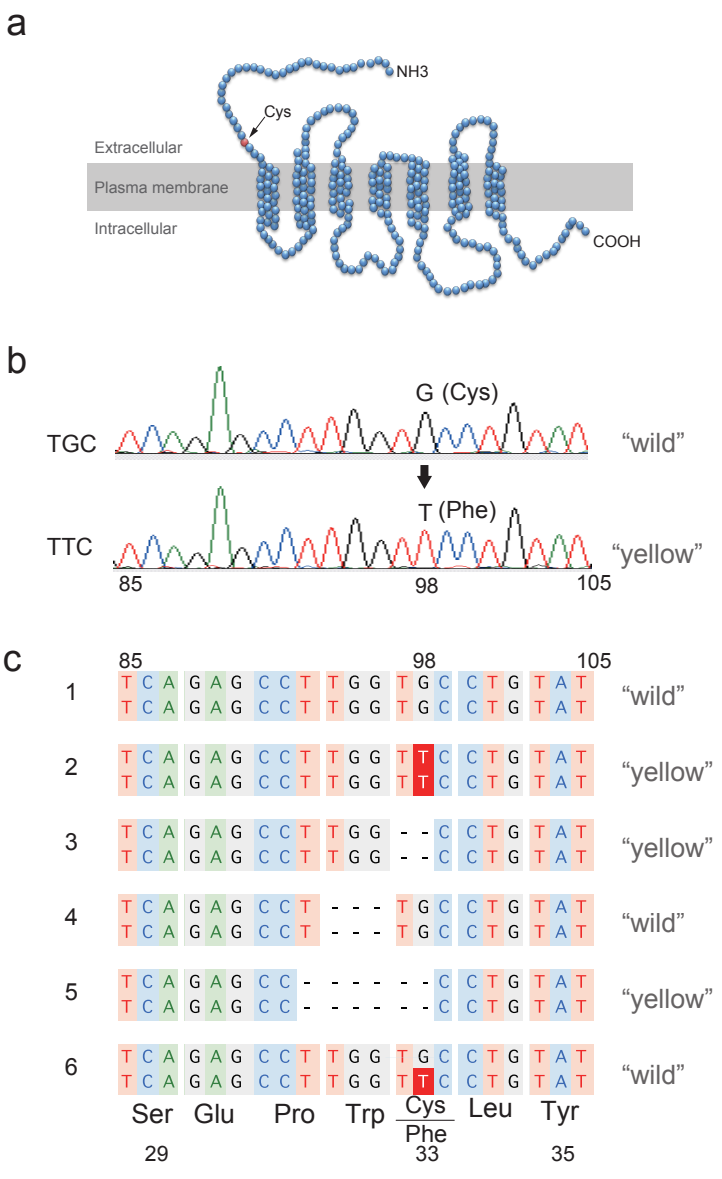
**ND, genotyping was failed to determine because of ambiguous results in the direct sequencing probably due to complex deletion and mosaicism.

Supplementary Table S2 Supplementary Table S1. Genotyping of Mc1r in G_0 mice in the C3H/HeJ background.

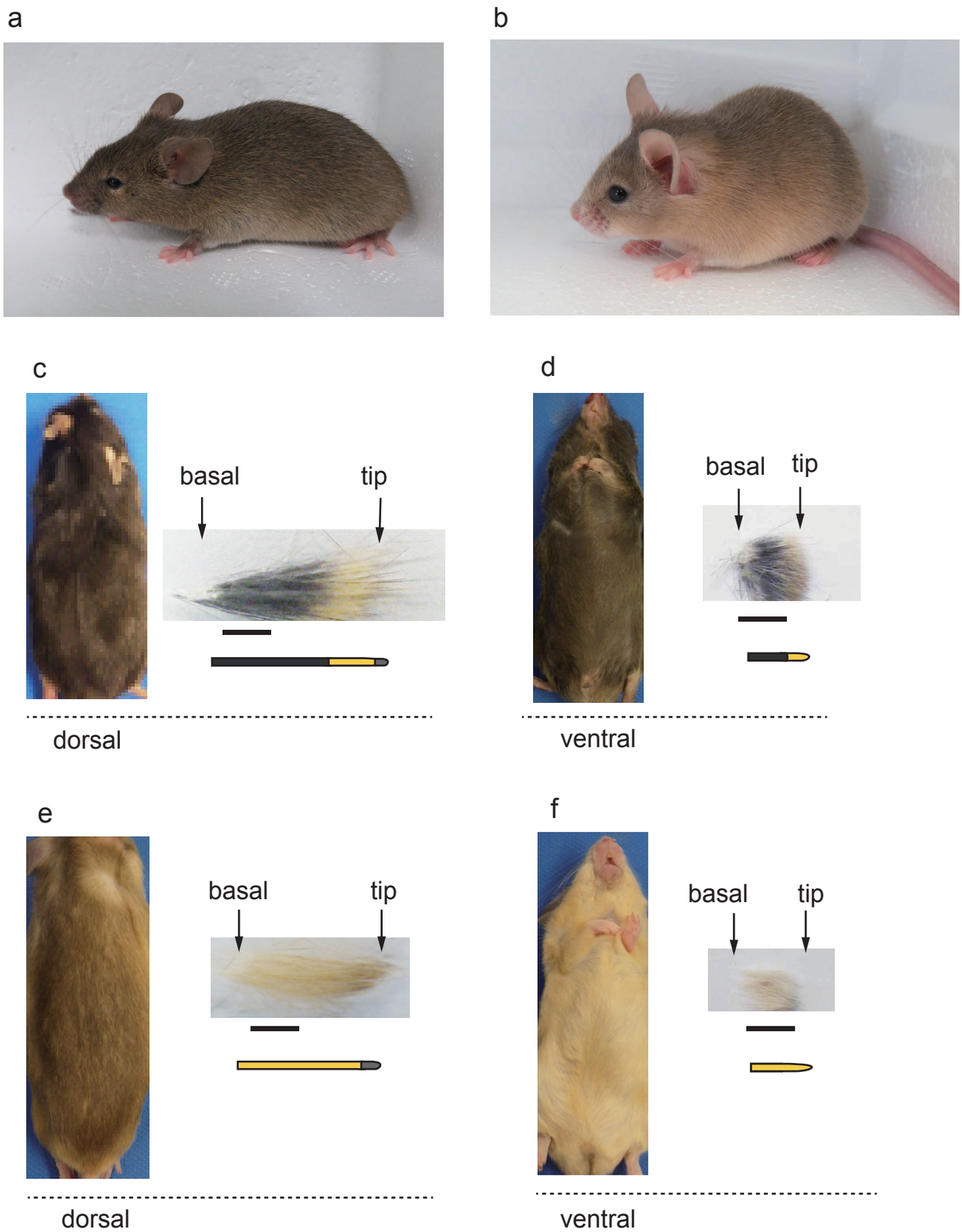
100 fertilized embryos from 5 female mice were subjected to electroporation. After cell culture, 99 injected eggs of the 2-cell stage were transferred to the oviducts of 5 pseudopregnant females, resulting in 28 offsprings.

*Coat color was temporarily categorized into three, yellowish (Y), wild (WT), and intermediate (I) types. See Supplementary Figure S1 for detail.

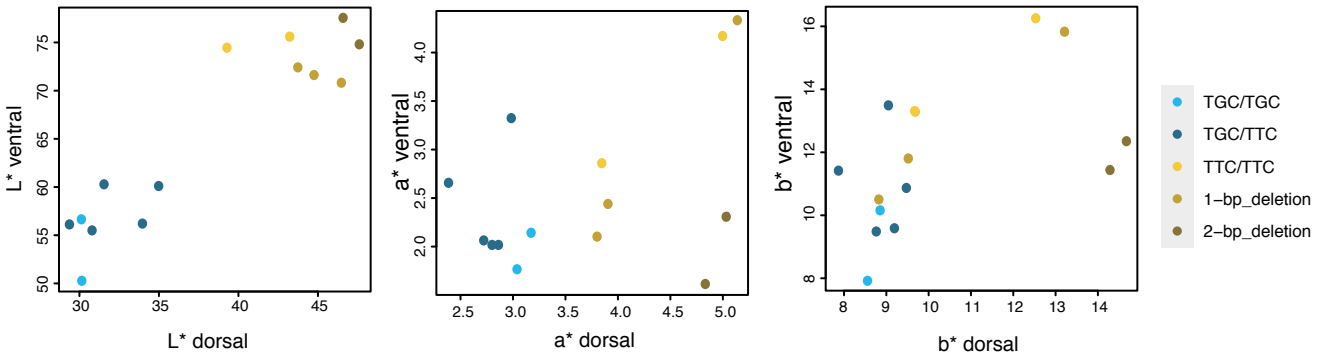
**ND, genotyping was failed to determine because of ambiguous results in the direct sequencing probably due to complex deletion and mosaicism.



Suzuki et al. Fig. 1



Suzuki et al. Figure 2.


L* ventral
L* dorsal
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1–bp_deletion
2–bp_deletion

Figure 3.