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## Kyoto Rhino Rats Derived by ENU Mutagenesis Undergo Congenital Hair Loss and Exhibit Focal Glomerulosclerosis

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**Abstract:** N-ethyl-N-nitrosourea (ENU) mutagenesis is an important tool for studying gene function and establishing human disease models. Here, we report the characterization of a novel hairless mutant rat strain that carries a recessive mutation called Kyoto rhino (*krh*), which was created by ENU-mutagenesis. We produced a F344-*krh* strain through inbreeding without backcrossing to F344 rats. The *krh/krh* rats lost their coat hair by eight weeks of age. They also developed wrinkled skin, cystic hair canals and long curved nails by four months of age. Markedly dilated hair follicles that contained keratin debris were observed during histological analysis of the skin. The *krh* locus was mapped near the hairless (*Hr*) gene on chromosome 15. Sequence analysis revealed a nonsense mutation (c. 1238 C>A, p. S413X) in the *Hr* gene. The truncated HR protein was deduced to lack a zinc-finger domain and repression domains. In aged *Hr<sup>krh</sup>/Hr<sup>krh</sup>* rats, focal glomerulosclerosis (FGS) was observed in which collapsed glomeruli contained protein exudates in Bowman's capsule. Mesangial matrices that had proliferated in segments and foot processes that were fused in podocytes were also observed. The *Hr<sup>krh</sup>/Hr<sup>krh</sup>* rats also suffered from significant proteinuria. Given its breeding history, the F344-*Hr<sup>krh</sup>* strain may harbor ENU-induced mutation(s) that underlie FGS in addition to having the *Hr<sup>krh</sup>* mutation. The F344-*Hr<sup>krh</sup>* rat is a useful model of skin disease and may provide a new model system for the examination of the pathogenesis of FGS.

**Key words:** disease model, hairless, mutation, nephrosis

### Introduction

Hairless mutant rodents are valuable models for studying molecular mechanisms that underlie hair growth control. They are particularly valuable when searching for the genetic basis of hereditary human hair disorders.

In mice, 43 mutations are responsible for primary genetic hairlessness [7]. Among them, the most important are allelic mutations of the hairless (*Hr*) gene. The best characterized allele is the hairless (*hr*). *Hr<sup>hr</sup>/Hr<sup>hr</sup>* mice have a striking total alopecia phenotype which appears between three and four weeks of age. The pheno-

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type originates in the periorbital region and propagates in a wave-like fashion in the rostral-to-caudal direction [20]. It has been determined, through comparative studies of several distinct mouse *Hr* mutations, that the *Hr* gene product plays a key role in controlling hair follicle transformation during the catagen phase [20]. The hairless phenotype of *Hr*-mutant mice ( $Hr^{hr}/Hr^{hr}$ ) is similar to that of the human disease atrichia. The disease phenotype comprises papular lesions (APL) and alopecia universalis congenital (ALUNC), complete hair loss after birth. It is a result of human *HR* mutation [2, 4, 9].

Another important mutant *Hr* of the mouse is the rhino (*rh*) mouse.  $Hr^{rh}/Hr^{rh}$  mice lose all of their hair by seven weeks of age, possess wrinkled skin and their nails overgrow. Additionally, they develop an autoimmune disease characterized by hypergammaglobulinemia, immunoglobulin deposits in the basement membrane of skin, spleen, liver, and kidney, and the presence of antinuclear antibodies which appear in young mice and increase with age [14].

Several rat hair loss mutations have been described. They are for the Charles River hairless rat [1], the Iffa Credo (IC) rat [6], the Hairless Wistar Yagi rat also known as the HWY/Slc rat [13], the Dundee experimental bald rat also known as the DEBR rat [22], the Bald rat [12], and the Hirosaki hairless rat (HHR) [17]. An intragenic deletion in the desmoglein 4 gene underlies the IC rat skin phenotype [6]. The absence of 80-kb of genomic DNA that contains five basic keratin genes is the cause of the HHR rat hairless phenotype [17].

Rat hair follicles are larger than those of mice. Therefore hairless mutant rats are attractive models for studying hair follicle development, differentiation, and cycling. Rat mutants are also good models for evaluating the effects of new drugs for treating human skin diseases. Therefore, it would be beneficial to establish new hairless rat models for these purposes.

We recently treated rats with *N*-ethyl-*N*-nitrosourea (ENU) to obtain different mutants [16]. Several hair loss phenotypes were identified by employing phenotype-driven screening. A hair loss mutant line was established by crossing mutant-type males with wild-type female littermates. Our analysis of the breeding record of this line can be used to prove that the hair loss phenotype is

autosomal recessive. Thus, the mutation was named Kyoto rhino (*krh*).

In this study, we identified the *krh* mutation using a positional candidate approach and characterized the *krh/krh* rats. *krh* is a nonsense mutation of the rat *Hr* gene. *krh/krh* rats develop renal failure with massive proteinuria and focal glomerulosclerosis (FGS).

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## Materials and Methods

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### Animals

ENU-treated F344/NSlc male rats were mated with F344/NSlc female rats to generate G<sub>1</sub> offspring [16]. The ENU-mutagenized G<sub>1</sub> rats (n=42) were used as founders for the phenotype-driven screening of recessive mutations. Briefly, the G<sub>1</sub> rats were crossed with two F344 rats to generate G<sub>2</sub> offspring. The female G<sub>2</sub> offspring were then backcrossed with their parental G<sub>1</sub> rats to generate G<sub>3</sub> offspring. The recessive mutations induced by ENU in the G<sub>1</sub> rats become homozygous in the G<sub>3</sub> rats. Among the G<sub>3</sub> offspring (n=11) from a G<sub>1</sub> male (#E2307), three rats showed a hair loss phenotype; these rats were probands (P generation). We mated the affected rats with the normal littermates to fix the hair loss phenotype. The phenotype was fixed at the F<sub>2</sub> generation and the mutation was called *krh*. A mutant line was established by employing brother-sister mating (homozygous male × heterozygous female). The generation of inbreeding had reached F<sub>6</sub> at the end of August, 2010. The animal care and experimental procedures that were used were approved by the Animal Research Committee, Kyoto University and were carried out according to the Regulation on Animal Experimentation at Kyoto University.

### Genetic mapping

Twenty N<sub>2</sub> rats were produced from a (BN/SsNSlc × F344-*krh/krh*)F<sub>1</sub> × F344-*krh/krh* backcross. The genotypes for the *krh* locus were identified on the basis of coat phenotype at four to five weeks of age. Genomic DNA was prepared from tail biopsies using an automatic DNA purification system (PI-200, Kurabo, Japan) and genotypes for *D15Rat10*, *D15Rat13*, and *D15Rat85* were determined. Linkage relationship was evaluated using the chi-square test of the Excel statistical package.



Confidence intervals ( $P < 0.05$ ) were calculated according to the method of a previous report [11].

#### *RT-PCR and direct sequencing*

Total RNA was isolated from the skin of five-week-old animals using ISOGEN (NIPPON GENE, Tokyo, Japan). RT-PCR and direct sequencing of the PCR product was carried out as described previously [15]. Rat *Hr* cDNA was amplified using the following eight primer sets: rHr-01&02 CACCTGTGGAAGGCTGCT and ACAGGGTCACTCTGGGATG; rHr-03&04 AGGGACTACGCTGGAAGGAA and CCCAAACGTTACCGAGAGTG; rHr-05&06 GCAGGCAGCAGAATCTTTG and TCCTGTGGATGTCTCTGGTG; rHr-07&08 ACTCAAGAGGGCAGGCAGT and GGTGTTGAAGAGTCCGTGGT; rHr-09&10 CTTCCAATCAACAAGGGCCTA and CTGGCTCTCTGTGGAGTCT; rHr-11&12 GGTCAGCA GAAGGAACCAAC and TTCCAGAATGCTGTGCTGTC; rHr-13&14 GACTTAGCCTGTGGGGAATG and CTCCAAGGTTCTGCTCCAG; rHr-15&16 GTCTCAGGTAGCCAGACCA and GTTCCCTGCTGTGACCCAAA. The PCR products overlapped each other and spanned the entire 3,624 bp *Hr* coding sequence (CDS).

#### *Morphological analysis*

Dorsal and ventral skin samples were collected from *krh/krh* and *krh/+* littermates at two, nine and seventeen weeks of age. Mouse anti-cytokeratin (AE1/AE3, Dako Japan, Tokyo, Japan) was used for immunohistochemical analysis of the skin samples. Bound antibody was detected using horseradish peroxidase conjugated anti-mouse antibody (Histofine Simplestain MAX-PO; Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine as a chromogen (Vector Laboratories, Burlingame, CA, USA). To detect lipids, frozen sections were made from specimens that had been fixed with formalin and they were stained with Oil red O.

Organ samples of the heart, lungs, liver, pancreas, kidneys, spleen, lymph nodes, salivary glands, lacrimal glands, thyroid gland, adrenal glands, small and large intestines, and knee and foot joints, were collected from three *krh/krh* rats and three F344 rats at 40 weeks of age. They were fixed using 10% neutral buffered formalin, embedded in paraffin, cut at 4  $\mu$ m in thickness, and then

stained with hematoxylin and eosin (HE). To study glomerular lesions, periodic acid-Schiff (PAS) or periodic acid-methenamine-silver (PAM) staining was employed. For immunofluorescence studies, kidney samples were frozen in 22-oxacalceitriol compound (Miles Inc., Elkhart, IN, USA).

#### *Electron microscopy*

Perfusion fixation through the left ventricle was conducted with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Kidneys that had been excised were stored in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB. They were fixed with 2% osmic acid for 2 h and embedded in epoxy resin. Ultra-thin sections were double-stained with uranyl acetate and lead citrate and examined using a Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan).

#### *Urine protein measurement*

To collect urine, six male *krh/krh* rats and six F344/NSlc (+/+) rats, 40 weeks of age, were caged individually in metabolic chambers after they had been orally loaded with physiological saline at 2.5 ml/100 g body weight. Six-hour urine samples were collected and their volumes, and protein concentrations were determined. Statistical differences were determined using the Mann-Whitney U test.

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## Results

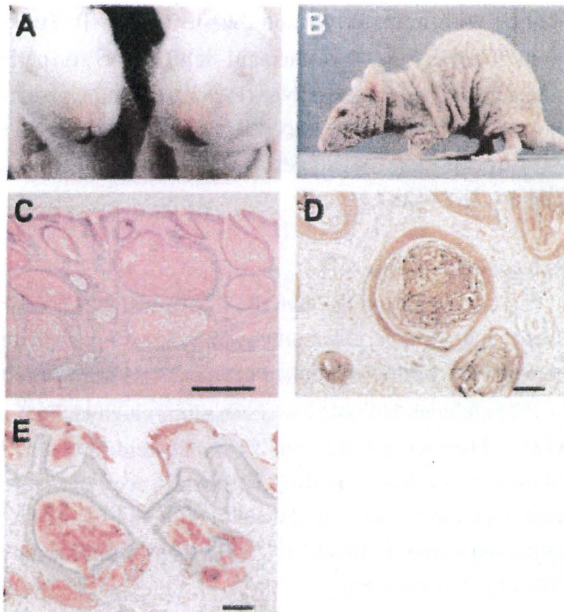
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#### *krh/krh rat hair loss phenotype and skin morphology*

For the *krh/krh* rats, hair loss first occurred around the nose around 2 weeks after birth and extended gradually from the anterior to the posterior of the body (Fig. 1A and 1B). At around four months of age they had wrinkled skin, cystic hair canals and long curved nails.

Through histopathological analysis, markedly dilated hair follicles were observed. These cystic follicles contained a lot of keratin debris (Fig. 1C), and they stained positive for cytokeratin (Fig. 1D). The cysts were lined by a thin layer of squamous epithelium and an easily identifiable granular cell layer. The sebaceous glands that surrounded the dilated cysts were hyperplastic. Staining with Oil red O revealed that a large amount of lipids was present in the lumen of each cyst and on the





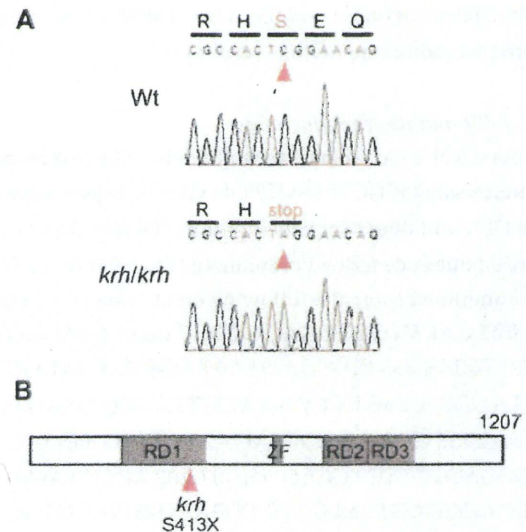
**Fig. 1.** Phenotypic and morphological characteristics of the Kyoto rhino rat. A: Right; a 2-week-old *krh/krh* rat with characteristic hair loss on the snout. Left; a littermate *krh/+* rat. B: Rhinocerotous appearance of a 10-week-old *krh/krh* rat. C: Histopathology of 9-week-old *krh/krh* rat skin. HE staining. Bar=500  $\mu$ m. D: IHC of keratin of a 9-week-old *krh/krh* rat. Bar=100  $\mu$ m. E: Oil Red O staining of a 17-week-old *krh/krh* rat. Bar=100  $\mu$ m.

surface of the epidermis (Fig. 1E). These findings are indicators that the *krh/krh* skin and hair phenotypes are similar to those of *rh* at the *Hr* locus of the laboratory mouse [7].

#### *krh* is an *Hr* nonsense mutation

*Hr* on Chr 15 was believed to be the best candidate for *krh* and therefore the genotype of the backcross progeny was determined using genetic markers for Chr 15. We obtained 12 *krh/krh* and 8 *krh/+* rats from the (BN/SsNSlc  $\times$  F344-*krh/krh*)F<sub>1</sub>  $\times$  F344-*krh/krh* backcross. A significant linkage relationship was observed between *krh* and *D15Rat10* (42.7 Mb) with no recombination ( $\chi^2=21.6$ ,  $P<0.01$ ), which is indicative that *krh* is located <13.9 cM away from *D15Rat10* with 95% probability [11]. *krh* was expected to span from 28.8 Mb to 56.6 Mb of Chr 15, within which the *Hr* locus (50.9 Mb) was mapped (RGSC v3.4).

Sequencing analyses of *Hr* cDNA obtained from *krh/*



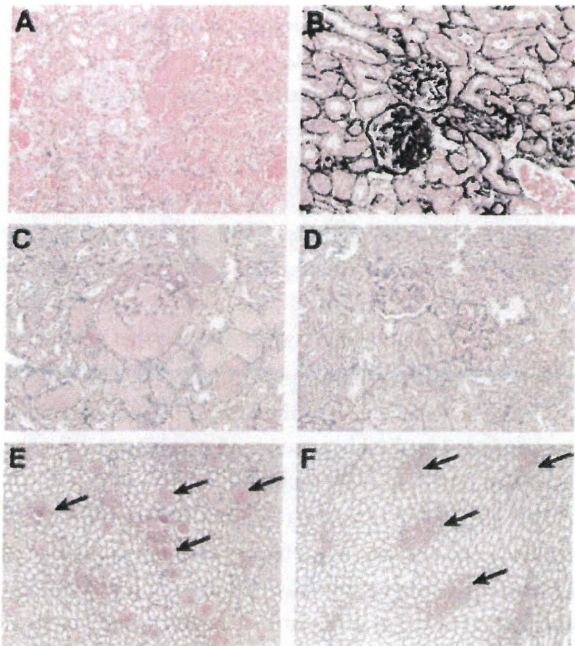
**Fig. 2.** Identification of the *krh* mutation. A: Results of direct sequencing of *Hr* cDNA of wild-type and *krh/krh* rats. The C-to-A nonsense mutation at position 1,238 is indicated by red arrowheads. The substitution produces a stop codon at amino acid residue 413 of protein HR. B: Schematic of the conserved domains in rat protein HR. RD1, RD2, and RD3 are used to denote the repression domains and ZF is used to denote the zinc-finger domain. The mutation site Ser413Ter is noted with a red arrowhead.

*krh* skin samples revealed that adenine (A) had been substituted for cytosine (C) at nucleotide position 1,238 from the start of the CDS (c. 1,238 C>A). This substitution resulted in a stop codon at amino acid residue 413 of the HR protein (p. Ser413Ter) (Fig. 2A). The truncated HR protein lacked a zinc-finger domain, a part of repression domain (RD) 1, and all of RD2 and RD3 (Fig. 2B). We characterized *krh* as an *Hr* nonsense mutation and called it *Hr<sup>krh</sup>*.

#### Focal glomerulosclerosis and proteinuria in the aged *Hr<sup>krh</sup>/Hr<sup>krh</sup>* rat

Histopathological examinations of organs that were taken from *Hr<sup>krh</sup>/Hr<sup>krh</sup>* rats at 40 weeks of age were performed. No lesions that are associated with autoimmune diseases were observed, however, prominent glomerular lesions were noted in the kidneys of the *Hr<sup>krh</sup>/Hr<sup>krh</sup>* rats. These lesions were focal lesions that had collapsed glomeruli and protein exudates in Bowman capsule and the renal tubules (Fig. 3A and 3C), and segmental prolifera-

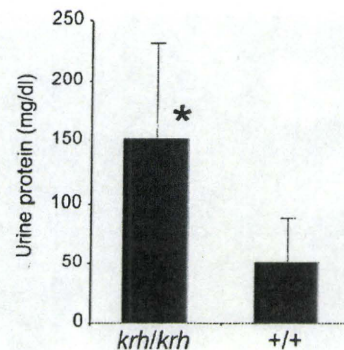




**Fig. 3.** Focal glomerular sclerotic lesions in 40-week-old  $Hr^{krh}/Hr^{krh}$  rat. Note that a collapsed glomerulus with protein exudates in Bowman's capsule and protein casts in renal tubules (A, C), and segmental proliferation of mesangial matrices (B, D) were seen. In the renal medulla, protein casts were notable in the collecting tubules (E) (arrows), but those in a wild-type F344 rat (+/+) were limited only to Henle's loop (F) (arrows). A: HE; B: PAM; C-F: PAS staining.

tion of the mesangial matrices (Fig. 3B and 3D). There was no inflammatory cell infiltration into the glomeruli and interstitium. In the renal medulla, protein casts were notably present in the collecting tubules (Fig. 3E). For the wild-type rats, protein casts were only observed in Henle's loop, possibly due to the effects of aging (Fig. 3F). These findings are indicators that the lesions that were observed in the F344- $Hr^{krh}/Hr^{krh}$  rat were caused by focal glomerulosclerosis (FGS). Moreover,  $Hr^{krh}/Hr^{krh}$  rats at 40 weeks of age had proteinuria. The  $Hr^{krh}$  homozygous rats had significantly higher urine protein concentrations than age-matched wild-type rats:  $152 \pm 80.3$  vs.  $51.0 \pm 38.5$  mg/dl, (average  $\pm$  SD),  $P < 0.02$  (Fig. 4).

From the electron microscopic observations, the segmental glomerular sclerotic lesions were characterized as having proliferating mesangial matrices (Fig. 5A). The proliferation was associated with the dendritic pro-



**Fig. 4.** Urine protein concentrations of  $Hr^{krh}/Hr^{krh}$  rats and wild-type F344 (+/+) rats. The  $Hr^{krh}$  homozygous rats had significantly higher urine protein concentrations than age-matched wild-type rats. Bars indicate standard deviation. \*:  $P < 0.02$ .

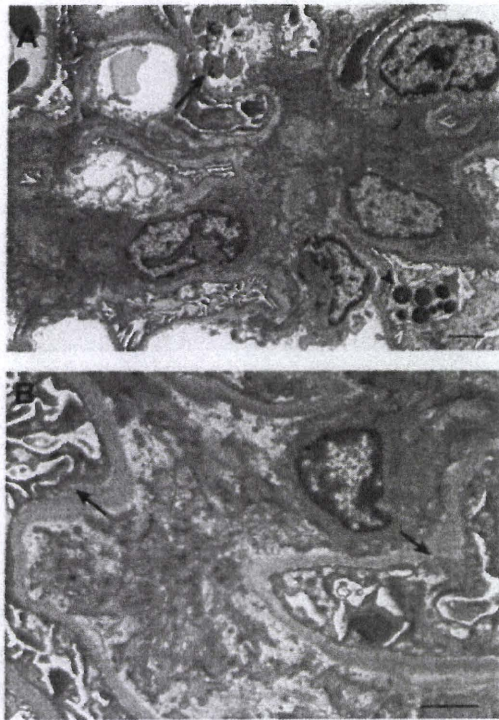
cesses of mesangial cells and on rare occasions with dense deposits in the mesangial regions. Foot process fusion was often observed in these glomeruli (Fig. 5B).

## Discussion

The *krh* mutation was identified as an *Hr* nonsense mutation and therefore called  $Hr^{krh}$ . Protein HR is a nuclear receptor co-repressor for multiple nuclear receptors, such as the thyroid hormone receptor and the vitamin D receptor [23]. In the hair follicle (HF), the absence of functioning HR proteins results in the synthesis of premature and dysregulated catagen. This results in the destruction of the normal HF architecture and abrogates the HF's ability to cycle [20]. The  $Hr^{krh}/Hr^{krh}$  rat has cystic hair follicles and suffers from a premature hair cycle (Fig. 1). The truncated HR protein that is encoded by the  $Hr^{krh}$  nonsense mutation is caused by a lack of functional domains which play important roles in regulating target genes [23]. Additionally, the mutation may cause nonsense mediated mRNA decay. Therefore, it is likely that  $Hr^{krh}$  may be a loss-of-function mutation. In humans, *HR* mutations are associated with congenital alopecia, such as ALUNC and APL [2, 4, 9]. Because rats are suitably sized for handling and manipulating [5, 21], the  $Hr^{krh}/Hr^{krh}$  rat may be a useful animal model for developing therapies for these human diseases.

The aged  $Hr^{krh}$  homozygous rat has FGS which is as-





**Fig. 5.** Fine structures of glomerular lesions in the  $Hr^{krh}/Hr^{krh}$  rat. A: Proliferative lesions of mesangial matrices associated with significant dendritic projections from mesangial cells. Platelet aggregation was observed in a capillary loop (arrow). Intracellular hyaline droplets were significant in a podocyte (arrowhead). B: Foot process fusion (arrows) and a proliferative lesion of mesangial matrices that was not associated with dense deposits in mesangial regions and the glomerular basement membrane. Bar: 2  $\mu$ m.

sociated with severe proteinuria. FGS is a descriptor for a pathological finding in the kidneys. When a nephritic range of proteinuria is observed, patients are diagnosed as having focal segmental glomerulosclerosis (FSGS). FSGS is classified by morphological variants including collapsed glomeruli, cellular proliferation, tip lesions, and diffuse mesangial proliferation [10]. The lesions that were observed in the F344- $Hr^{krh}$  rat are characteristic of collapsed glomeruli associated with protein exudates in Bowman's capsule and may involve a collapsed variant of FSGS. Thus, the F344- $Hr^{krh}$  rat may have potential as a model of nephritic FSGS.

FGS has not been reported for the APL or ALUNC family, and  $Hr$ -mutant mice, [2, 3, 7]. It is believed that

FGS has a heterogeneous etiology and that it may manifest through multiple genetic factors [10]. The F344- $Hr^{krh}$  rats were derived by employing ENU mutagenesis. The founder animals ( $G_1$  generation) were expected to carry no more than four ENU-induced mutations in their CDS, if the CDS occupies 1% of the genome [16]. The F344- $Hr^{krh}$  rats were mated by inbreeding without backcrossing to F344 rats to eliminate ENU-induced mutations other than the  $Hr^{krh}$  mutation. Thus, it is likely that the F344- $Hr^{krh}$  rat may harbor mutation(s) that may play a role in the pathogenesis of FGS. FGS in  $Hr^{krh}/Hr^{krh}$  rats might be caused by unidentified mutation(s) that were induced by ENU or the combined effects of such mutation(s) with the  $Hr^{krh}$  mutation.

The  $Hr^{rh}/Hr^{rh}$  mouse has the nonsense mutation (R597X) [8] and develops hypergammaglobulinemia. The excess immunoglobulins that are produced due to this disease are deposited in the basement membranes of the skin, spleen, liver, and kidney, and antinuclear antibodies are produced. These symptoms appear in young mice and increase in severity with age [14]. Although the F344- $Hr^{krh}/Hr^{krh}$  rat has a nonsense mutation (S413X), the mutation is not associated with an autoimmune disease or IgM, IgG, and C3 deposition in the kidneys (data not shown). Generally, pathological phenotypes that are associated with this disease are often influenced by a predisposed genetic background [18, 19]. Therefore, genes that are predisposed to causing autoimmune diseases in  $rh/rh$  mice may be absent in F344- $Hr^{krh}$  rats. By replacing the genetic background of F344- $Hr^{krh}$  with those of other rat strains, we might find autoimmune disease in  $Hr^{krh}/Hr^{krh}$  rats.

In summary, a novel rat mutant strain, F344- $Hr^{krh}$ , was established that carries an  $Hr$  nonsense mutation (S413X). In addition to the hair loss phenotype,  $Hr^{krh}$  homozygous rats suffer from proteinuria and FGS. Therefore, F344- $Hr^{krh}$  may have potential as a model of skin disease as well as nephritic FSGS.

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# A Mutation in the Gene Encoding Mitochondrial Mg<sup>2+</sup> Channel MRS2 Results in Demyelination in the Rat

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## Abstract

The rat demyelination (*dmy*) mutation serves as a unique model system to investigate the maintenance of myelin, because it provokes severe myelin breakdown in the central nervous system (CNS) after normal postnatal completion of myelination. Here, we report the molecular characterization of this mutation and discuss the possible pathomechanisms underlying demyelination. By positional cloning, we found that a G-to-A transition, 177 bp downstream of exon 3 of the *Mrs2* (MRS2 magnesium homeostasis factor (*Saccharomyces cerevisiae*)) gene, generated a novel splice acceptor site which resulted in functional inactivation of the mutant allele. Transgenic rescue with wild-type *Mrs2*-cDNA validated our findings. *Mrs2* encodes an essential component of the major Mg<sup>2+</sup> influx system in mitochondria of yeast as well as human cells. We showed that the *dmy/dmy* rats have major mitochondrial deficits with a markedly elevated lactic acid concentration in the cerebrospinal fluid, a 60% reduction in ATP, and increased numbers of mitochondria in the swollen cytoplasm of oligodendrocytes. MRS2-GFP recombinant BAC transgenic rats showed that MRS2 was dominantly expressed in neurons rather than oligodendrocytes and was ultrastructurally observed in the inner membrane of mitochondria. Our observations led to the conclusion that *dmy/dmy* rats suffer from a mitochondrial disease and that the maintenance of myelin has a different mechanism from its initial production. They also established that Mg<sup>2+</sup> homeostasis in CNS mitochondria is essential for the maintenance of myelin.

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## Introduction

Myelin is an essential component of the nervous tissue of higher vertebrates. It acts as a natural insulator of axonal segments allowing, at the same time, the maintenance of axonal integrity and the fast conduction of action potentials. It also reduces ionic currents across the axonal membrane and stabilizes the extracellular milieu within rapidly-firing axon bundles.

In the central nervous system (CNS), myelin is produced by oligodendrocytes, while in the peripheral nervous system (PNS), this function is achieved by Schwann cells. Myelination is completed within a relatively short period of time during mammalian development and requires a high rate of production and transport of different kinds of molecules, mostly proteins and lipids. In adult life, myelin is constantly remodeled and the maintenance of functional myelin sheaths requires a careful balance of *de novo* synthesis and turnover. It is quite clear that any event generating an imbalance in the myelination or remyelination process has the greatest chance of inducing dys- or demyelination of either the central or peripheral nervous system.

Our knowledge of the myelination process has benefited from careful observations conducted on human patients affected by one of

the many defects of myelination or myelin turnover. It has also benefited from researches carried out on animal models, mostly mutant mice and rats, including those that have been induced by transgenesis or genetic engineering in ES cell lines [1,2]. Some of these models have even allowed therapies to be developed in a preclinical setting [3]. Unfortunately, only a small number of the many genes that are directly or indirectly involved in the myelination process have been identified and only a few of these genes have been functionally annotated, for example, by the characterization of one or more mutant alleles. For this reason, any new mutation occurring spontaneously or after mutagenesis is of potential interest for unraveling the molecular mechanisms involved in myelin assembly.

In an earlier paper we reported the discovery and pathology of a rat mutation designated *demyelination* (symbol *dmy*), which is characterized by severe and progressive myelin breakdown in the CNS. We mapped the locus responsible for this myelin disorder to rat chromosome (Chr) 17, very close to the prolactin (*Pr*) locus, in a region homologous to human Chr 6p21.1-22.3 and mouse Chr 13 [4,5]. Based on its pathological features, as well as its genetic localization, this demyelination syndrome appeared to be unique, with no homologue so far reported in any other mammalian species, including humans.



## Author Summary

The myelin sheath that surrounds the axon of a neuron acts as a biological insulator. Its major function is to increase the speed at which impulses propagate along myelinated fibers in the central nervous system, as well as the peripheral nervous system. Alterations or damage affecting this structure (demyelination) result in the disruption of signals between the brain and other parts of the body. In the rat, mutations producing demyelination have been frequently identified and characterized and have contributed to a better understanding of the genetics of myelin development, physiology, and pathology. This paper reports the molecular characterization of a recessive allele responsible for the progressive disruption of myelin that was initially observed in mutant rats, previously named demyelination (*dmy*). This mutation generates an additional splicing acceptor site in an intron of the mitochondrial  $Mg^{2+}$  transporter gene (*Mrs2*), resulting in the insertion of a 83-bp genomic DNA segment into the *Mrs2* transcript and complete functional inactivation of the mutant allele. We firstly defined the biological function of MRS2 in mammals and demonstrated the crucial and unexpected role of MRS2 in myelin physiology. Our findings might be helpful in the development of new therapeutic strategies for demyelinating syndromes.

In this report we demonstrate that the causative gene (*Mrs2*) encodes a protein that is an essential component of the major electrophoretic  $Mg^{2+}$  influx system in mitochondria [6]. This gene has orthologues in other organisms, including lower eukaryotes and plants [7,8]. The protein shares many of the properties of bacterial CorA and yeast Alr1 proteins but its specific involvement in the myelination process was not known or even suspected.

## Results

### *dmy/dmy* rats exhibit a phenotype with typical demyelination

The pathology of homozygous *dmy/dmy* rats has been reported in detail previously [4]. Mutant rats exhibit no significant differences from their control littermates until 4 weeks of age. From 5 weeks on, flaccidity of the hind limbs becomes noticeable and evolves towards complete paralysis around 7–8 weeks of age. Progressive demyelination is observed in several parts of the CNS (Figure 1), namely the corpus callosum, the capsula interna, the striatum and the cerebellar peduncle, with major effects on the ventral and lateral parts of the spinal cord. Astrogliosis, which is a major feature of myelin disorder, is observed in demyelinated areas but motor neurons remain normal and there is no sign of associated inflammation in the white matter. The *dmy* mutation can then be regarded as a mutation affecting the maintenance and turnover of myelin rather than its initial production: this is typical demyelination [9].

### The *dmy* syndrome is associated with a mutation in a splicing site of *Mrs2*, a gene encoding a mitochondrial $Mg^{2+}$ channel

Out of 687 *dmy/dmy* mutant rats, collected from the 3,252 offspring of an intercross segregating for the *dmy* mutation, 23 individuals were found to carry a recombinant haplotype between the two loci that were used for the initial genetic mapping, namely; *Prl* (prolactin) and *Hh1ts* (testis-specific histone, H1t). Further investigation of these animals, using three novel informative SSLP markers, allowed us to narrow the genetic interval containing *dmy*

down to 0.22 cM, between markers *D17Kur17* and *D17Gol45*. Within this critical section, we found no recombination between the *dmy* locus and either *Aldh5a1* (aldehyde dehydrogenase family 5, subfamily A1) or *Mrs2* (mitochondrial 118 RNA splicing2) loci, among  $687 \times 2 = 1,374$  meioses. The rat genome databases revealed that *D17Kur17* and *D17Gol45* were at position 46.78-Mb and 47.26-Mb, respectively, on rat Chr 17, yielding a physical size of 0.48 Mb of DNA for the interval containing the *dmy* locus. This stretch of DNA contained 6 genes (Figure 2A).

Analysis by RT-PCR of the transcription products of these 6 genes revealed that the cDNA transcribed from the *Mrs2* gene was larger in *dmy/dmy* mutants than in the controls (Figure 2B). After sequencing, we found that the larger size of the *dmy* cDNA was due to the insertion of an 83 bp intronic sequence between exons 3 and 4. Comparison of the two genomic sequences revealed a G-to-A transition, 177 bp downstream of the end of exon 3 (Figure 2C, Figure S1), generating a novel splice acceptor site, which accounted for the addition of the 83bp stretch of intronic sequence to the mutant transcript. In addition, while the *Mrs2* gene normally encodes a 434 amino-acid protein, the intronic insertion leads to a shorter protein (106 amino acids) due to the occurrence of a stop codon as a consequence of frame shifting within the novel pseudo-exon X. The new protein consisted of the first 91 amino acids of normal (wild-type) MRS2 protein followed by an additional 15 amino acids transcribed from the intronic stretch (Figure 2D) [10]. No nucleotide alteration was observed between normal and mutant haplotypes in the cDNA transcribed from the other 5 genes (*Vmp*, *Dcdc2*, *Gpld1*, *Aldh5a1*, and *KIAA0319*). These findings strongly suggested that the G-to-A mutation in intron 3 of *Mrs2* in *dmy/dmy* rats was very likely causative of the neurological phenotype.

### *dmy/dmy* rats exhibit morphological and biochemical features characteristic of mitochondrial deficiencies

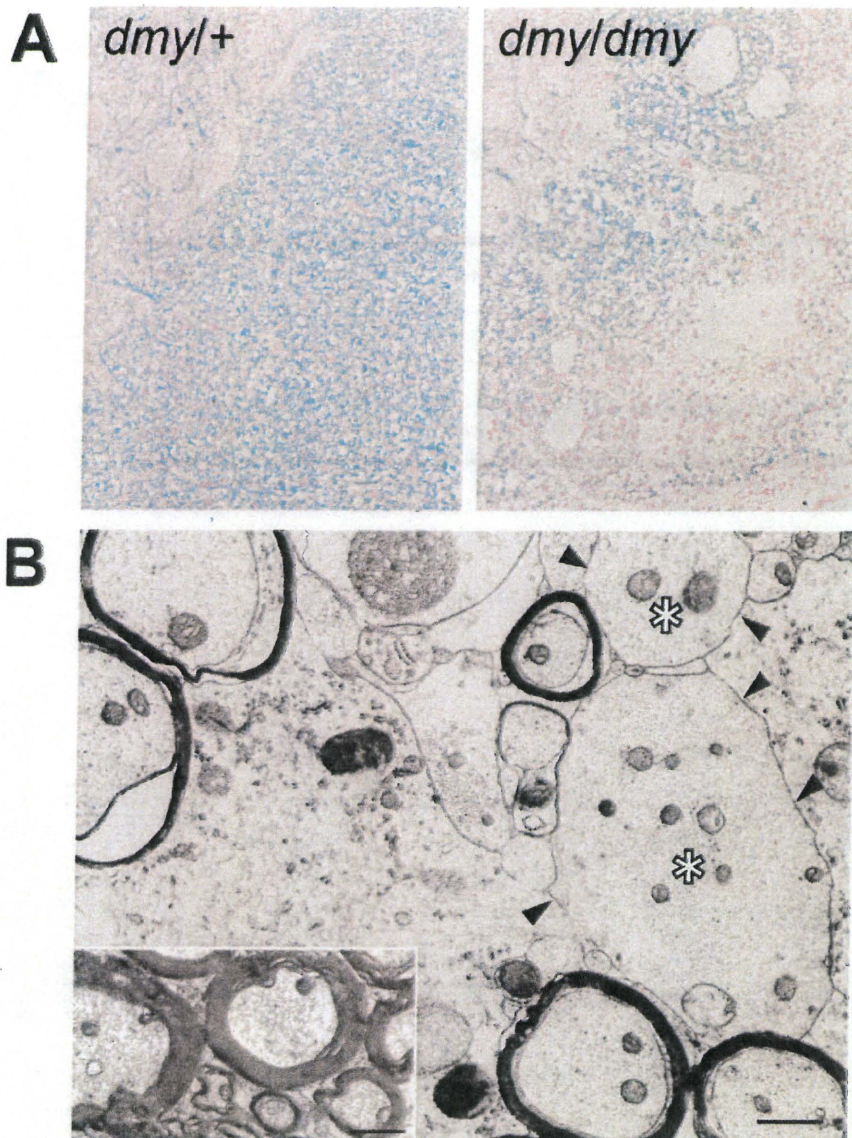
The MRS2 protein functions as a major transporter protein ( $Mg^{2+}$ ,  $Ni^{2+}$  and  $Co^{2+}$ ) in yeast as well as in human cells [10,11]. When this protein is functionally defective this leads to the "petite" phenotype in yeast and to cell death in human HEK 293 cells [11,12]. Because mitochondrial diseases in mammals are often accompanied by elevated lactic acid, reduced ATP, increased cytochrome oxidase (COX) activity, and the morphological alteration of mitochondria [13–15], we measured lactic acid levels and ATP contents in the CNS and performed morphological analyses of the CNS of *dmy/dmy* rats.

Lactic acid concentration in the cerebrospinal fluids was significantly elevated in *dmy/dmy* rats when compared with normal littermates:  $126 \pm 43.7$  mg/dL vs  $25 \pm 9.6$  mg/dL (average  $\pm$  SD),  $P < 0.002$  (Figure 3A). The ATP concentration was markedly reduced in *dmy/dmy* rats:  $265 \pm 79$   $\mu$ M/mg vs  $99 \pm 46$   $\mu$ M/mg (average  $\pm$  SD),  $P < 0.005$  (Figure 3B). In the affected *dmy/dmy* rats, swollen oligodendrocytes were often observed in the white matter, showing the increased COX reaction products (Figure 3C). Ultrastructurally, their cytoplasm contained many mitochondria and Golgi apparatus-like membrane structures (Figure 3D). These findings indicated that the mitochondria of *dmy/dmy* rats were functionally defective.

### Rescue of *dmy/dmy* mutant phenotypes by transgenic complementation

To ascertain that the molecular defect (*i.e.* G-to-A transition) observed in the *dmy* mutant haplotype was causative of the abnormal phenotype observed in *dmy/dmy* rats, we attempted to rescue the mutant phenotype by transgenic complementation. We





**Figure 1. Demyelination in *dmy/dmy* rats.** A. Histopathology of the cervical part of the spinal cord of *dmy/+* (left) and *dmy/dmy* (right) rats aged 10 weeks. Luxol fast blue-HE staining. Original magnification:  $\times 100$ . B. Electron microscopy of the cervical part of spinal cord of *dmy/dmy* rats (10 weeks). Naked axons with demyelination (arrowheads) are indicated by asterisks. Inset: control image of the spinal cord from the age-matched wild type rat. Axons are normally myelinated. Bar = 1  $\mu\text{m}$ . doi:10.1371/journal.pgen.1001262.g001

established two independent WTC.DMY-*dmy* lines, expressing each *Mrs2* wild-type cDNA under the control of a cytomegalovirus (CMV) promoter (Figure S2A), and found that all *dmy/dmy* transgenic rats exhibited a completely normal phenotype, with no paralysis of the hind limbs. Histopathological analyses demonstrated that both transgenic lines no longer exhibited any sign of demyelination of the CNS (Figure S2B). In addition, lactic acid levels of the cerebrospinal fluid of transgenic *dmy/dmy* rats had returned to the normal range (Figure S2C). Electron microscopic observations revealed that mitochondria of the oligodendrocytes in transgenic rats were normal in their morphology and number (Figure S2D). These findings confirmed that the molecular changes reported above and observed in the *Mrs2* gene were

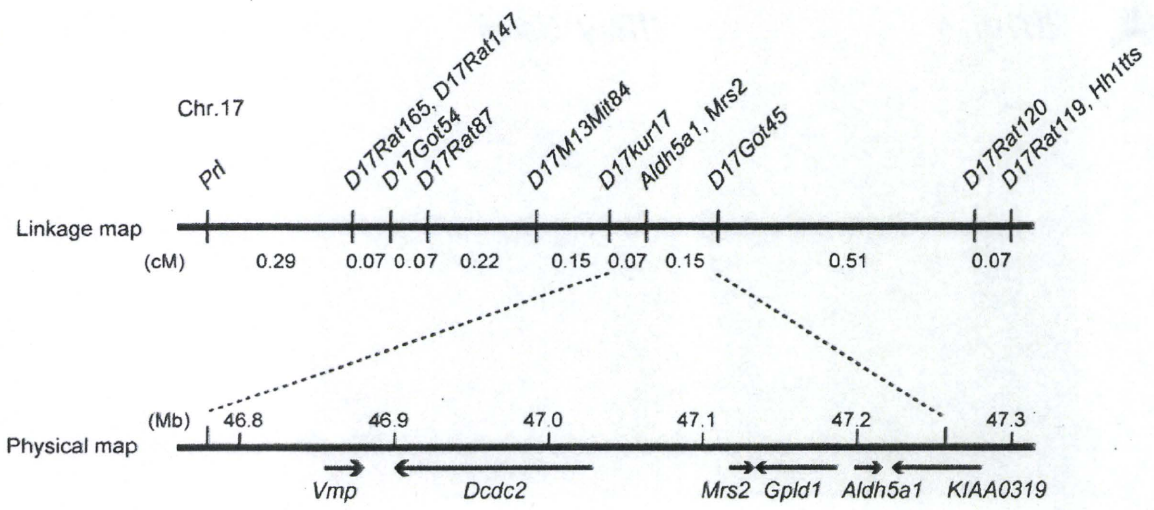
indeed causative of the *dmy*-mutant phenotypes. For this reason we decided that the symbol of the mutant allele should, from now on, be changed to *Mrs2<sup>dmy</sup>*.

#### MRS2-GFP recombinant protein is expressed in the mitochondria

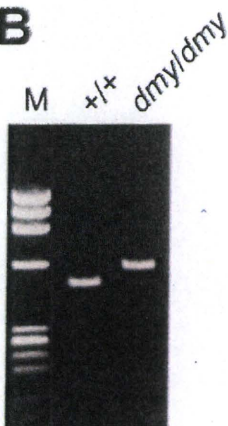
To characterize the tissues and cell types expressing MRS2 as well as the subcellular localization of this protein in the CNS, we generated a strain of rats transgenic for a recombinant MRS2-GFP BAC clone. These transgenic rats were expected to express recombinant protein under the control of the endogenous, normal *Mrs2* promoter. We found that cytoplasmic dot-like MRS2-GFP



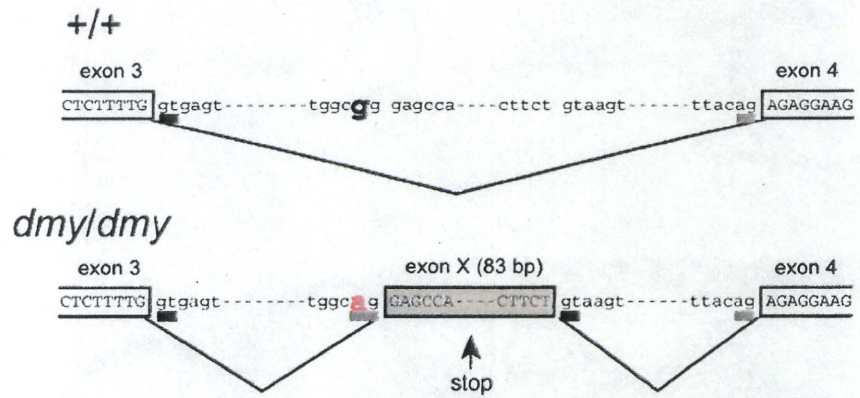
**A**



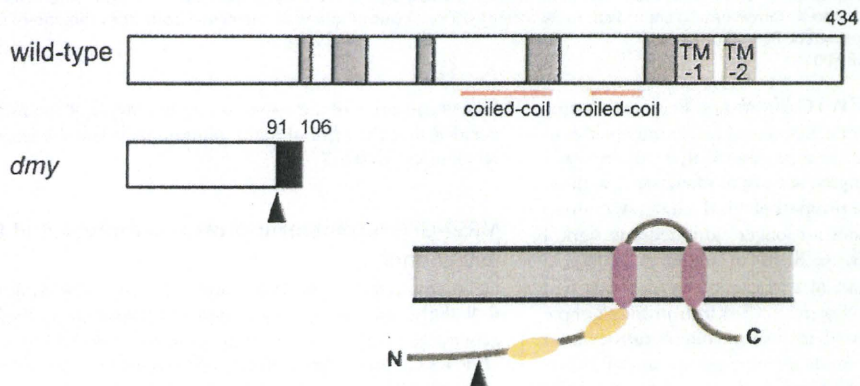
**B**



**C**



**D**



**Figure 2. Positional cloning of the *dmy* mutation.** A. The *dmy* locus was localized within a 0.22-cM region of chromosome 17 between *D17Kur17* and *D17Got45* and no recombination was observed with SSLP markers designed from *Aldh5a1* and *Mrs2* genomic sequences in 1,374 informative meioses. Within the 0.48-Mb physical interval between *D17Kur17* and *D17Got45*, harboring the *dmy* locus, 6 genes: *Vmp* (vesicular membrane protein p24), *Dcdc2* (doublecortin domain containing 2), *Mrs2* (MRS2 magnesium homeostasis factor (*S. cerevisiae*)), *Gpld1* (glycosylphosphatidylinositol specific phospholipase D1), *Aldh5a1* (aldehyde dehydrogenase family 5, subfamily A1), and *KIAA0319*, were previously mapped. B. A larger RT-PCR product was obtained when amplifying the 5' region of *Mrs2* cDNAs from *dmy/dmy* rats with a primer set of rMrs2l-3&4 (5'-TGTAAGTACTACCGAGTCC-3' and 5'-TCTGGAGTTATCACAGCCTCA-3'). M: molecular marker,  $\Phi$ X174-*Hae*III digest. C. Upper: Genomic organization in the vicinity of intron 3 of the *Mrs2* wild-type allele. Lower: Genomic rearrangements in the same intron 3 of the *Mrs2<sup>dmy</sup>* mutant allele. In the *Mrs2<sup>dmy</sup>* mutant allele, a novel splice acceptor site was generated as a consequence of a G-to-A transition at 177 bp downstream of the end of exon 3. An 83-bp genomic sequence (boxed in gray), downstream of the recently generated acceptor site (tgccag), is then inserted into the *Mrs2* mutant transcript. This sequence contains a premature stop codon (vertical arrow), which truncates the protein almost immediately downstream of exon 3. D. Schematic representations of the wild-type and *dmy* MRS2 proteins. Conserved amino acid residues and transmembrane domains are indicated by grey and purple boxes, respectively. Coiled-coil regions are indicated by horizontal orange lines. The position of the *dmy* mutation is indicated by an arrowhead, and the additional 15 residues (GATWTPRILEECLES), indicated by a black box, are deduced to be added subsequently. Bottom: Schematic representation of the topology of MRS2. Purple: transmembrane domains, Orange: coiled-coil regions. The position of the *dmy* mutation is indicated by an arrowhead.

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signals were observed in neurons throughout the CNS. To a lesser extent, astrocytes and oligodendrocytes also exhibited occasional expression of MRS2 (Figure S3). Confocal microscopy demonstrated that MRS2 is located in the mitochondria (Figure 4A–4C). Moreover, immunoelectron microscopic examinations with anti-GFP antibody revealed that MRS2 is localized in the inner membrane of the mitochondria (Figure 4D). MRS2 expression was also observed in the myocardium, liver, testis and skeletal muscles (Figure S4).

#### Microglia activation and high expression of inflammatory cytokines were observed in *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats

Microglial activation, characterized by cellular hypertrophy, has been reported in various dysmyelinating and demyelinating pathologies. To assess microglial activation, we performed immunohistochemistry for IBA1, a specific marker of microglia. In *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats, prolonged activation of microglia was prominently observed at 6–7 weeks of age (Figure 5A and 5B), the stage at which clinical symptoms such as flaccid paralysis were commonly observed. Expression levels of proinflammatory cytokines, such as *Il1b* and *Il6*, were also significantly higher in *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats than in wild-type littermates at 6 weeks of age (Figure 5C).

#### Discussion

Characterization, by positional cloning, of the molecular defect responsible for the demyelinating phenotype observed in adult *dmy/dmy* rats led us to incriminate a mutation in the *Mrs2* gene. No mutant allele before *Mrs2<sup>dmy</sup>*, which we report here, has ever been reported at this locus in any mammalian species.

*Mrs2* encodes an inner membrane  $Mg^{2+}$  channel in mitochondria and belongs to a family with orthologous copies in a wide range of species [10,12]. *Mrs2* was originally identified in yeast, and orthologous copies of this gene have been identified in a variety of organisms, including bacteria (*CorA*), fungi (*Alr1*), and plants (*AtMrs2*). All proteins in the family have the same substrate selectivity: they transport  $Mg^{2+}$ ,  $Co^{2+}$  and some other divalent cations across the mitochondrial membrane. Even if these proteins exhibit relatively low sequence similarities, they all have a few important domains at the same relative position and can functionally complement each other over a wide range of phylogenetic distances [16,17]. In mammals, the normal protein MRS2 has two universally conserved transmembrane domains (TMs) and a conserved Gly-Met-Asn (GMN) motif close to the first TM domain that forms part of the pore and is essential for  $Mg^{2+}$  transport [18] (Figure 2D, Figure S5). As we demonstrated, the protein is truncated in *dmy/dmy* mutant rats, having lost both of its

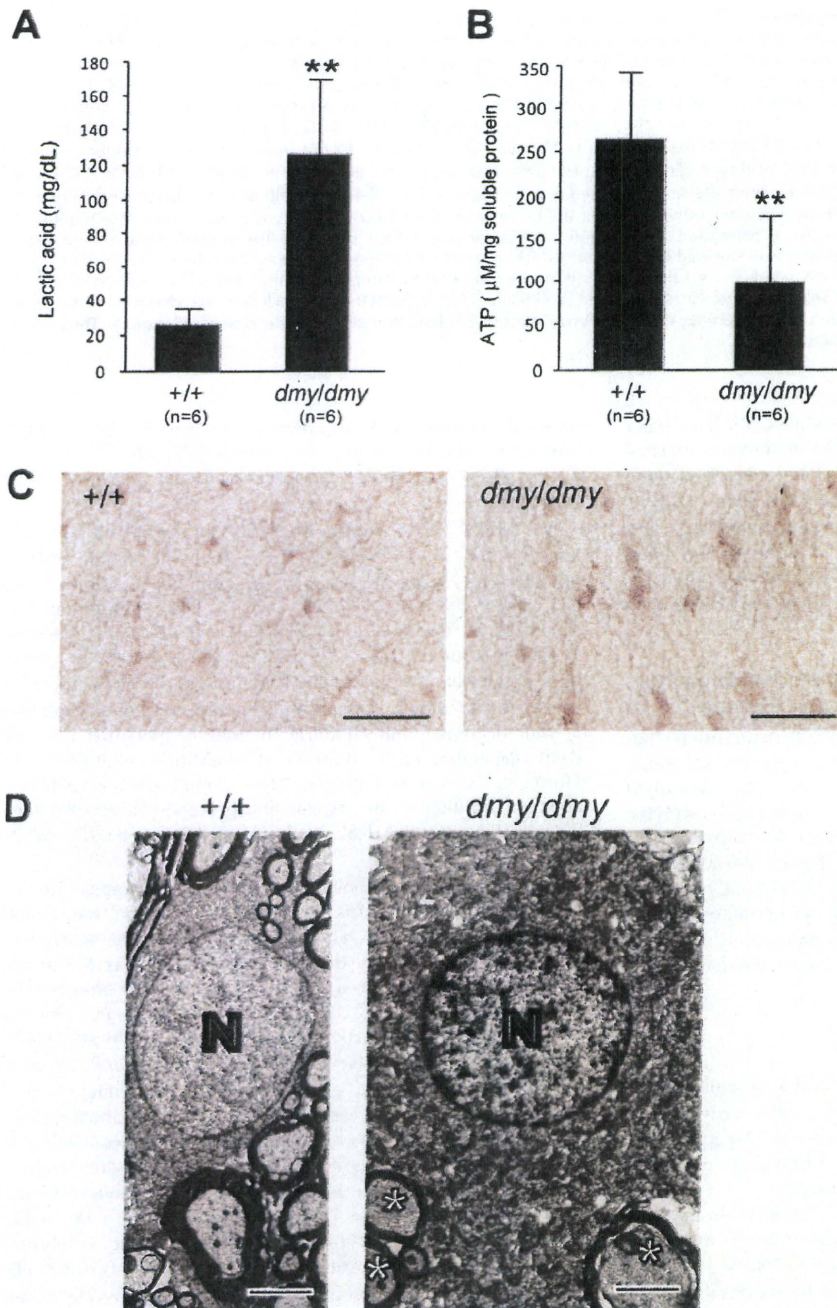
essential domains and accordingly its function of an  $Mg^{2+}$  transmembrane transporter. In other words, *Mrs2<sup>dmy</sup>* is a null allele, which is totally consistent with its recessive allelic interaction.

An MRS2 is a major transport for  $Mg^{2+}$  uptake into mitochondria, its function would be expected to be important, if not essential, for the maintenance of respiratory complex I and accordingly for cell viability [6,11]. This assumption was supported by the analysis of MRS2 knock-down, mediated by shRNA in a human HEK-293 cell line, which resulted in a series of physiological changes ranging from transient reduction of  $Mg^{2+}$  uptake to the complete loss of mitochondrial respiratory complex I, with decreased mitochondrial membrane potential and cell death, depending on the duration of knock-down treatment [11]. However, if we consider the phenotype of our mutant rat, which is apparently limited to the myelination process with a rather long lifespan, the role of MRS2 in the maintenance of cell integrity should be reconsidered.

Considering the pathological features that appear to be characteristics of the *Mrs2<sup>dmy</sup>* allele on the one hand, and MRS2-specific functions, as described above on the other, it is logical to consider that the demyelinating syndrome in mutant rats results from a mitochondrial disease. This assertion is supported by the observation of an elevated rate of lactic acid in the cerebrospinal fluid, reduced ATP in the brain, increased COX activity, and the morphological alteration of mitochondria, which is generally considered a major characteristic of mitochondrial diseases [13–15]. An increase in mitochondria is characteristic of cells with reduced respiratory capacity [19]. The association of mitochondrial dysfunction with demyelination (or leukodystrophy) has been already reported in Leigh syndrome and mitochondrial DNA depletion syndrome [20–23]. The tissues most frequently affected in these mitochondrial diseases are the cerebrum, peripheral nerves, and skeletal muscles, presumably because cells of these tissues require more energy than any other cells in the body. Unfortunately, the detailed pathophysiological mechanism(s) leading to demyelination in these diseases has not yet been unraveled. We consider that our mutant rat could be an interesting tool for investigating this matter.

Mitochondrial dysfunction has also been observed in multiple sclerosis (MS), one of the most common demyelination diseases, but here again many aspects of the pathophysiology require further investigation [24,25]. This difficulty of linking gene functions with a specific syndrome is not so surprising if we consider that, according to the most recent estimates, there may be as many as 1,500 nuclear-encoded mitochondrial proteins [26] and that less than half have been identified with experimental support. Clearly, a complete protein inventory of this organelle





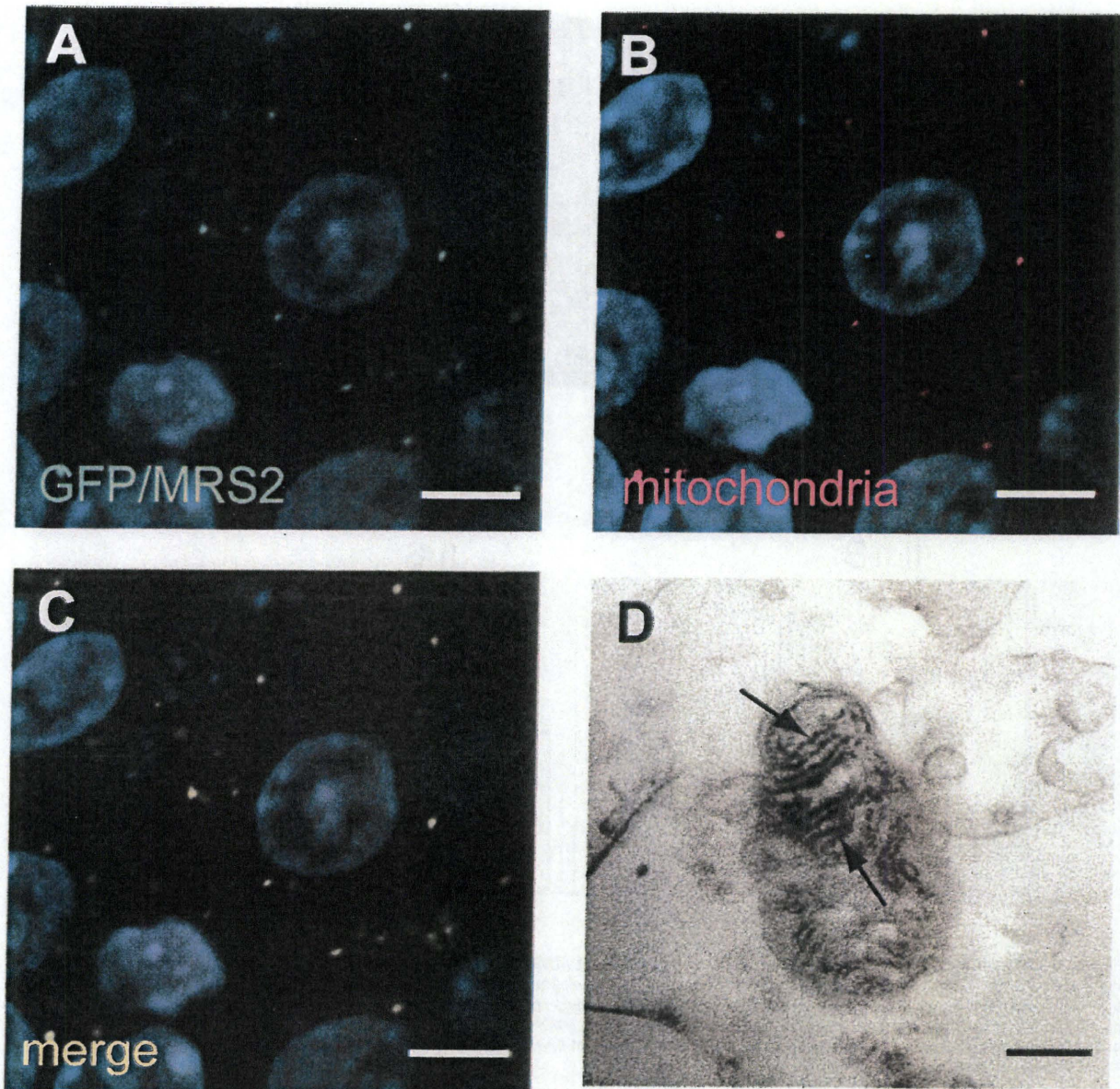
**Figure 3. Biochemical and morphological abnormalities in the mitochondria of *dmy/dmy* mutant rats.** A. Lactic acid concentration in cerebrospinal fluid of 6–7-week-old *dmy/dmy* rats and age-matched wild-type rats. \*\*,  $P < 0.002$ . B. ATP levels in the brain of 6–7-week-old *dmy/dmy* rats and age-matched wild-type rats. \*\*,  $P < 0.005$ . C. Cytochrome oxidase staining of the spinal cords of 6–7-week-old *dmy/dmy* (right) and age-matched wild-type (left) rats. Swollen oligodendrocytes were often seen they showed increased COX reaction product. Bar = 50 µm. D. Electron microphotographs of a swollen oligodendrocyte in a *dmy/dmy* rat (right) and an oligodendrocyte in a control wild-type rat. White matter of thoracic spine at 6 weeks of age. N: Nucleus of the oligodendrocyte. Axons adjacent to the oligodendrocyte are indicated by asterisks. Bar = 2 µm.  
doi:10.1371/journal.pgen.1001262.g003

across tissues would provide a molecular framework to relate mitochondrial biology and pathogenesis [27].

A point concerning *Mrs2* gene expression in the CNS that is worth noting after our experiments and observations is that the

gene in question is expressed at a higher rate in neurons than in oligodendrocytes (Figure 4, Figure S3). This was rather unexpected if we consider that oligodendrocytes are the cells actually responsible for myelination of the CNS. At this time, it remains





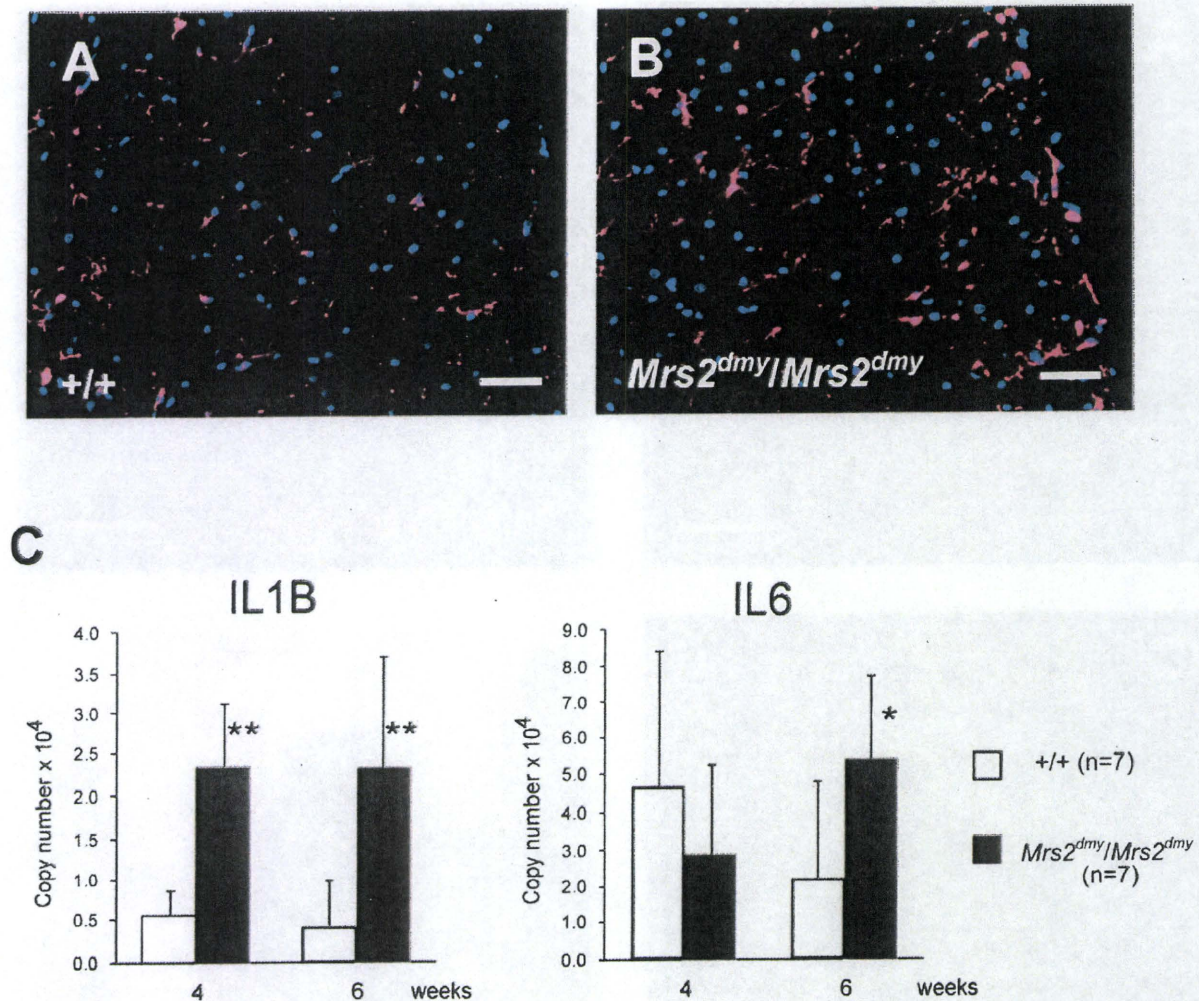
**Figure 4. Expression of MRS2 protein in the mitochondria.** MRS2-GFP recombinant protein (Green) was seen in the cytoplasm of pyramidal cells (A). MRS2-GFP signals were colocalized with the mitochondria (B), as shown in the confocal image of GFP and mitochondrial immunohistochemistry (C). Nuclei were stained with DAPI (Blue). Bar: 5  $\mu$ m. Immunoelectron microscopy using anti-GFP antibody revealed that MRS2-GFP signals were localized in the inner membrane of the mitochondria (arrows) (D). Bar: 200 nm.  
doi:10.1371/journal.pgen.1001262.g004

unclear whether the demyelination in *dmy/dmy* rats is triggered cell-autonomously or cell-nonautonomously. Instead, it is likely that demyelination is enhanced by the surrounding cells, such as activated microglia and astroglia. At 6 weeks of age, when *dmy/dmy* rats began to exhibit ataxia [9], cytokine levels were elevated and microglia were activated (Figure 5), and it is considered that activated microglia cause neuronal damage through the release of potentially cytotoxic molecules, such as proinflammatory cytokines, reactive oxygen intermediates, proteinases, and complement proteins [28]. Oligodendrocytes show greater vulnerability to such

molecules [29,30]. Additionally, Kuwamura and co-workers reported prominent astrogliosis and many ED-1-positive macrophages in myelin-destroyed areas [9]. When considered together, these morphological observations led us to believe that the demyelination observed in *dmy/dmy* rats is probably enhanced by activated microglia and astroglia.

In summary, we identified *Mrs2<sup>dmy</sup>* as a loss-of-function mutation of the *Mrs2* gene that normally encodes  $Mg^{2+}$  transporter protein of the mitochondrial inner membrane. Our observations also demonstrate that the mechanisms underlying the





**Figure 5. Activation of microglia in the central nervous system of *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats.** Immunohistochemistry for Iba1 in the lumbar part of the spinal cord of wild-type (A) and *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats (B) at 6 weeks of age. Signals of Iba1 (AlexaFluor 546 nm; red), which is upregulated during the activation of microglia, are seen in *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats much more than the wild-type control. Nucleus is stained with DAPI (blue). C, Inflammatory cytokine mRNA expression in the CNS of wild-type (□) and *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* (■) rats. IL1b expression was elevated in *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats at 4 and 6 weeks of age. IL6 was elevated in *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats at 6 weeks of age. \*  $P < 0.05$ , \*\*  $P < 0.005$ . doi:10.1371/journal.pgen.1001262.g005

initial development of myelin (myelination) are different from those that are involved in its maintenance and turnover since, in *Mrs2<sup>dmy</sup>/Mrs2<sup>dmy</sup>* rats, myelin development is normal while its maintenance is defective. Our mutant rats also appear to be an excellent animal model, not only to evaluate the causal relationships between primary mitochondrial dysfunction and subsequent demyelination, but also for the development of therapies making use, for example, of cell transplantation.

## Materials and Methods

### Genetic fine mapping of *dmy*

Congenic strains WTC (NBRP#0020) and WTC.DMY-*dmy* (NBRP#0021) were both from the National BioResource Project - Rat, Kyoto University (Kyoto, Japan). (WTC.DMY-*dmy* × BN/SsNSlc)F1(+/*dmy*) rats were intercrossed to produce F2 progeny.

*dmy/dmy* homozygotes were identified at 7–8 weeks of age, when paralysis of the hind limbs was obvious. 687 *dmy/dmy* rats were collected out of 3,252 F2 animals (~21%) and used for fine mapping of the *dmy* locus. Simple sequence length polymorphisms (SSLPs) from the *Prl* (prolactin) and *Hh1ts* (Testis-specific histone, H1t and H4t) genes were used for genotyping as described [31]. To refine the limits of the recombinant interval between *Prl* and *Hh1ts*, two gene-specific and one anonymous SSLP markers were used: *Mrs2* (5'-TCTCCCTTGCCTCTATCTCTCGTCT-3', 5'-CCTGCAGTACTGGGTAAGCCTGATG-3'), *Aldh5a1* (5'-GT-TAACTGCACAAGAGCAAGCCAGT-3', 5'-GCTAATGTTA-AGTCAATGGGGTGAGG-3'), and *D17Kur17* (5'-ACCTCTTT-TGCCAGCAITG-3', 5'-CCCTGGGATTGGTCCATA-3').

All animal experiments were approved by the Animal Research Committee of Kyoto University and were conducted according to the Regulations on Animal Experimentation of Kyoto University.



### RT-PCR and direct sequencing

Total RNA was isolated from the brain of 5-week-old animals using ISOGEN (NIPPON GENE, Tokyo, Japan). RT-PCR and direct sequencing of the PCR products were carried out as described previously [32].

### Transgenic rescue and recombinant BAC transgenics

A construct containing the CMV promoter, 1.45-kb of the *Mrs2* coding sequence, and SV40 polyA signal was excised from the vector (pCMV-Script; Agilent Technologies, CA, USA) and used as a transgene, which was microinjected into the pronuclei of fertilized oocytes collected from Crj:Wistar rats. Transgenic offspring founder rats were then crossed with WTC- *+ / dmy* rats and then backcrossed again to WTC- *+ / dmy* rats to obtain *dmy / dmy* homozygous and also hemizygous for the transgene (*dmy / dmy*, *tg / -*). Expression of the transgene was confirmed by RT-PCR with primers (5'-GCCAATGGAGATCCAAATTTT-3', 5'-GGGAG-GTGTGGGAGGTITTT-3') to detect SV40 polyA sequence. Brain RNA was treated with DNase I (New England BioLabs) to remove contaminating genomic DNA and then subjected to cDNA synthesis.

A rat BAC clone, CHORI-230-9K13, including the rat *Mrs2* gene was modified to express MR2SL-EGFP fusion protein under the endogenous promoter by ET recombination technology [33]. Modified genomic DNA was excised from the vector and then used for *in ovo* transgenesis.

### Quantitative PCR

Real-time PCR was performed using the Thermal Cycler Dice Real Time System (Takara Bio Inc., Otsu, Japan) with SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan). By monitoring amplification curves of a test sample and reference samples that contained 101–106 molecules of the gene of interest, the number of target molecules in the test sample was analyzed. The number of target molecules was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an internal control. The primers used are as follows: 5'-GCTGTGGCAGCTACC-TATGTCITG-3' and 5'-AGGTGGTTCATCATCCCACGAG-3' for the rat Interleukin-1b (*Il1b*), 5'-CCACTTCACAAGTCCG-GAGGCTTA-3' and 5'-GTGCATCATCGCTGTTTCATACA-ATC-3' for the rat interleukin-6 (*Il6*), 5'-GGCACAGT-CAAGGCTGAGAAATG-3' and 5'-ATGGTGGTGAAGACGC-CAGTA-3' for rat *Gapdh*.

### Electron microscopy

Perfusion fixation through the left ventricle was conducted with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains and spinal cords were dissected and stored in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB, then post-fixed with 2% osmic acid for 2 hours and embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined by a Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan).

### Immunohistochemistry

Immunohistochemistry was performed as described previously [9]. The following primary antibodies were used: monoclonal anti-2', 3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) for oligodendrocytes (1:1,000; Sigma, St. Louis, MO, USA), monoclonal anti-mitochondria (1:100; Abcam, Cambridge, MA, USA), polyclonal anti-GFAP for astrocytes (1:1,000; Dako, Carpinteria, CA, USA), polyclonal anti-Iba1 for microglia/macrophages (1:200; Wako Pure Chemical Industries, Osaka, Japan). Cy3-

conjugated anti-mouse IgG (1:500; Jackson Laboratories) or Alexa 588-conjugated anti-rabbit IgG (1:500; Molecular Probes) antibody was reacted. Nuclei were counterstained with DAPI (Vector Laboratories). Signals were detected with a fluorescence microscopy (Olympus, Tokyo, Japan) or a confocal imaging system (C1Si; Nikon, Tokyo, Japan).

For immunoelectron microscopy, PFA-perfused frozen sections were incubated with rabbit antibody against fluorescent protein (1:2,000; Molecular Probes) at 4 °C overnight. After washing in PBS, peroxidase-conjugated anti-rabbit IgG Fab fraction (Jackson Laboratories, 1:1,000) and immunoreactions were reacted 3,3'-diaminobenzidine substrate kit (Vector Laboratories), postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, and then embedded in epoxy resin. Ultrathin sections were examined by electron microscopy (Hitachi, Tokyo, Japan).

### Lactic acid measurements

Cerebrospinal fluid was collected from *dmy / dmy*, wild-type littermates, and *dmy / dmy* with the normal *Mrs2* transgene at 6–7 weeks of age under isoflurane anesthesia. They were then mixed with 0.8N perchloric acid to inactivate proteins. After centrifugation, lactic acid concentrations of the supernatants were measured by Determiner LA (KYOWA MEDEX Co., Ltd., Tokyo, Japan).

### Cytochrome oxidase histochemistry

Frozen spinal cord sections were prepared. Then, 100 µl of freshly prepared reaction buffer [50 mM Tris/HCl (pH 7.4), 0.5 mg/ml diaminobenzidine, 20 µg/ml catalase and 0.50 mg/ml cytochrome C] was added to each section and slides were incubated for 30 min at 37°C.

### ATP measurements

Rats were sacrificed by cervical dislocation and the brains were immediately excised, frozen in liquid nitrogen, and stored at -80°C until measurement. In order to release cellular ATP, frozen tissue (25 mg) was boiled for 2 min after the addition of 300 µl water containing 100 mM Tris/HCl (pH 7.75) and 4 mM EDTA. Samples were placed on ice and homogenized by sonification (micro tip, 1 s × 10 pulse). ATP concentrations were determined using the ATP bioluminescence assay kit HS II (Roche) according to the manufacturer's protocol. Data were standardized to the protein concentration which was determined by Coomassie Plus – the better Bradford assay kit (Pierce).

### Statistical analysis

Statistical differences in lactic acid, ATP and mRNA expressions between wild-type and *dmy / dmy* rats were evaluated using the Mann-Whitney U test.

### Supporting Information

**Figure S1** Detection of the *Mrs2<sup>dmy</sup>* mutation. A. Chromatograms showing the *Mrs2<sup>dmy</sup>* G-to-A mutation. Upper: wild-type genome. Lower: *Mrs2<sup>dmy</sup> / Mrs2<sup>dmy</sup>* genome. The *Mrs2<sup>dmy</sup>* mutation disrupted *AclI* restriction site (GGCG) in the *Mrs2<sup>dmy</sup> / Mrs2<sup>dmy</sup>* genome. B. Molecular diagnosis of the *Mrs2<sup>dmy</sup>* mutation. In the wild type, the 349-bp PCR product amplified with primers rMrs2-31&32 (5'-AAAGTTTGACAAAGAAGGAAACG-3' and 5'-GGGGATGGAGGGCTATGTAA-3') is digested with *AclI* but not in *Mrs2<sup>dmy</sup> / Mrs2<sup>dmy</sup>* mutant rats. M:  $\Phi$ X174-*HaeIII* digests. Found at: doi:10.1371/journal.pgen.1001262.s001 (1.15 MB TIF)

**Figure S2** Transgenic rescue experiment. A. Expression of the transgene in the brain of a transgenic rat. Brain cDNA from Tg-



positive rats (Lanes 2 and 3) and Tg-negative rats (Lanes 1 and 4) was used as templates. Brain RNA was treated with DNaseI to remove contaminating genomic DNA. M:  $\Phi$ X174 *Hae*III digests. B. Histopathology of the cervical part of the spinal cord of *dmy/dmy* transgene-negative rats (left) and *dmy/dmy* transgene-positive (right) rats aged 10 weeks. Luxol fast blue-HE staining. Original magnification:  $\times 100$ . C. Lactic acid concentration in cerebrospinal fluid of 6-7-week-old *dmy/dmy* rats and age-matched *dmy/dmy* *Mrs2* cDNA-transgenic rats. Elevated lactic acid ( $126 \pm 43.7$  mg/dL) was reduced to normal level ( $22 \pm 3.1$  mg/dL). \*\*,  $P < 0.002$ . D. Electron microphotograph of an oligodendrocyte in a *dmy/dmy* transgene-positive rat. Densely packed mitochondria (arrowheads) were found in the cytoplasm. Bar: 2  $\mu$ m.

Found at: doi:10.1371/journal.pgen.1001262.s002 (5.52 MB TIF)

**Figure S3** MRS2 expression in the CNS of *Mrs2*-GFP recombinant BAC transgenic rats. MRS2 signals were mainly found in neurons (A), and occasionally in GFAP-positive astrocytes (B) and CNP-positive oligodendrocytes (C). Left: Bar: 50  $\mu$ m. Center, Right: Bar: 20  $\mu$ m.

Found at: doi:10.1371/journal.pgen.1001262.s003 (3.15 MB TIF)

**Figure S4** MRS2 expression in *Mrs2*-GFP recombinant BAC transgenic rats. MRS2 signals were observed in the myocardium (A), liver (B), testis (C) and skeletal muscles (D). Bar: 50  $\mu$ m.

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## OXIDATIVE STRESS-INDUCED TUMORIGENESIS IN THE SMALL INTESTINES OF DNA REPAIR-DEFICIENT MICE

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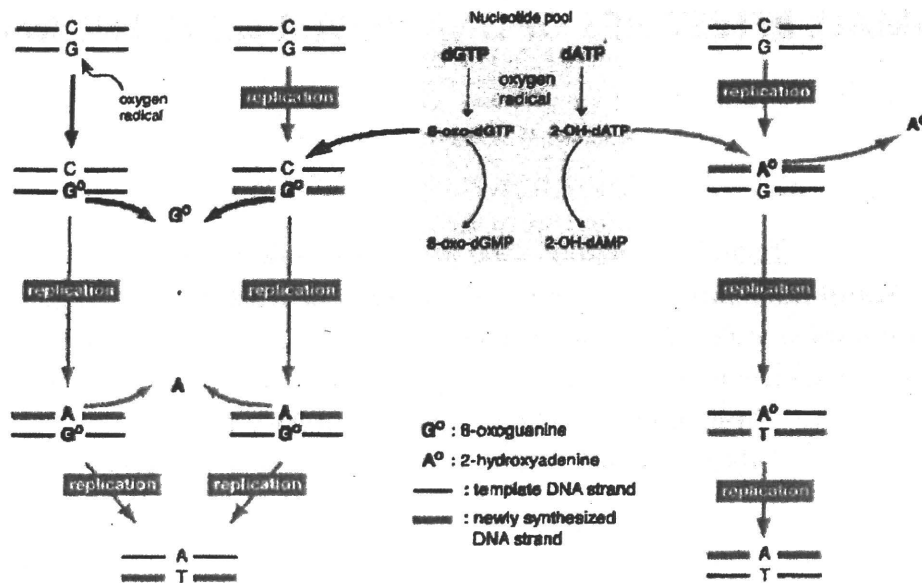
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Oxygen radicals are produced through normal cellular metabolism, and the formation of such radicals is further enhanced by radiation and by various chemicals. Oxygen radicals attack DNA and its precursor nucleotides, and consequently bases with various modifications are introduced into the DNA of normally growing cells. One such modified base, 8-oxo-7, 8-dihydroguanine (8-oxoG) is highly mutagenic because of its ambiguous pairing property. Three enzymes, MTH1, OGG1, and MUTYH, play important roles in avoiding 8-oxoG-related mutagenesis in mammalian cells (Figure 1)<sup>1-3</sup>.

We have established an experimental system for oxidative DNA damage-induced mutagenesis and tumorigenesis in the gastrointestinal tracts of mice<sup>4</sup>. Oral administration of an oxidizing reagent, potassium bromate (KBrO<sub>3</sub>), effectively induced G:C to T:A transversions and epithelial tumors in the small intestines of *Mutyh*-deficient mice, implying the significance of *Mutyh* in the suppression of mutagenesis and tumorigenesis induced by oxidative stress (Figure 2, 3). We performed mutation analysis of the tumor-associated genes amplified from the intestinal tumors developed in four mutant mice that had been treated with KBrO<sub>3</sub>. Many tumors had G:C to T:A transversions in either *Apc* or *Cttnb1*. No mutations were found in either *K-ras* (exon 2) or *Trp53* (exon 5-8). Our findings confirm the association between MUTYH-deficiency and the recessive form of hereditary multiple colorectal adenoma/carcinoma in humans, known as MUTYH-associated familial adenomatous polyposis<sup>5</sup>, with the characteristic feature; G:C to T:A transversions in the GAA sequence context. Also, our results suggest that the abnormality in Wnt signal transduction pathway is causatively associated with oxidative stress induced tumorigenesis in the small intestines of *Mutyh*-deficient mice (Figure 4). In addition, the





**Figure 1** Mutagenesis induced by oxidative DNA and its avoiding mechanisms in mammals.

Among the various types of oxidative damage in DNA, the oxidized forms of guanine and adenine, 8-oxo-7,8-dihydroguanine and 1,2-dihydro-2-oxoadenine, can form relatively stable base pairs with either adenine or guanine in DNA, respectively. During DNA replication, they are thought to induce spontaneous mutagenesis, such as A: T to C: G and G: C to T: A transversions. The direct oxidation of DNA by reactive oxygen species has been reported to generate a substantial amount of 8-oxo-7,8-dihydroguanine but little 1,2-dihydro-2-oxoadenine. In contrast, 1,2-dihydro-2-oxoadenine is generated exclusively by the oxidation of dATP in the nucleotide pool. Studies on mutator mutants have revealed that *Escherichia coli* has several error-avoiding mechanisms that minimize the deleterious effects of 8-oxo-7,8-dihydroguanine, and in which MutT, MutM and MutY proteins play important roles. MutT protein hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate, thus avoiding the occurrence of A: T to C: G transversion mutations during DNA replication. MutM and MutY proteins are DNA glycosylases, the former excises 8-oxoG paired with cytosine whereas the latter removes adenine paired with 8-oxo-7,8-dihydroguanine. Mammalian cells are also equipped with elaborate error-preventing mechanisms similar to those found in prokaryotes; MTH1 as a MutT homolog, OGG1 as a functional homolog for MutM, and MUTYH (MYH) as a MutY homolog. Recent studies showed that MTH1 effectively hydrolyzes 2-OH-dATP as well as 8-oxo-dGTP, while MUTYH has the ability to excise 1,2-dihydro-2-oxoadenine inserted opposite guanine in the template strand as well as the ability to remove adenine incorporated opposite 8-oxo-7,8-dihydroguanine in the template. As a result of the cooperative action among MTH1/OGG1/MUTYH and other repair pathways, mammalian cells effectively protect the occurrence of spontaneous mutations such as A: T to C: G and G: C to T: A transversions, which are caused by 8-oxo-7,8-dihydroguanine and 1,2-dihydro-2-oxoadenine.