A Novel Kit Gene Mutation in CF1 Mice Involved in the Extracellular Domain of the KIT Protein

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Abstract: We screened for natural mutations in Crl:CF1 closed colony mice using an ordinary backcrossing system. Five of 30 CF1 males carried novel genes that caused white spots on colored coats. Their backcross progenies showed a white spot phenotype. The white spot gene was mapped to approximately 39 cM on chromosome 5, where the Kit gene is known to reside. Allelism testing between this spot gene and the Kit gene was performed using two already known Kit alleles, KitW and KitW-v. We demonstrated that the spot mutation was semidominant and a novel allele of the Kit gene, which was tentatively named KitW-Ham. No infertility or anemia was observed in KitW-Ham homozygotes. However, a reduced number of germ cells and mast cells was observed in KitW-Ham/KitW and KitW-Ham/KitW-v transheterozygotes. Sequencing of the 21 exons of the Kit gene in the KitW-Ham mutants revealed that a unique guanine-to-adenine (G-A) transition at nucleotide position 545 (c.545G>A) of exon 3 changes arginine (R) to glutamine (Q) at position 182 in the extracellular domain of the KIT protein (p.R182Q). This extracellular KIT domain is a binding site for stem cell factors (SCF). It was concluded that the KitW-Ham mutant may serve as a new model of human piebaldism.

Key words: CF1 closed colony, dominant white spotting, germ cells, Kit gene

Introduction

Piebaldism [OMIM ID: 164920; http://omim.org/] is a known inheritable disorder characterized by congenital leukoderma observed on the forehead, abdomen, and knees in humans [10, 22, 26]. About 75% of the patients show Kit gene mutations [7].

In mice, dominant white spot mutations have been found in colored strains such as C57BL/6 and C3H/He [15, 19]. The Kit (synonym c-Kit) gene, a kind of oncogene [4, 9], is known to be the gene responsible for the dominant spot mutation. At the Kit locus, 138 mutations, including 66 spontaneously occurring mutations, 17 chemically- and 2 radiation-induced mutations, 15 targeted mutations, and 38 gene-trap mutations, have been reported (http://www.informatics.jax.org/javawi2/service/WIFetch?page=markerDetail&key=10603). Sequencing revealed that Kit mutations mainly occur in regions encoding the transmembrane and cytoplasmic domains of the KIT receptor protein. Kit mutations are pleiotropic in their phenotypes. Typical phenotypes are leukoderma, macrocytic anemia, mast cell depletion, sterility, and a white spot on a colored coat depending on the mutation sites [13, 19].

Recently, we developed a system to find natural mutations in Jcl:ICR closed colony mice that are maintained by random mating [12]. In this study, we applied a natural mutation screening system to the Crl:CF1 closed
colony. The Crl:CF1 mouse, an albino outbred mouse strain, was produced by Carworth Farms (New City, NY, USA) from mice obtained from a Missouri laboratory (Columbia, MO, USA), which are thought to have been derived from wild albino mice (Charles River, http://www.criver.com/). As a result, we found a novel spot mutation in Crl:CF1 closed colony mice. The results of gene mapping and allelism testing suggest that the spot mutation is a novel Kit allele. This novel mutation was named KitW-Ham according to international nomenclature guidelines.

In this study, we describe the phenotypes and DNA sequencing of the novel Kit mutation, which demonstrated a novel G-to-A transition in exon 3 of the Kit gene (c.545G> A). This mutation led to an arginine (R) to glutamine (Q) substitution at amino acid 182 (p. R182Q) within a conserved second immunoglobulin (Ig)-like domain (D2) in the extracellular domain of the Kit protein.

Materials and Methods

Mice

Thirty Crl:CF1 (CF1) male mice were purchased from Charles River Laboratories (Wilmington, MA, USA). DBA/2JCl (DBA) and C57BL/6JCl (B6) were purchased from CLEA Japan (Tokyo, Japan). To perform the allelism testing, WB-KitW/+ and C57BL/6 (B6)KitW−/− mice were purchased from Japan SLC (Hamamatsu, Japan). Mice were maintained under temperature-controlled conditions (24 ± 2°C) with a 12L/12D light cycle and free access to food and water. The animal facility was maintained under specific pathogen-free (SPF) conditions. In-house monitoring was performed every 3 months using a Monilisa IVA kit (Wakamoto Pharmaceutical Co., Ltd., Tokyo, Japan) that detects four major organisms, Sendai virus, mouse hepatitis virus, mycoplasma, and Tyzzer’s organism. No infections were detected in any of the rooms in which mice used in this study were maintained.

Genetic crosses

Backcrossing was performed to detect possible spontaneous recessive mutations carried by CF1 mice. Four randomly selected (DBA female × CF1 male) F1 females were crossed with CF1 males to obtain F2 progeny homozygous for recessive mutations that showed abnormal phenotypes.

Establishment of a congenic strain

A congenic strain carrying the novel spot gene was bred using B6 as the recipient strain. The spot gene carried by male CF1 mouse #23 was introduced into B6 mice by backcrossing. Genotypes of the spot gene in mice used to obtain the next generation were confirmed by a progeny test using a heterozygote.

Gene mapping

Linkage between the spot gene and microsatellite markers on autosomes was studied using F2 progeny. Fifty-eight markers on 19 autosomes that showed genetic polymorphisms between CF1 #23 and DBA were selected (Table 1). Primer sets for the microsatellite markers were purchased from Invitrogen (Carlsbad, CA, USA). Microsatellite DNA markers were amplified by PCR followed by agarose gel electrophoresis. The procedures that were used have been described elsewhere [24].

Allelism testing

To study the genetic relationship between the novel spot gene and Kit gene, WB-KitW/+ and B6-KitW−/− mice were mated with mice homozygous for the novel spot gene, and the frequencies of mice showing a white coat color and spot were observed.

Phenotyping

The body and tissues of 8-week-old male mice were weighed using an electronic balance. Skin, ears, and testes were dissected and fixed in Bouin’s fixative. Organs were dehydrated in increasing concentrations of ethanol, embedded in paraffin, and sectioned at 4 µm. Skin and testis sections were stained with hematoxylin and eosin (HE) according to the standard procedures. The number of normal seminiferous tubules per testis section was counted in several independent sections and averaged. Melanin in the skin was histochemically visualized using Masson-Fontana (MF) stain. Mast cells in ear tissues were identified by staining with toluidine blue. The number of mast cells per visual field (approximately 340 µm²) was counted using a microscope. The spot area of the dorsal and ventral hair coat was measured using the NIH image software.

Fertility testing was carried out for a period of 6 months using five adult males of each genotype mated with two B6 wild-type adult females. Females were checked for pregnancy and changed every 2 months.

Blood collection was performed from the hearts of
## Table 1. Mapping of the novel spot gene using linkage analyses with microsatellite DNA markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Chr</th>
<th>cM</th>
<th>Position</th>
<th>Recombination ratio</th>
<th>(\chi^2)</th>
</tr>
</thead>
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<tr>
<td>D1Mit215</td>
<td>1</td>
<td>39.91</td>
<td>78,202,934–78,203,082</td>
<td>49:72</td>
<td>9.5</td>
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<tr>
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<td>1</td>
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<td>130,447,674–130,447,818</td>
<td>40:72</td>
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<tr>
<td>D1Mit291</td>
<td>1</td>
<td>88.97</td>
<td>186,554,196–186,554,340</td>
<td>31:72</td>
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<tr>
<td>D2Mit45</td>
<td>2</td>
<td>50.63</td>
<td>91,783,198–91,783,315</td>
<td>25:72</td>
<td>6.8</td>
</tr>
<tr>
<td>D2Mit258</td>
<td>2</td>
<td>63.22</td>
<td>130,233,353–130,233,457</td>
<td>23:72</td>
<td>9.5</td>
</tr>
<tr>
<td>D2Mit145</td>
<td>2</td>
<td>86.75</td>
<td>166,220,487–166,220,630</td>
<td>29:72</td>
<td>2.8</td>
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<tr>
<td>D2Mit213</td>
<td>2</td>
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<td>174,372,705–174,372,832</td>
<td>47:72</td>
<td>6.8</td>
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<tr>
<td>D3Mit151</td>
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<td>36:72</td>
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<td>D3Mit11</td>
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<td>43.71</td>
<td>100,360,598–100,360,744</td>
<td>31:72</td>
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<tr>
<td>D4Mit178</td>
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<tr>
<td>D4Mit37</td>
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<td>34:72</td>
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<tr>
<td>D5Mit1</td>
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<td>18:72</td>
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<td>64,813,426–64,813,544</td>
<td>1:72</td>
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<td>65,814,213–65,814,361</td>
<td>1:72</td>
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<td>D5Mit213</td>
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<td>43.71</td>
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<td>31:72</td>
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<td>33:72</td>
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<td>56:72</td>
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<td>43:72</td>
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<td>11.43</td>
<td>24,374,124–24,374,271</td>
<td>29:72</td>
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<tr>
<td>D9Mit205</td>
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<td>20.75</td>
<td>37,105,909–37,106,102</td>
<td>33:72</td>
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<td>9</td>
<td>32.76</td>
<td>60,421,238–60,421,384</td>
<td>24:72</td>
<td>9.8</td>
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<tr>
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<td>114,838,220–114,838,331</td>
<td>27:72</td>
<td>9.9</td>
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<tr>
<td>D10Mit4</td>
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<td>13.08</td>
<td>25,732,780–25,732,912</td>
<td>27:72</td>
<td>12.0</td>
</tr>
<tr>
<td>D10Mit162</td>
<td>10</td>
<td>55.73</td>
<td>106,528,846–106,528,949</td>
<td>24:72</td>
<td>12.0</td>
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<tr>
<td>D11Mit16</td>
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<td>2.94</td>
<td>4,169,563–4,169,684</td>
<td>31:72</td>
<td>17.9</td>
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<tr>
<td>D11Mit29</td>
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<td>42.88</td>
<td>69,607,483–69,607,623</td>
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<tr>
<td>D12Mit170</td>
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<td>7.79</td>
<td>3,410,212–3,410,347</td>
<td>22:72</td>
<td>18.0</td>
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<tr>
<td>D12Mit110</td>
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<td>15.68</td>
<td>39,861,164–39,861,310</td>
<td>24:72</td>
<td>19.1</td>
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<tr>
<td>D13Mit60</td>
<td>13</td>
<td>14.44</td>
<td>35,968,794–35,968,936</td>
<td>42:72</td>
<td>6.0</td>
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<td>D13Mit142</td>
<td>13</td>
<td>32.53</td>
<td>60,758,437–60,758,582</td>
<td>46:72</td>
<td>5.6</td>
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<tr>
<td>D13Mit78</td>
<td>13</td>
<td>67.21</td>
<td>119,618,032–119,618,260</td>
<td>37:72</td>
<td>2.8</td>
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<tr>
<td>D14Mit54</td>
<td>14</td>
<td>20.88</td>
<td>36,137,026–36,137,157</td>
<td>40:72</td>
<td>1.3</td>
</tr>
<tr>
<td>D14Mit165</td>
<td>14</td>
<td>56.16</td>
<td>106,982,674–106,982,807</td>
<td>32:72</td>
<td>1.3</td>
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<tr>
<td>D15Mit13</td>
<td>15</td>
<td>1.84</td>
<td>3,410,212–3,410,347</td>
<td>22:72</td>
<td>18.0</td>
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<tr>
<td>D15Mit235</td>
<td>15</td>
<td>34.11</td>
<td>73,817,161–73,817,283</td>
<td>27:72</td>
<td>9.9</td>
</tr>
<tr>
<td>D15Mit34</td>
<td>15</td>
<td>45.31</td>
<td>90,583,806–90,583,952</td>
<td>28:72</td>
<td>25.3</td>
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<tr>
<td>D16Mit152</td>
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<td>48.23</td>
<td>85,804,079–85,804,183</td>
<td>32:72</td>
<td>2.7</td>
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<tr>
<td>D17Mit164</td>
<td>17</td>
<td>2.11</td>
<td>3,924,615–3,924,747</td>
<td>24:72</td>
<td>15.1</td>
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<tr>
<td>D17Mit177</td>
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<td>24.51</td>
<td>48,698,276–48,698,388</td>
<td>23:72</td>
<td>22.8</td>
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<tr>
<td>D18Mit233</td>
<td>18</td>
<td>17.17</td>
<td>29,961,139–29,961,260</td>
<td>29:72</td>
<td>8.2</td>
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<td>D18Mit87</td>
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<td>20.18</td>
<td>33,507,010–33,507,247</td>
<td>31:72</td>
<td>4.2</td>
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<td>D19Mit68</td>
<td>19</td>
<td>3.38</td>
<td>3,645,155–3,645,286</td>
<td>30:72</td>
<td>3.8</td>
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<td>33,009,697–33,009,809</td>
<td>29:72</td>
<td>8.2</td>
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<td>D19Mit40</td>
<td>19</td>
<td>20.38</td>
<td>25,410,811–25,410,922</td>
<td>33:72</td>
<td>3.3</td>
</tr>
</tbody>
</table>
8-week-old male mice using a syringe with a 26 G needle. Blood analysis was performed with a Sysmex SF-3000 hematology analyzer (Sysmex, Hyogo, Japan). The parameters measured were red blood cell (RBC) count and hematocrit (HCT).

Sequencing
All 21 exons of the mouse Kit gene were amplified using 18 primer pairs (Table 2). Nucleotide sequences of these primers were designed using the Kit genomic sequence from Ensembl assembly NCBI M37 as the reference sequence. PCR products were directly sequenced using the dideoxy chain-termination method with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and then analyzed on an ABI PRISM 3100 (Applied Biosystems) automated DNA sequencer.

PCR-restriction fragment length polymorphism (RFLP)
To perform conventional genotyping of the novel spot mutation, PCR-RFLP analysis was performed as described elsewhere [23]. Primer pairs were designed to overlap the mutation site on exon 3 of the Kit gene. The primer pair, forward primer (F) 5’- ttgttcttctccagctgatcct-3’ and reverse primer (R) 5’- ctgtgtccttcagatgccctag-3’, yielded a 392 bp product. Four microliters of PCR product was digested with AluI (Toyobo, Osaka, Japan) and then electrophoresed in a 3% agarose gel.

Statistics
Statistical analysis was performed by analysis of variance (ANOVA) and a Student’s t-test using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Nomenclature
The gene, allele, and protein symbols used here were named according to the international nomenclature of mice and rats.

Approval of the experiment
This study was approved by the Animal Care and Use Committee of the Hamamatsu University School of Medicine, Japan.

| Table 2. Primers used to amplify 22 exons of the Kit gene |
|---|---|---|---|
| Exon | Primer pair | Sequence (5’–3’) | Product size (bp) |
| 1 | Kit-E1-F  
Kit-E1-R | cccggatcagcttattgcag 
agttgagctcctgtaac | 574 |
| 2 | Kit-E2-F  
Kit-E2-R | agctacacatcgtgagtt 
tgtaggtgctcctgtaac | 483 |
| 3 | Kit-E3-F  
Kit-E3-R | aacccagtctgctttaatcc 
atgacctgaacagatagg | 531 |
| 4 | Kit-E4-F  
Kit-E4-R | gtcttctcatcaggctctac 
cagacagctgataaagtgg | 453 |
| 5 | Kit-E5-F  
Kit-E5-R | cagctcttctcagctcctc 
gttctgtgcaagaagtc | 499 |
| 6 | Kit-E6-F  
Kit-E6-R | ctaaggccacatccttctc 
caccacatccttaatggg | 539 |
| 7 | Kit-E7-F  
Kit-E7-R | ggtctttatagtctgt 
cagagtaagttcttcctc | 643 |
| 8 | Kit-E8-F  
Kit-E8-R | gctgcttcagctcagtt 
tctcagatcgaattc | 629 |
| 9 | Kit-E9-F  
Kit-E9-R | ctagaaaggaggtgctag 
catcgaagcatcactag | 476 |
| 10 and 11 | Kit-E10,11-F  
Kit-E10,11-R | tcctagagtttcagat 
tcctagagtttcagctac | 501 |
| 12 and 13 | Kit-E12,13-F  
Kit-E12,13-R | ctagagtttcagctc 
tcctagagtttcagctac | 625 |
| 14 | Kit-E14-F  
Kit-E14-R | gacatcctcagttcaggg 
cagcagcaactgaagtc | 630 |
| 15 | Kit-E15-F  
Kit-E15-R | tggagccatcagtagag 
tctgtctgctcctgaagg | 475 |
| 16 | Kit-E16-F  
Kit-E16-R | cccctcattacatcagtt 
gagcagcagaattgcct | 539 |
| 17 | Kit-E17-F  
Kit-E17-R | gctcggagaccccatctc 
ccctgctcctgctcctg | 601 |
| 18 and 19 | Kit-E18,19-F  
Kit-E18,19-R | cctcgtcctgccttcctc 
tctgtctgctcctgaagg | 674 |
| 20 | Kit-E20-F  
Kit-E20-R | ccacgggtttacatcagc 
tagcagcctgctcctga | 455 |
| 21 | Kit-E21-F  
Kit-E21-R | attcagcagctttgtag 
attcagcagctttgtag | 545 |

Results

Inheritance of the novel spot and establishment of a congenic strain
In (DBA × CF1#23) F1 progeny, no spot mice were observed. In F2 mice obtained by intercrossing F1 mice, the ratio of spot to wild-type mice, with sex ratios in parentheses (female: male), was 36 spot (15:21) to 104 wild-type (49:55) (1:3, \( \chi^2 \) value=0.038, \( P=0.91 \). The
genetic crosses suggested that this spot gene is autosomal and recessive.

A congenic strain with the novel spot gene derived from male CF1 mouse #23 on a B6 genetic background was generated using serial backcrossing. The relationship between genotype and phenotype was studied using the congenic strain carrying the spot gene.

Of the 32 N6 mice obtained by crossing heterozygotes of the N5 generation, 7 (21.9%) showed no spots, 17 (53.1%) had a small spot, and 8 (25.0%) had larger spots (Fig. 1a, 1b, and 1c, respectively). These frequencies were significantly concordant ($\chi^2$ value=0.187, $P=0.91$) with the ratio for wild homozygotes:heterozygotes:mutant homozygotes=1:2:1, as expected according to Mendelian inheritance. These results strongly suggested that the spot gene is autosomal and semidominant. As described above, some incidences of the spot phenotype were affected by the genetic background.

Gene mapping

Using linkage analyses with 36 mice homozygous for the spot gene obtained in the F2 generation, gene mapping was performed. As shown in Table 1, a significant linkage was observed between the spot gene and microsatellite markers on chromosome 5. No recombination was observed between the white spot gene and the $D5Mit355$ (38.34 cM), $D5Mit134$ (38.44 cM), and $D5Mit135$ (39.53 cM) markers that are closely linked to the Kit gene (Table 1).

Allelism testing

An allelism testing was performed to determine whether the spot gene was an allele of the Kit locus. B6-congenic male mice homozygous for the spot gene were crossed with females heterozygous for either Kit$^W$ or Kit$^{W-v}$. Of the 38 F1 mice obtained by crossing B6-congenic and B6-Kit$^{W-v}$/+ mice, 17 (44.7%) showed a white coat color with black eyes (Fig. 1D), and 21 (55.3%) had small spots. Of the 36 F1 mice that were obtained by crossing the B6-congenic and WB-Kit$^W$/+ mice, 17 (47.2%) had a white coat color with black eyes (Fig. 1E), and 19 (52.8%) had small spots. These results revealed that the novel spot gene is an allele of the Kit locus and that Kit$^W$ and Kit$^{W-v}$ are dominant for the novel spot gene. The novel allele was tentatively named Kit$^{W-Ham}$ according to the international nomenclature guideline.
Phenotypic characterization of spot mice homozygous for KitW-Ham

Phenotypes of B6-+/KitW-Ham and B6-KitW-Ham/KitW-Ham were compared with those of WBB6F1-KitW/KitW-Ham, B6-KitW+/KitW-Ham, and WBB6F1-KitW/KitW-v. KitW-Ham heterozygotes showed a small spot on the midline of the ventral area (Table 3 and Fig. 1B). However, KitW-Ham homozygous mice showed spots both in dorsal and ventral areas (Table 3 and Fig. 1C). Three transheterozygotes (WBB6F1-KitW/KitW-Ham, B6-KitW+/KitW-Ham, and WBB6F1-KitW/KitW-v) showed a white coat color with black eyes and an almost 100% white area in dorsal and ventral regions (Table 3, Fig. 1D and 1E).

There were no differences among the six genotypes (Table 3) in terms of body weight, the number of RBCs and HCT. However, testis weight, spermatogenesis, and the number of mast cells were significantly lower in the three transheterozygotes than in the other genotypes (Table 3). However, spermatogenesis was found in approximately 28 and 1% of the seminiferous tubules of KitW-v/KitW-Ham and KitW/KitW-v mice, respectively, and these mice showed infertility (Fig. 3D and Table 3).

Sequencing of the KitW-Ham gene

Sequencing of the KitW-Ham gene revealed that the KitW-Ham mutation was a nucleotide change from G to A at position 545 (c.545G>A) in exon 3. This missense mutation led to an amino acid substitution from arginine to glutamine at amino acid residue 182 (p.R182Q) in the KIT protein (Fig. 4). In addition, a silent mutation (T to C) was found at position 1602 (c.1602T>C) in exon 10 (Fig. 4).

Development of PCR-RFLPs for genotyping

The c.545G>A KitW-Ham mutation generated an AluI recognition site (AGCT). Therefore, a primer pair was designed to amplify a 392 bp fragment from exon 3 of the Kit gene (Fig. 5A). PCR products were digested with AluI, and the digests were electrophoresed in a 3% agarose gel. The following profiles were produced: i) the homozygous wild-type (+/+) with a 392 bp band; ii) the heterozygous genotype (KitW-Ham/+) with three bands of 392, 222, and 170 bp; and iii) the mutant homozygous genotype (KitW-Ham/KitW-Ham) with two bands of 222 and 170 bp (Fig. 5B). These results indicated that PCR-based RFLP genotyping is a reliable and efficient method to determine the genotype of albino (e.g., CF1) and heterozygous mice with incomplete penetrance. Individual genotyping was performed on 30 mice of the CF1 colony using RFLP. Surprisingly, 5 of the 30 mice showed the KitW-Ham heterozygous genotype. The KitW-Ham allele frequency was calculated as 0.083 (5/60) in this colony.

### Table 3. Phenotypes of 8-week-old KitW-Ham mice

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>No. mice</th>
<th>White area (%)</th>
<th>Body weights (g)</th>
<th>Number of RBCs (× 10⁶/μl)</th>
<th>HCT (%)</th>
<th>Number of mast cells (per 340 μm²)</th>
<th>Testis weights (mg)</th>
<th>Spermatogenesis (active/total tubules)</th>
<th>Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>5</td>
<td>0.0</td>
<td>24.7±1.9</td>
<td>8.19±0.87</td>
<td>41.4±3.8</td>
<td>15.45±0.82</td>
<td>189.8±9.2</td>
<td>100%</td>
<td>Yes</td>
</tr>
<tr>
<td>KitW-Ham/KitW-Ham</td>
<td>6</td>
<td>0.0</td>
<td>23.6±2.9</td>
<td>8.48±0.84</td>
<td>43.2±4.9</td>
<td>16.48±0.64</td>
<td>188.4±8.8</td>
<td>100%</td>
<td>Yes</td>
</tr>
<tr>
<td>KitW-Ham/KitW-Ham</td>
<td>8</td>
<td>11.9</td>
<td>23.6±1.1</td>
<td>8.25±0.98</td>
<td>40.7±7.0</td>
<td>14.06±0.78</td>
<td>191.0±9.3</td>
<td>99.77%</td>
<td>Yes</td>
</tr>
<tr>
<td>KitW-v/KitW-Ham</td>
<td>7</td>
<td>99.1</td>
<td>23.6±0.7</td>
<td>8.09±0.27</td>
<td>44.9±0.9</td>
<td>3.30±0.50**</td>
<td>135.9±22.4</td>
<td>82.76%</td>
<td>Yes</td>
</tr>
<tr>
<td>KitW/KitW-v</td>
<td>5</td>
<td>98.9</td>
<td>24.6±1.9</td>
<td>8.77±0.30</td>
<td>44.7±4.3</td>
<td>3.25±0.46**</td>
<td>49.6±6.7**</td>
<td>28.41%</td>
<td>No</td>
</tr>
<tr>
<td>KitW/KitW-v</td>
<td>4</td>
<td>100.0</td>
<td>24.2±0.6</td>
<td>7.38±0.74</td>
<td>39.8±2.2</td>
<td>2.63±0.49**</td>
<td>36.0±5.0**</td>
<td>9.89%</td>
<td>No</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM of each number of mice. Asterisks (* and **) indicated P<0.05 and P<0.01 vs. +/+ mice, respectively.
Fig. 2. Melanocytes in hair follicles of mice homozygous for the +/+ (A) and Kit<sup>W-Ham</sup>/Kit<sup>W-Ham</sup> (B) genotypes. Wild-type (+/+) hair follicles with pigmented melanocytes are detectable (A1 and A2). Note the absence of pigment granules in the homozygous hair follicles of the white spot area (B1 and B2). HE, hematoxylin and eosin staining; MF, Masson-Fontana stain.

Fig. 3. Histological analysis of the testes in +/+ (A), Kit<sup>W-Ham</sup>/Kit<sup>W-Ham</sup> (B), Kit<sup>W-v</sup>/Kit<sup>W-Ham</sup> (C), and Kit<sup>W</sup>/Kit<sup>W-Ham</sup> (D) 8-week-old mice. Testes were sectioned and stained with HE and showed a normal appearance and organization in +/+ and Kit<sup>W-Ham</sup>/Kit<sup>W-Ham</sup> homozygous mice. Conversely, transheterozygous (Kit<sup>W-v</sup>/Kit<sup>W-Ham</sup> and Kit<sup>W</sup>/Kit<sup>W-Ham</sup>) mice show some abnormal seminiferous tubules (asterisk).

Fig. 4. Schematic representation of the Kit<sup>W-Ham</sup> mutation in the KIT protein. Two point mutations, missense and silent mutations, were found in Kit<sup>W-Ham</sup> mice. The missense mutation is located at position c.545G>a in exon 3 of the mouse Kit gene, which substitutes Arg for Gln at amino acid 182 (p.R182Q). The silent mutation is located at position c.1602T>C in exon 10. Abbreviations: SP, signal peptide; D1-D5, Ig-like domains; TM, transmembrane domain; K1, kinase domain I; and K2, kinase domain II.
Discussion

White spotting has been associated with several mutations in genes such as Kit [4, 9, 27], Kitl (kit ligand) [2, 5], Ednrb (endothelin receptor type B) [11], and Pax3 (paired box gene 3) [6]. To investigate which gene was involved in the spot mutation found in this study, we carried out gene mapping, sequencing, and phenotyping to demonstrate that the novel spot gene is an allele of the Kit locus and that a mutation occurred in exon 3 that encodes the extracellular domain of the Kit protein.

It is well known that Kit mutations show pleiotropic effects. KitW and KitW-/- are representatives of Kit mutations that occur in transmembrane and intracellular domains, respectively. The novel spot mutation (KitW-Ham) reported in this study is the first mutation found to occur in an exon that encodes the extracellular domain of the KIT protein.

Numerous Kit mutations occur in the tyrosine kinase domain of the KIT receptor protein [28]. These mutations lead to inactivation of signaling cascades and pleiotropic developmental defects. In KitW, KitW37F, and KitW42I mutant mice, the kinase activity is extremely reduced, which is lethal to the embryo [17, 25]. On the other hand, mice homozygous for the KitWv mutation have residual kinase activity and are viable. In the KitW-Ham mutant, the typical phenotype is spotting. Therefore, we showed that the p.R182Q variation in the KITW-Ham protein did not affect signaling cascades.

The active KIT protein is a dimer composed of two identical peptide chains and is a member of the type III family of receptor tyrosine kinases [1, 16, 18, 30]. The KIT protein consists of three domains, the extracellular, transmembrane, and cytoplasmic domains [21]. The extracellular domain contains five Ig-like subdomains (D1–D5). D1, D2, and D3 play important roles in recognizing and binding the KITL protein [3, 14]. A previous study revealed that three amino acids, Y125, R181 (R182 in mouse), and K203 (K204 in mouse), in the D2 subdomain of the human KIT receptor are important for binding to KITL [31]. On the other hand, the D4 and D5 subdomains play important roles in stabilizing the dimer [29, 31]. The cytoplasmic domain of KIT contains a tyrosine kinase subdomain. Binding of KITL to KIT leads to receptor dimerization, intermolecular autophosphorylation, and protein tyrosine kinase activation.

Arginine 182 is highly conserved in inbred mouse strains and eutherian mammalian KIT proteins. Binding of KIT to stem cell factor (SCF) may not be completely inhibited by the p.R182Q variation. However, this substitution may account for the reduced affinity of SCF toward the mutant KITW-Ham receptor protein.

A white spot on colored coats or an entirely white coat are typical phenotypes of Kit mutations [13, 20]. Phenotypes such as infertility, anemia, and mast cell depletion have also been observed in Kit mutants. Fleischman et al. reported that mild piebaldism observed in humans may be linked to missense mutations in exon 3 of the Kit gene. This study also suggested that the KIT receptor defect associated with loss of the D2 subdomain is less severe than the defects that result from some of the null or deletion mutations or from the majority of kinase subdomain mutations [8].

Here, we report a missense mutation in the extracellular ligand-binding domain (D2) of the KIT receptor, which is associated with a piebald phenotype. This mutation is a novel allele of the Kit locus, which we named KitW-Ham. KitW-Ham mutant strains will be useful for analyzing the function of the Kit gene and future investiga-
tions into the mechanism of SCF binding to the KIT receptor.

Finally, we found that 5 out of the 30 Crl:CF1 mice may strongly exhibit the KitW-Ham/+/ genotype. CF1 mice are albinos, making it unlikely that this mutation would be found in them. We propose that the frequency of occurrence of the KitW-Ham allele in the CF1 colony should be fully investigated and that, if needed, the KitW-Ham allele should be excluded from the CF1 colony. PCR-RFLP will be a useful tool for the identification of KitW-Ham mutants in CF1 colonies.

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References


