A 5-bp Insertion in *Mip* Causes Recessive Congenital Cataract in KFRS4/Kyo Rats

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Abstract

We discovered a new cataract mutation, *kfrs4*, in the Kyoto Fancy Rat Stock (KFRS) background. Within 1 month of birth, all *kfrs4/+* homozygotes developed cataracts, with severe opacity in the nuclei of the lens. In contrast, no opacity was observed in the *kfrs4/+* heterozygotes. We continued to observe these rats until they reached 1 year of age and found that cataractogenesis did not occur in *kfrs4/+* rats. To define the histological defects in the lenses of *kfrs4* rats, sections of the eyes of these rats were prepared. Although the lenses of *kfrs4/+* homozygotes showed severely disorganised fibres and vacuolation, the lenses of *kfrs4/+* heterozygotes appeared normal and similar to those of wild-type rats. We used positional cloning to identify the *kfrs4* mutation. The mutation was mapped to an approximately 9.7-Mb region on chromosome 7, which contains the *Mip* gene. This gene is responsible for a dominant form of cataract in humans and mice. Sequence analysis of the mutant-derived *Mip* gene identified a 5-bp insertion. This insertion is predicted to inactivate the MIP protein, as it produces a frame shift that results in the synthesis of 6 novel amino acid residues and a truncated protein that lacks 136 amino acids in the C-terminal region, and no MIP immunoreactivity was observed in the lens fibre cells of *kfrs4*/+ heterozygous rats using an antibody that recognises the C- and N-terminus of MIP. In addition, the *kfrs4/+* heterozygotes showed reduced expression of *Mip* mRNA and MIP protein and the *kfrs4/+* homozygotes showed no expression in the lens. These results indicate that the *kfrs4* mutation conveys a loss-of-function, which leads to functional inactivation through the degradation of [*Mip* mRNA by an mRNA decay mechanism. Therefore, the *kfrs4* rat represents the first characterised rat model with a recessive mutation in the *Mip* gene.

Introduction

Kyoto Fancy Rat Stock (KFRS) strains are inbred strains derived from fancy rats to collect new rat mutations and increase the value of the rat model system. The founder rats are six fancy rats imported to Kyoto University from a fancy rat colony in the USA and six inbred lines (KFRS2/Kyo, KFRS3A/Kyo, KFRS3B/Kyo, KFRS4/Kyo, KFRS5A/Kyo, and KFRS6/Kyo), including two sublines that were produced by brother-sister mating after the initial cross with a laboratory strain, TM/Kyo or PVG/Seac. The KFRS strains are a potential source of novel rat mutations because mutations, such as those affecting coat and eye colour, occur frequently in fancy rats. Indeed, we have identified 16 mutations that affect coat colour, eye colour, and hair pattern in the KFRS strains [1]. In addition, fancy rat colonies are thought to have been maintained relatively independently of laboratory rats [2]. This characteristic suggests that fancy rats have a unique genetic background that is more similar to that of rat strains that were recently derived from wild rats than to that of laboratory rats; therefore, KFRS strains are likely to become a new powerful tool for forward genetic studies of various pathogenic phenotypes among human populations and for providing valuable biological information regarding human disease.

We found a recessive *kfrs4* mutation in a KFRS4/Kyo strain that exhibits bilateral congenital cataract with progressive severe degeneration of the lens fibre cells. Using a positional cloning approach, we discovered a mutation in the major intrinsic protein of eye lens fibre gene (*Mip*, also known as aquaporin 0 or *Aqp0*), which is the most abundant membrane protein in the lens fibre cells, constituting more than 60% of the total membrane protein content of these cells [3,4]. Spontaneous mutations in *Mip* (seven in humans and four in mice) have been associated with congenital cataract [5–14]. *Mip* mutant mice exhibit cataract as a result of disrupted lens differentiation [5,7,8,15,16]. These pathological observations suggest that MIP has essential roles in the establish-
ment and maintenance of a uniform lens fibre structure and in fibre organisation. All of the characterised Mip mutations are associated with cataract as the dominant phenotype, which could be explained by a specific dominant negative effect of the Mip mutant allele [17–19]. However, the cataract phenotype in KFRS4/Kyo rats is inherited in a recessive fashion, in contrast to known Mip mutations in humans and mice. In this study, we performed genetic, phenotypic and expression analyses of the KFRS4/Kyo rats. Our results suggest that this mutant should be classified as the first identified recessive mutant allele of Mip.

Materials and Methods

Rats

We used KFRS4/Kyo, BN/CrlJ, WIAR/lar and DOB/Oda rats as the wild-type rats in all of the experiments. KFRS4/Kyo and DOB/Oda rats were supplied by The National BioResource Project for the Rat in Japan (NBRP Rat: http://www.anim.med.kyoto-u.ac.jp/nbr/Default.aspx), Kyoto University (Kyoto, Japan). The BN/CrlJ and WIAR/lar rats were purchased from Charles River Japan (Yokohama, Japan) and from the Institute for Animal Reproduction (Kasumigaura, Japan), respectively. All of the procedures involving animals met the guidelines described in the Proper Conduct of Animal Experiments, as defined by the Science Council of Japan, and were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Medical Science, Tokyo University of Agriculture and Kyoto University.

Phenotypic Analysis

The rat pupils were dilated using Mydrin-P (Santen Pharmaceutical, Osaka, Japan), and both eyes were observed after 5 min. The diagnosis of cataract was performed by macroscopic examination, as described previously [20]. Both the right and left eyeballs from embryonic and postnatal wild-type, kfrs4/+ heterozygous and kfrs4/kfrs4 homozygous rats were examined for histological analysis. The rats were sacrificed, and both eyeballs were enucleated and fixed in Superfix (Kurabo, Tokyo, Japan) overnight at room temperature. After fixation, specimens were transferred to methanol, dehydrated, embedded in paraffin, and sectioned (5 μm). After removing the paraffin, the sections were stained with haematoxylin and eosin and observed under a Leica DM2500 light microscope.

Genetic Mapping

Genetic mapping of the kfrs4 mutant locus was performed by intercrossing progeny derived from the mating of (KFRS4/Kyo × DOB/Oda) F1 × KFRS4/Kyo. The backcrossed progeny with a mutant phenotype were easily identified by the overt lens opacity induced by mydriatic instillation. DNA samples from 58 offspring, including 31 cataract-presenting rats of a KFRS4/Kyo and DOB/Oda cross, were genotyped using 108 polymorphic microsatellite markers selected from The NBRP Rat (Table S2) and six microsatellite markers (Table S2) developed from the rat genomic sequence (Ensembl: http://asia.ensembl.org/Rattus_norvegicus/Info/Index). Genotyping was carried out using PCR (Table S2) and 4% agarose gel electrophoresis. The map position was refined using the Map Manager computer program [21].

Mutation Analysis

A genomic fragment covering the four coding exons of Mip was amplified from genomic DNA isolated from wild-type rats (DOB/Oda, BN/CrlJ and WIAR/lar), kfrs4/+ heterozygous rats (F1 rats from a kfrs4/kfrs4 and wild-type cross), and kfrs4/kfrs4 homozygous rats. The primers Mip_F and Mip_R were used for amplification, and the following primers were used for sequencing: Mip_F1, Mip_F2, Mip_F3, Mip_F4, Mip_R1, and Mip_R2 (Table S2). The PCR products were purified using the QiAquick Gel Extraction Kit (Qiagen, Valencia, CA), sequenced using a BigDye Terminator kit (Life Technologies, Grand Island, NY) and analysed using an Applied Biosystems 3130xl Genetic Analyzer.

The kfrs4 allele was genotyped using PCR to amplify genomic DNA and fluorescently labelled using Mip_del_F and Mip_del_R (Table S2). DNA samples from 20 inbred rat strains (ACI/NKy, DON/Kyo, IS/Kyo, RCS/Kyo, SHR/Kyo, TM/Kyo, W/Kyo, WAG/Kyo, WTC/Kyo, ZI/Kyo, KDP/Tky, LE/Sm, F344/Sm, DOB/Oda, KFRS2/Kyo, KFRS3A/Kyo, KFRS3B/Kyo, KFRS4/Kyo, KFRS5A/Kyo, and KFRS6/Kyo) for control of genotyping were supplied by NBRP Rat. The PCR products were separated using a Beckman CEQ8000 instrument (Beckman Coulter, Fullerton, CA) with a size standard, and the fragment sizes were determined using fragment analysis software.

RT-PCR

Total RNA was isolated from the eyes of 7-week-old wild-type and kfrs4/kfrs4 rats using TRIzol (Life Technologies) and the TRIzol Plus Purification Kit (Life Technologies) according to the manufacturers’ protocols. The Superscript VILO cDNA synthesis kit (Life Technologies) was used to generate cDNA using 2 μg of DNase-pretreated total RNA. We also prepared cDNA from wild-type, kfrs4/+ and kfrs4/kfrs4 rats at 7 and 10 weeks for quantitative RT-PCR (qRT-PCR). Primers for Mip and Gapdh were purchased from Qiagen (Valencia, CA). A total of 9 specific transcripts (Cryaa, Crygb, Crygd, Casp6, Lin2, Bfsp1, Bfsp2, Gap43, and Gap53) for lens fibre cells were used as the initial controls and to verify the quality of the fibre cells (Table S2). Subsequently, qRT-PCR was performed as previously described [20].

Antibodies

An anti-MIP rabbit polyclonal antibody (MIP-Cter) that targets a 17-amino acid (aa) peptide in the C-terminal cytoplasmic domain of MIP was previously characterised [8,22] and commercially acquired from Alpha Diagnostic International (San Antonio, TX). We also generated an anti-MIP rabbit polyclonal antibody (MIP-Nter) to a peptide (C+TPPAVRNLALNT) in the N-terminal region of the MIP peptide from aa 108 to 129 (NM_001105719). We used N-cadherin (CHD2) and β-catenin (CTNNB1) as markers of the lens. Both proteins were evenly expressed in lens fibre cells [23–26]. Mouse monoclonal anti-CDH2 and anti-CTNNB1 antibodies were purchased from BD Biosciences (San Jose, CA). For a secondary antibody, Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit and HRP-sheep anti-mouse IgG antibodies (GE Healthcare Life Science, Piscataway, NJ) were used for Western blotting. Alexa Fluor-conjugated secondary antibodies were obtained from Invitrogen.

Immunoblotting

Membrane proteins from the eyes of wild-type, kfrs4/+ and kfrs4/kfrs4 rats at 7 and 8 weeks of age were purified as described by Sidjianin et al. [7]. Samples containing approximately 500 ng of protein were separated on 15% SDS-polyacrylamide gels, and the separated proteins were transferred onto a Hybond-P PVDF membrane (GE Healthcare Life Science). MIP protein bands were detected using the anti-MIP-Cter (1:1,000) and -Nter (1:5,000) antibodies, followed by
rats showed normal lens phenotypes (Table 1). Crossbreeding of DOB/Oda, BN/CrlCrlj, and WIAR/lar rats with KFRS4/Kyo F1 rats with Kyo rats produced 31 offspring with cataract and 27 offspring without cataract. These results indicate that the kfrs4 mutation is recessive (Figure 1E, Table 1). 

While characterising rats from the KFRS4/Kyo strain, we observed that all of the rats developed severe bilateral lens opacity throughout the eye within 1 month of birth (Figure 1A). To classify the cataract type caused by the kfrs4 mutation, lenses from wild-type and KFRS4/Kyo rats were dissected and observed under a light microscope (Figure 1B). The opacity of the KFRS4/ Kyo rat lenses was observed in the anterior nuclear regions at P0 (Figure 1C). The cataract progressed to a nuclear cataract and then to an anterior nuclear cataract at P1 (Figure 1D). The F1 progeny from the crossbreeding of DOB/Oda, BN/CrlCrlj, and WIAR/lar rats with KFRS4/Kyo rats showed normal lens phenotypes (Table 1). Crossbreeding between the (KFRS4/Kyo × DOB/Oda) F1 rats and KFRS4/ Kyo rats produced 31 offspring with cataract and 27 offspring without cataract. Moreover, mating (KFRS4/Kyo × WIAR/lar) F1 rats with kfrs4/+ rats produced 23 offspring with cataract and 29 offspring without cataract. These results indicate that the kfrs4 mutation is recessive (Figure 1E, Table 1).

Isolation and Phenotypic Characterisation of the Spontaneous kfrs4 Mutant

While characterising rats from the KFRS4/Kyo strain, we observed that all of the rats developed severe bilateral lens opacity throughout the eye within 1 month of birth (Figure 1A). To classify the cataract type caused by the kfrs4 mutation, lenses from wild-type and KFRS4/Kyo rats were dissected and observed under a light microscope (Figure 1B). The opacity of the KFRS4/ Kyo rat lenses was observed in the anterior nuclear regions at P0 (Figure 1C). The cataract progressed to a nuclear cataract and then to an anterior nuclear cataract at P1 (Figure 1D). The F1 progeny from the crossbreeding of DOB/Oda, BN/CrlCrlj, and WIAR/lar rats with KFRS4/Kyo rats showed normal lens phenotypes (Table 1). Crossbreeding between the (KFRS4/Kyo × DOB/Oda) F1 rats and KFRS4/ Kyo rats produced 31 offspring with cataract and 27 offspring without cataract. Moreover, mating (KFRS4/Kyo × WIAR/lar) F1 rats with kfrs4/+ rats produced 23 offspring with cataract and 29 offspring without cataract. These results indicate that the kfrs4 mutation is recessive (Figure 1E, Table 1).

To define the histological defects in the lenses of kfrs4 mutants, sagittal sections of the eye were prepared from wild-type, kfrs4/+, and kfrs4/kfrs4 rats at various embryonic and postnatal stages (Figure 2). At E15.5, the phenotypes of the lens in kfrs4/+ kfrs4 homozygotes showed a pattern of development similar to that of kfrs4/+ heterozygotes (Figure 2A, B). However, there were signs of disorganisation in the fibre cells in the nuclear region of the kfrs4/+ kfrs4 homozygous lens; the nuclei of the fibre cells remained in this region to a significant degree in comparison to the kfrs4/+ heterozygotes (Figure 2C, D). Degeneration of the lens fibre was pronounced in both the anterior and posterior regions in the kfrs4/+ kfrs4 homozygotes at P0, with lens fibre swelling (Figure 2E, F). Over the next few weeks, the fibre cells became progressively more disorganised (Figure 2G, H), and irregularly shaped cells and huge vacuoles were present by 9 weeks of age (Figure 2I). In contrast, the lenses of kfrs4/+ heterozygotes exhibited compactly packed and uniform fibre cells at 19 weeks of age, similar to the lenses of wild-type rats (Figure 2J, K).

Identification of the kfrs4 Mutation

DNA samples from 58 [(KFRS4/Kyo × DOB/Oda) × KFRS4/Kyo] N2 progeny (Table 1) were genotyped using 114 microsatellite markers (Table S1) on rat chromosome 1–20 to determine the location of the kfrs4 mutation. Using this linkage analysis, we mapped the kfrs4 mutation to an interval of approximately 9.7-Mb between markers Casf1912 and D7Wox43 on chromosome 7 (Figure 3A). This region contains more than 550 protein coding genes, including the kfrs4 candidate region [Ensembl]. Given its genomic locus and its involvement in a similar mutant pathology in humans and mice, Mip was the strongest candidate gene for the kfrs4 mutation. The kfrs4 phenotype was non-recombinant with a microsatellite marker in the 3’ region of Mip in 58 [(KFRS4/Kyo × DOB/Oda) × KFRS4/Kyo] N2 progeny (Figure 3A).

By sequencing Mip, we identified a 5-bp insertion at nucleotide position 360 in exon 1 of Mip in the (KFRS4/Kyo × DOB/Oda) F1 and KFRS4/Kyo rats (Figure 3B). This 5-bp insertion, AACAC, is present as a tandem repeat, most likely resulting from the duplication of the same 5 bp 5’ of the insertion (Figure 3B). We designed a genotyping PCR primer pair to detect a 104-bp fragment for the wild-type Mip allele and a 109-bp fragment for the kfrs4 allele. Based on these PCR amplicons, we were able to confirm the genotypes of the +/+, kfrs4/+, and kfrs4/kfrs4 rats (Figure 3B). Moreover, the deletion was only observed in the kfrs4 mutants and was not present among a set of 21 rat strains, including other KFRS strains (Figure S1).

The kfrs4 5-bp insertion is predicted to cause a frameshift mutation that results in truncation of the peptide chain by generating a stop codon at amino acid position 127, and this kfrs4 frameshift mutation causes a truncation of the MIP protein that removes three transmembrane domains (H4, H5 and H6), the HE hemichannels, cytoplasmic loop D, and extracellular loop E (Figure 4A, B). To confirm this truncation of the C-terminal domain in kfrs4 rats, we performed immunoblot and immunohistochemistry analyses using a rabbit polyclonal antibody, anti-MIP-Cter, that targets a 17-aa peptide in the C-terminal cytoplasmic domain of MIP [Alpha Diagnostic International]. The 28-kDa (previously reported as the MIP band [27]) bands were abundant in extracts from the wild-type and kfrs4/+ heterozygote eyes but were not detectable in the eye extracts from kfrs4 homozygotes (Figure 4C). Moreover, we generated an antibody, anti-MIP-Nter, to a peptide within an LC extracellular domain in the N-terminal region of the kfrs4 mutation to detect the presence of the mutant MIP protein in kfrs4 mutants. In the eye extracts of wild-type and kfrs4/+ rats, this antibody, as well as anti-MIP-Cter antibody, detected the 28-kDa band (Figure 4C). In addition, we detected a variant of approximately 23 kDa. Although we have not yet identified whether these bands represent nonspecific antibody binding or a degradation product, these bands could not be detected in the eye extracts of kfrs4/kfrs4 homozygotes.
As reported previously [27], MIP was expressed throughout the fibre cells of the lens in wild-type rats; we also observed strong signals in the anterior fibre cells of adult rats (Figure 4D). The MIP staining was abolished in the \textit{kfrs4} homozygous rats, confirming the truncation of the C-terminal domain in rats with the \textit{kfrs4} mutation (Figure 4D). Immunohistochemistry using the anti-MIP-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Strain or cross & Phenotype & Age (W) & n & Cataract (n) & Normal (n) & Incidence (%) \\
\hline
KFRS4/Kyo & & 8 & 55 & 55 & 0 & 100 \\
(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 12 & 33 & 33 & 0 \\
 & $F_1$ & & & & \\
 & $KFRS4/Kyo$ & & 44 & 3 & 0 & 0 \\
 & $KFRS4/Kyo$ & & 12 & 58 & 31 & 27 & 53.4 \\
 & (KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 14 & 2 & 0 & 2 & 0 \\
 & (KFRS4/Kyo $\times$ BN/Crlj) $F_1$ & & 19 & 31 & 0 & 31 & 0 \\
 & (KFRS4/Kyo $\times$ WIAR/lar) $F_1$ & & 12 & 52 & 23 & 29 & 44.2 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 55 & 55 & 0 & 100 \\
(KFRS4/Kyo $\times$ WIAR/lar) $N_2$ & & 12 & 58 & 31 & 27 & 53.4 \\
(KFRS4/Kyo $\times$ BN/Crlj) $N_1$ & & 14 & 2 & 2 & 0 & 0 \\
(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 19 & 31 & 0 & 31 & 0 \\
(KFRS4/Kyo $\times$ WIAR/lar) $F_1$ & & 12 & 52 & 23 & 29 & 44.2 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 55 & 55 & 0 & 100 \\
(KFRS4/Kyo $\times$ WIAR/lar) $N_2$ & & 12 & 58 & 31 & 27 & 53.4 \\
(KFRS4/Kyo $\times$ BN/Crlj) $N_1$ & & 14 & 2 & 2 & 0 & 0 \\
(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 19 & 31 & 0 & 31 & 0 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 52 & 23 & 29 & 44.2 \\
(KFRS4/Kyo $\times$ WIAR/lar) $F_1$ & & 12 & 55 & 55 & 0 & 100 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 58 & 31 & 27 & 53.4 \\
(KFRS4/Kyo $\times$ BN/Crlj) $N_1$ & & 14 & 2 & 2 & 0 & 0 \\
(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 19 & 31 & 0 & 31 & 0 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 52 & 23 & 29 & 44.2 \\
(KFRS4/Kyo $\times$ WIAR/lar) $F_1$ & & 12 & 55 & 55 & 0 & 100 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 58 & 31 & 27 & 53.4 \\
(KFRS4/Kyo $\times$ BN/Crlj) $N_1$ & & 14 & 2 & 2 & 0 & 0 \\
(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 19 & 31 & 0 & 31 & 0 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 52 & 23 & 29 & 44.2 \\
(KFRS4/Kyo $\times$ WIAR/lar) $F_1$ & & 12 & 55 & 55 & 0 & 100 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 58 & 31 & 27 & 53.4 \\
(KFRS4/Kyo $\times$ BN/Crlj) $N_1$ & & 14 & 2 & 2 & 0 & 0 \\
(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 19 & 31 & 0 & 31 & 0 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 52 & 23 & 29 & 44.2 \\
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(KFRS4/Kyo $\times$ BN/Crlj) $N_1$ & & 14 & 2 & 2 & 0 & 0 \\
(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ & & 19 & 31 & 0 & 31 & 0 \\
(KFRS4/Kyo $\times$ KFRS4/Kyo) $N_2$ & & 12 & 52 & 23 & 29 & 44.2 \\
\hline
\end{tabular}
\caption{Incidence of cataractogenesis in \textit{kfrs4} $F_1$ rats from crosses of KFRS4/Kyo and 3 inbred lines and in [(KFRS4/Kyo $\times$ DOB/Oda) $F_1$ $\times$ KFRS4/Kyo] $N_2$, and [(KFRS4/Kyo $\times$ WIAR/lar) $F_1$ $\times$ KFRS4/Kyo] $N_2$ rats.}
doi:10.1371/journal.pone.0050737.t001
\end{table}
Nter antibody revealed a nearly identical staining pattern to that of anti-MIP-Cter (Figure 4E). We found that the staining of MIP is completely ablated in the \(kfrs4/kfrs4\) homozygotes.

Reduction in \(Mip\) mRNA and MIP Protein Expression by the \(kfrs4\) Mutation

As mentioned above, the frameshift in the \(kfrs4\) mutant produces a truncated MIP protein that is missing several domains in the C-terminal region (Figure 4). To examine the effect of the \(kfrs4\) mutation on \(Mip\) mRNA expression, we carried out real-time qRT-PCR using RNA from wild-type, \(kfrs4/\)heterozygous, and \(kfrs4/kfrs4\) homozygous rats. The relative abundance of \(Mip\) transcripts in the eyes of \(kfrs4/\) and \(kfrs4/kfrs4\) rats was approximately 34.6 and 7.1% of wild-type levels, respectively (Figure 5). To confirm this reduction of \(Mip\) mRNA in \(kfrs4/\) and \(kfrs4/kfrs4\) eyes and determine whether it caused a loss of lens fibre cells to induce cataracts, we performed qRT-PCR analysis of nine other lens-specific transcripts. Five of these transcripts, \(Casp6\), \(Lim2\), \(Bfsp1\), and \(Gja8\), did not exhibit significant expression changes among the wild-type, \(kfrs4/\), and \(kfrs4/kfrs4\) rats (Figure 5). The expression of three crystallins, \(Cyna\), \(Crygd\), and \(Crygd\), exhibited similar
levels between the wild-type rats and the kfrs4/+ heterozygous rats, but significant reductions were detected in the kfrs/kfrs homozygous rats, suggesting the possibility that the reduction of these crystallins caused structural defects in the lens fibre cells of kfrs/kfrs homozygous rats. Although the expression of Bfsp2 (a code lens structural protein) was lower in kfrs4/+ rats relative to wild-type, a change in expression was not detected in the kfrs/kfrs rats (Figure 5). These results may confirmed that the reduction of Mip expression in kfrs4/+ and kfrs4/kfrs4 rats is not the result of cataract formation but is instead caused by the expression of certain genes that were affected in the kfrs4 mutant. Gja3 (also known as connexin 46) showed a significantly lower expression in kfrs4/+ and kfrs4/kfrs4 rats compared with wild-type (Figure 5). Although we could not explain why the expression of Gja3 is also reduced in kfrs4/kfrs4 homozygous rats, the GJA3 protein directly interacts with MIP, mediated by the C-terminal region [28]; therefore, it may be an effect of the reduction in Mip mRNA as a result of the kfrs4 mutation.

Quantitative immunoblotting and immunohistochemistry were also performed to confirm the MIP protein expression levels in wild-type and kfrs4/+ heterozygous rats. Although the difference in protein levels was not significant according to a statistical analysis, MIP was 1.59 times less abundant in kfrs4/+ eyes than in wild-type eyes (Figure 6A). Quantitative immunohistochemical analysis of MIP in the fibre cells of the anterior lens revealed that the expression level of MIP significantly decreased in the kfrs4/+ rat, whereas the level of CDH2 did not decrease (Figure 6B). As shown in Figure 6C, the amount of MIP in kfrs4/+ eyes was 33.1% of the level observed in wild-type eyes. Similar results were obtained using the anti-MIP-Nter antibody.
Figure 4. The kfrs4 mutation produces a frameshift and the generation of a C-terminally truncated MIP protein. A, B. Predicted structures of the MIP proteins encoded by the wild-type (A) and kfrs4 mutant (B) alleles. A schematic diagram showing the presumed membrane topology of MIP (modified from Francis et al. [17]). The locations of the six transmembrane domains (H1, H2, H3, H4, H5, and H6), two hemichannels
and CTNNB1 as control for quantitative analysis (Figure 6D–F, Figure S2).

**Discussion**

We present several lines of evidence demonstrating that a mutation in the Mip gene underlies congenital cataract in kfrs4 mutant rats. First, Mip is located within the candidate region identified for the kfrs4 mutation (Figure 3A). Second, the kfrs4 mutant phenotypes were consistent with the Mip mutation genotypes in all of the rats examined (Figure 3B, Figure S1). Third, the absence of MIP-positive bands in the Western blots of eye tissue from kfrs4 mutants and the lack of MIP-specific immunofluorescence in eye sections from kfrs4 mutants indicate the absence of the normal Mip gene product (Figure 4B–G). The identified mutation is a 5-bp nucleotide insertion within the coding region of the amino terminus of MIP, adding to the catalogue of known Mip mutations that cause cataract in mammals. In humans and in mice, point mutations [5,6,10–14], intragenic in-frame deletions [7,8] and frameshift mutations [5,9] in Mip have been characterised to cause dominant phenotypes (Figure S3). The cataract phenotype in MipHfi/+ heterozygous mice suggested that a 76-bp deletion in Mip is a gain-of-function mutation, consistent with data showing that a normal level of wild-
type MIP protein expression observed in MipHfi/+ could not protect the lens from cataract formation [7]. Another Mip mutation in mice, MipCat-Tohm is a 12-bp deletion in Mip that does not alter the open reading frame, but these mutant mice exhibited a more severe cataract phenotype than did Mip knockout mice [15,16] with a null mutation in Mip [8]. Interestingly, the lens fibre cells of a transgenic mouse that expressed both the wild-type and the MipCat-Tohm mutated Mip showed more severe degeneration at birth than did the lens cells of MipCat-Tohm/Cat-Tohm homozygotes, which expressed only MipCat-Tohm. In addition, the E134G/T138R mutant of human MIP leads to congenital cataract and results in a loss of water permeability owing to a failure in protein trafficking to the plasma membrane in homozygotes [6]; however, when the E134G/T138R mutant is co-expressed with wild-type MIP protein, the mutant protein reaches the plasma membrane but causes tetramer instability and a loss-of-function of wild-type MIP [17]. Moreover, FRET analysis of human MipADC2 and wild-type MIP coexpressed in mammalian cells demonstrated that this mutation is dominant because the hetero-oligomerisation of the wild-type and mutant MIP molecules traps the wild-type MIP in the endoplasmic reticulum [18]. These studies indicate that mutated versions of the MIP protein have a strong dominant-negative effect on lens transparency and that cataract in these mouse and human cases is caused by gain-of-function mutations.

However, we characterised kfrs4 as a recessive mutation because kfrs4/+ heterozygous rats exhibit a lens structure that is similar to that of wild-type rats until the late stages of development (Figure 1, 2, Table 1), and the kfrs4 mutation was effectively mapped in a linkage study using backcrosses to follow the recessive phenotype (Figure 3A). The kfrs4/+ heterozygous and kfrs4/kfrs4 homozygous rats appear to have reduced Mip mRNA expression (Figure 5). Moreover, the mutant MIP protein in kfrs4 rats presumably lacks 136 aa from the C-terminus because MIP signals were not detected by Western blotting and immunohistochemistry when an

Figure 6. Quantitative analysis of MIP protein expression in lenses from +/+ and kfrs4/+ rats. A, D. Densitometric quantification of MIP expression levels detected by Western blot analysis using the anti-MIP-Cter (A) and anti-MIP-Nter (D) antibodies in the eyes of +/+ and kfrs4/+ rats at 7 weeks of age. B, E. Immunofluorescence labelling of CDH2 (top), MIP (middle), and merged images (bottom) in the lens fibres from +/+ (left) and kfrs4/+ (right) rats at 8 weeks of age. The sections are stained by both anti-MIP-Cter (B) and anti-MIP-Nter (E) antibodies. Scale bar = 20 μm. C, F. Quantification of MIP intensities in B and E. The values shown in each graph (A, C, D, and F) indicate the mean relative expression levels and the standard errors of triplicate samples (n = 3). **P<0.01, ***P<0.001, n.s., not significant.
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anti-MIP-Cter antibody that recognise C-terminal region of MIP was used (Figure 4, 6). These results suggest that the frameshift caused by the kfrs4 mutation leads to functional inactivation though the rapid degradation of mRNA by nonsense-mediated mRNA decay; this observation supports the characterisation of kfrs4 as a loss-of-function mutation in Mip, which may be explained by a partial loss of MIP function via dosage effects. Indeed, Mip null mutations may be characterised as semi-dominant because heterozygous mutants present with a milder cataract phenotype than do homozygotes [22,27]. In addition, Mip<sup>kfrs4+/+</sup> (in which aa 203–263 at the C-terminus has been replaced with a transposon sequence) and Mip<sup>kfrs4+/−</sup> (A51P) heterozygous mutants also show mild phenotypes compared with the corresponding homozygous mutants [5]. Furthermore, the phenotype can be rescued in chimeric mice, which have lenses containing equal numbers of mutant and wild-type fibre cells [29].

Finally, the difference in heterozygous phenotypes and the inheritance mode in kfrs4 rats may result from the diversity of the genetic background and species differences between rats and mice compared to humans, but we lack the evidence to make this determination. In humans, individuals with MIP mutations have disorders of multiple genetic origins that exhibit marked phenotypic heterogeneity [6,10–14,30]. In particular, it is known that cataracts caused by different types of mutant MIP have different phenotypes, suggesting that Mip mutations cause phenotypic heterogeneity [6]. The diverse phenotypes exhibited by the Mip gene suggest that other genetic modifiers are likely to influence the expression and function of MIP in lens development and in lens fibre formation.

Supporting Information

Figure S1 Gain of the 5-bp insertion in exon 1 of Mip in a KFRS4/Kyo strain. Genotyping of various wild-type strains of rat, including other KFRS strains (asterisks), revealed an absence of the 5-bp insertion.

Figure S2 Quantitative analysis of MIP protein expression in lenses from +/- and kfrs4/+ rats. A, D. Densitometric quantification of MIP expression levels detected by Western blot analysis using the anti-MIP-Cter (A) and anti-MIP-Nter (D) antibodies in the eyes of +/- and kfrs4/+ rats at 7 weeks of age. B, E. Immunofluorescence labelling of CTNNB1 (top), MIP (middle), and merged images (bottom) in the lens fibres from +/- (left) and kfrs4/+ (right) rats at 8 weeks of age. The sections are stained by both anti-MIP-Cter (B) and anti-MIP-Nter (E) antibodies. Scale bar = 20 μm. C, F. Quantification of MIP intensities in B and E. The values shown in each graph (A, C, D, and F) indicate the mean relative expression levels and the standard errors of triplicate samples (n = 3). **P<0.01, n.s., not significant.

Table S1 Microsatellite markers used in genetic mapping of the rat kfrs4 locus.

Table S2 Primers and PCR conditions used for the genetic mapping, mutation analysis, and qPCR in this study.

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Author Contributions

Conceived and designed the experiments: K. Watanabe, K. Wada, J-IH, TS, TK, YK. Contributed reagents/materials/analysis tools: TS, TK. Wrote the paper: K. Watanabe, K. Wada, J-IH, TS, TK, YK.

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