Progressive Hair Loss and Myocardial Degeneration in Rough Coat Mice: Reduced Lysyl Oxidase-Like (LOXL) in the Skin and Heart

Kimiko Hayashi,*, Tongyu Cao,*, Howard Passmore,† Claude Jourdan-Le Saux,*, Ben Fogelgren,*, Subarna Khan,† Ian Hornstra,† Youngho Kim,§ Masando Hayashi,*, and Katalin Csiszar*†

*John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii, USA; †Department of Genetics, Rutgers University, Piscataway, New Jersey, USA; ‡Department of Medicine, Division of Dermatology, Barnes-Jewish Hospital, Washington University School of Medicine, St Louis, Missouri, USA; §Genome Research Center for Birth Defects and Genetic Diseases, Asan Medical Center, Seoul, Korea

The rough coat (rc) is a spontaneous recessive mutation in mice. To identify the mutated gene, we have characterized the rc phenotype and initiated linkage mapping. The rc mice show growth retardation, cyclic and progressive hair loss, hyperplastic epidermis, abnormal hair follicles, cardiac muscle degeneration, and reduced amount of collagen and elastin in the skin and heart. The rc locus was mapped at 32.0 cM on chromosome 9, close to the loxl gene. Lysyl oxidase-like (LOXL) protein is a novel copper-containing amine oxidase that is required for the cross-linking of elastin and collagen in vitro. LOXL is expressed at high levels in the skin and heart, where the rc mice show strong phenotype. The expression pattern and the genetic proximity to rc suggested loxl as a potential candidate gene. In rc mice, the loxl mRNA was reduced in the skin and the LOXL protein in the heart, dermis, atrophic hair follicles, and sebaceous glands. No mutations, however, were identified within the coding region of loxl, and offspring from rc/rc and loxl null mice crossing were phenotypically normal. Based on these results, loxl appears non-allelic to rc. Heart- and skin-specific downregulation of LOXL in rc mice, however, may contribute to the extracellular matrix alterations and the rc phenotype.

Key words: hair follicle/sebaceous gland/amino oxidase/collagen/elastin


The rough coat (rc) was first described at The Jackson Laboratory as a spontaneous mutation. Weanling mice with unkempt looking coats were observed in several litters of one family of C57BL/6J mice. The rc mutation was recessive, and homozygotes of both sexes were fertile (Dickie, 1966). The initial breeding experiments showed that rc was not allelic with several other mutations with skin and hair defects, including ichthyosis (ic), plucked (pk), fuzzy (fz), and rough (ro) (Dickie, 1966). By more refined linkage analysis, the rc locus was assigned to 32.0 cM on chromosome 9 close to the Mpi-1 gene (Eicher et al, 1977), indicating no allelic association with the Brindled mouse (mop, chromosome X), the closest homologue of Menkes kinky hair, Balding (bal, chromosome 18), matted (ma, chromosome 3) or Nackt (nkt, chromosome 13). Nc/nga (nc, chromosome 9) is a model for human atopic dermatitis with hyperplastic epidermis, increased number of mast cells, and eosinophils and degranulation in the ears and neck. It has some similarities to the rc phenotype, but was linked to several markers between D9Mit103 at 33 cM and D9Mit209 at 35 cM (Kohara et al, 2001) and is not allelic with rc. Abnormal feet and tail (Aft) are closely linked to D9Mit48 at 32 cM, but allelism of rc with Aft was excluded as Aft confers kinky tail, syndactyly in the hind limbs and dominant late onset hair loss (Ruvinsky et al, 2002), whereas the rc mutation has no feet and tail abnormality and demonstrates recessive early onset hair loss.

Previously, the mouse loxl (lysyl oxidase-like) gene was mapped to chromosome 9 (Tchernev et al, 1997; Wydner et al, 1997). The loxl gene is listed at 33.0 cM according to The Jackson Laboratory Mouse Genome Informatics database (MGI http://www.informatics.jax.org), close to rc, which has some similarity to the phenotype of the Menkes kinky hair syndrome resulting from mutations in a copper-binding ATP-ase, ATP7A (MIM #309400) (Mercer et al, 1994; Das et al, 1995; Grimes et al, 1997; Levinson et al, 1997; Reed and Boyd, 1997). In addition, LOXL was highly expressed in the heart (Kim et al, 1995) and skin (Liu et al, 2004), where the rc mice show strong phenotype. LOXL is a catalytically active secreted protein closely related to lysyl oxidase (LOX), and a member of a newly characterized family of copper-dependent amine oxidases (Csiszar, 2001). LOXL shares extensive homology with LOX and the other LOX-like proteins, LOXL2, LOXL3, and LOXL4, in the C-terminal region. This conserved region includes the copper-binding domain, residues surrounding the lysyl and tyrosine residues that form the quinone co-factor, and the cytokine receptor-like domain. The amino-terminal region of LOXL has a unique proline-rich region that is absent in LOX and other LOX-like proteins (Kenyon et al, 1993; Kim et al, 1995; Csiszar, 2001).

Abbreviations: LOXL, lysyl oxidase-like; rc, rough coat

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LOXL was detected in various human tissues (Kenyon et al., 1993; Kim et al., 1995). Increased LOXL expression was associated with remodeling of the extracellular matrix in fibrotic diseases of the liver and lung and the stromal reaction of bronchio-alveolar carcinoma and breast cancer (Decitre et al., 1998). At these locations, LOXL was reported to fulfill a function similar to LOX, which is traditionally known for the extracellular oxidative deamination of lysyl residues in fibrillar collagens and elastin that subsequently results in cross-link formation (Kagan, 2000). In induced liver fibrosis, however, the loxl gene was co-expressed with type III pro-collagen and preceded the expression of lox, which was closely co-regulated with the expression of the collagen type I gene, suggesting that LOXL might play a distinct role with different substrate specificity from LOX (Kim et al., 1999). These observations, taken together, plausibly suggested that loxl might be a positional and functional candidate gene for rc.

In an effort to identify the rc gene and evaluate the association of loxl with the rc, we have initiated mapping of the rc locus, characterized the rc phenotype, determined LOXL distribution in normal and rc mouse tissues, and analyzed the coding region of the loxl gene for mutations in rc mice.

**Results**

**Linkage analysis of rc and the loxl gene** In an initial linkage analysis of loxl we have used a loxl intron length polymorphism that distinguished between BALB/cJ and C57BL/6J. The position of loxl at 58.82 Mb has since been identified by Ensembl Mouse Genome database (http://www.ensembl.org/Mus_musculus/). To define the position of the rc locus, backcross offspring were used for linkage analysis. A linkage map was generated by the analysis of 129 F2 backcross animals. There was one recombination event between the rc locus and D9Mit162 (50.07 Mb in the Ensembl Mouse Genome Database) and three recombination events between rc and D9Mit104 (66.13 Mb in the Sanger Mouse Genome database) among the 129 backcross animals. We therefore concluded that the rc locus is proximal to D9Mit104, consistent with a previous report that mapped rc close to the Mpi-1 gene (58.08 Mb, Ensembl Genome Database) (Eicher et al., 1977). The genetic data derived from these crosses and mapping confirmed that the loxl gene was located within the rc chromosomal region. Therefore we have evaluated the rc phenotype and the tissue and cellular expression pattern of loxl in normal and rc mice and investigated the possibility of loxl as a candidate gene for rc.

**The rc phenotype** Recording of the rc phenotype development started on the day of birth and continued until the time of death. Phenotypic rc pups started to show disorganized hair and hair loss on the back at approximately 2 wk of age. The hair grew back when the follicles entered anagen phase, as revealed by the change in back skin color from gray/pink to black, and was lost again during catagen and/or telogen. Gradually, the hair of rc mice became disoriented and depigmented, accompanied by progressive hair loss (Fig 1).

The rc mice displayed growth retardation compared with normal C57BL/6J mice. In two independent experiments, the body weight of 24 rc/rc mice over a 17-wk period (Fig 2) and 31 rc/+ mice over an 18-wk period (data not shown) was monitored and compared with non-phenotypic littermates. In both experiments, the rc/rc mice had significantly lower body weight, and this difference increased progressively with age. Homozygous rc mice obtained through breeding between rc/rc parents displayed an average survival rate of 28% (43 of 156 born) at weaning age, with most deaths occurring within the first 48 h after birth. This low rate of survival is likely due to abnormalities in the nursing females, as rc/+ pups from rc/rc female and +/- male breeding also suffered a low survival rate, and rc/rc pups and rc/+ littermates from rc/+ female and rc/rc male breeding survived at a similar rate.

**Histopathological findings** In the skin of adult rc mice, multifocal, moderate to marked follicular atrophy with
epidermal ulceration was observed on the ventral neck and trunk. Locally extensive granulation tissue formations were noted with neutrophilic, mastocytic and lymphoplasmacytic dermatitis. In the hairless areas, the epidermis became hyperplastic. Abnormal and dilated hair follicles that contained amorphous material were frequently observed. Melanocyte pigments in hair follicles changed in color from black to light brown (Fig 3A–C). In the heart of adult, rc mice multifocal myocardial degeneration was present. Cardiac muscle fibers in rc mice appeared disoriented (Fig 3D, E). The bone marrow showed diffused hyperplasia. In the femur sections of normal mice, a large amount of basophilic amorphous deposit was present in the bone matrix along the marrow cavity. This basophilic material was probably the residue of calcium deposition. In contrast, there was no such deposit in the femur of rc mice (Fig 3F, G). Increased calcium level in the blood of rc mice correlated with the loss of calcium from the bone. Although the calcium level in normal mouse blood ranges between 3.2 and 8.5 mg per mL, the blood calcium level was higher than 8.5 mg per mL in all five rc mice tested and averaged at more than 8.8 mg per mL. In the liver, numerous erythrocytes, lymphocytes, polymorphonuclear leukocytes (neutrophils) and enlarged Kupffer cells were found in the hepatic sinusoids of rc mice compared to normal mice (not shown). In the trabeculae of the spleen numerous macrophages loaded with hemosiderin granules were observed (Fig 3H, I).

Reduced collagen and elastin in the skin and heart of rc mice To evaluate the changes in the extracellular matrix of the skin and heart of rc and normal mice, tissue sections were stained with Elastic fiber-van Gieson stain. In the skin of rc mice within the papillary layer of the dermis, collagen and elastic fibers appeared thinner and disorganized. The reticular layer was much thinner compared to normal mouse skin. On the contrary, the adipose layer in rc mice was thicker than in normal mice. Both collagen and elastic fibers were reduced in amount and disoriented in many places (Fig 4B). In the heart of adult rc mice, cardiac muscle fibers were degenerated and disoriented. This may result from the alterations of the connective tissue that separate the bundles of cardiac muscle (Fig 4D). In the heart of rc mice, blood vessels were distorted and the amount of elastic fibers were markedly reduced in the aorta, large arteries, muscular arteries and veins. The wall of blood vessels appeared thinner (Fig 4F).

Decreased loxl and lox mRNA in the skin of rc mice We wanted to determine whether the reduced collagen and elastin in the extra cellular matrix correlated with changes in loxl expression. Quantitative real-time PCR analysis was carried out using total RNA isolated from the dorsal skin, heart, liver and kidney of rc/rc and age-matched normal C57BL/6J mice. The relative abundance of the loxl mRNA in rc mouse skin samples was significantly reduced, on average to 13.5% of normal (p < 0.01, Fig 5A). In the heart, kidney and liver of rc mice, loxl mRNA levels were comparable to normal controls. In order to determine if the expression of the closely related lox has also been affected, we have quantitated the lox mRNA in the same tissue samples. In the skin of rc mice the relative abundance of the lox mRNA was also significantly reduced (Fig 5A).

Reduced amine oxidase activity in rc skin To investigate the consequence of this significant reduction in loxl and lox mRNA, we carried out amine oxidase activity analysis using skin protein extracts from three rc/rc and three wild-type mice. The mean activity in rc skin was almost half of the mean activity in the wild-type skin (Fig 5C). There was a large variation among the activities of the wild-type samples, whereas, all rc skin samples demonstrated constantly low activity.
To further evaluate the possible involvement of LOXL2 in the phenotype, we compared its expression pattern in normal and rc mouse tissues using immunohistochemistry. In normal mouse skin, LOXL showed an intense staining in the epidermis, follicular root sheaths and sebaceous glands (Fig 6A). In the skin of rc mice, LOXL immunostaining was significantly reduced compared to normal controls. Reduced staining was most obvious in the deformed, atrophic hair follicles and hypertrophic sebaceous glands. (Fig 6C). LOXL immunostaining was dramatically reduced in the cardiac muscle in the ventricle of rc mouse heart (Fig 6F). Comparable levels of LOXL immunostaining were present in the kidney and liver of rc and normal mice. Consistent with the very low level of loxl2 mRNA in the liver, LOXL immunostaining was only noted in a small number of cells.

Mutational analysis of the loxl2 mRNA in rc mice Because loxl2 is a positional candidate for rc and its expression is significantly reduced in the rc mouse skin, we carried out northern blot analysis to determine whether there is an increase in the size of the loxl2 mRNA in rc mice. Analysis of total RNA from the skin, heart, kidney, and liver of normal C57BL/6J and rc mice detected a single loxl2 mRNA in all tissues examined, that appeared to be 2.7 kb both in normal and rc mice (Fig 5D). Very low loxl2 mRNA expression was also noted in the liver. The normal size of the loxl2 mRNA suggested that there was no gross insertion or deletion within the loxl2 gene in rc mice. These results, however, did not exclude the possibility that a point mutation, small insertion, deletion or inversion within the loxl2 gene contribute to altered expression and the rc phenotype. We therefore analyzed the coding sequence of the loxl2 cDNA obtained from rc mice, and compared it to normal C57BL/6J mice, the strain from which rc arose. Comparison of these sequences did not reveal any mutations within the coding region of the loxl2 gene (data not shown).
Discussion

In this study, we have characterized the phenotype of the rough coat mice and tested the hypothesis that the loxl gene is a positional and functional candidate for rc. The rc mice display an unkempt-looking coat, suffer from early onset and progressive hair loss and demonstrate growth retardation, which persists throughout their lifetime. In addition to the skin and hair phenotype, the rc mice show a range of abnormalities in multiple tissues, suggesting that the gene mutated in these mice might be involved in the development and maintenance of several tissues and organs.

Histopathology of the skin of rc mice revealed follicular atrophy, sebaceous gland hypertrophy, epidermal hyperplasia, and granulation tissue formation with leukocyte invasion. Elastic fiber-van Gieson staining demonstrated altered deposition and reduced amount of collagen and elastic fibers. In the heart of rc mice, cardiac muscle was degenerated and disorganized and blood vessels were distorted with reduced amount of elastin. In this study, we have examined only adult male homozygous rc mice of 12–20 mo of age. The age- and sex-related severity of the observed alterations of cardiac muscle and blood vessels need to be further refined as previous studies noted both age- and sex-related variation in pathologies, including altered copper metabolism in the macular mutant mouse, a model for Menkes disease (Shiraishi et al., 1993).

The loxl gene is located on mouse chromosome 9, in the same region as the rc locus. It is expressed in organs where the rc mice show severe abnormalities. Loxl encodes a copper-binding amine oxidase that is closely homologous to LOX. Some of the changes we have observed in the rc mice, including reduced amount of collagen and elastic fibers, appeared similar to known pathological conditions with reduced collagen and elastin cross-linking due to LOX deficiency or inhibition by β-aminopropionitrile (Csizsar, 2001). Furthermore, functional impairment of LOX is also known to result in tortuosity of blood vessels, similar to what we have observed in rc mouse heart (Hornstra et al., 2003). The rc mutant mice also exhibit some similarities to the Mottled/Brindled mutant mice, that are homologous to Menkes disease. Menkes disease is caused by mutations in a copper-transporting ATPase, which results in abnormal hair shaft and loss of pigmentary and significantly reduced LOX activity (Vulpe et al., 1993; Mercer et al., 1994; Das et al., 1995; Grimes et al., 1997; Levinson et al., 1997; Reed and Boyd, 1997). These observations collectively supported loxl as a positional and functional candidate for rc.

The mutational analysis demonstrated that loxl primary mutations are likely not the cause of the rc phenotype. Consistent with these results, offspring from rc/rc and the recently available loxl null mice breeding were phenotypically indistinguishable from normal mice and further confirmed that loxl and rc are not allelic.

We noted, however, the secondary LOXL changes in certain tissues. Analysis of loxl expression by northern blot detected the same size loxl mRNA in both rc and normal mice, but the expression of the loxl mRNA in the skin of rc mice appeared lower than in normal mice. Subsequent quantitative PCR data demonstrated significant reduction of the loxl mRNA in the skin with individual variations between 0.1% and 10% of normal.

Catalytic activity measurements detected significantly reduced BAPN-inhibitable amine oxidase activity in the skin. As these assays were only able to measure total amine oxidase activity, we also determined if any changes occur in lox expression in rc mice that may contribute to this reduced activity. We found that the relative abundance of the lox mRNA was also significantly reduced in the rc skin samples but demonstrated no change in other tissues. Therefore, it is likely that the reduction in total catalytic activity in the skin resulted from the reduced amount of the LOXL and LOX proteins in adult rc mice.

In the heart of rc mice, focal myocardial degeneration and disoriented cardiac muscle fibers paralleled the reduced...
amount of the LOXL protein, but there was no change in the loxl mRNA. In the heart and skin of normal and rc mice, LOXL appeared in the same areas where collagen and/or elastic fibers are found. In a recent publication LOXL was also reported in the dermis and co-localized with elastic fibers both in the dermis and in blood vessels. Furthermore, LOXL null mice demonstrated fragmented and reduced elastic fibers in the dermis and aorta and the authors concluded that LOXL was essential for elastic fiber homeostasis (Liu et al, 2004). These observations support the hypothesis that reduced LOXL may contribute to some of the extracellular matrix phenotypes noted in the skin, myocardium, and blood vessels in rc mice.

Similar extracellular matrix and fat deposition changes were noted in the SPARC null mice. These mice demonstrate aberrations in the structure and composition of the dermal ECM, and among other features, a greater deposition of fat without change in the overall body weight (Brads haw et al, 2003). Notable differences, however, exist between SPARC null and rc mice in the lack of overall fat deposition and progressive weight loss.

In addition to the skin, hair follicle, and heart abnormalities, the rc mice exhibited skin lesions in which the dermis was infiltrated by leukocytes. Several studies indicated that mouse mutations with skin and hair follicle abnormalities were associated with immunological defects. Homozygous nude mice (nu/nu) were congenitally athymic and hairless throughout life (Flanagan, 1966; Gershwin, 1977). The hairless mouse (hr/hr) displayed hereditary immunodeficiency, which resulted in the high incidence of spontaneous lymphomas (Heiniger et al, 1974; Reske-Kunz et al, 1979). The hair loss in Nckt (nkt) mice was associated with CD4 deficiency (Benavides et al, 1999). Skin lesions of NC/Nga, a model for human atopic dermatitis with elevated IgE levels, histologically resembled skin lesions of rough coat mice (Vestergaard et al, 2000). Further studies are needed to determine if some of the phenotypes of the rc mice are similarly related to immunodeficiency.

As loxl appears to be not the gene mutated in rc mice, additional positional candidates need to be evaluated. Protein kinase Cik3, a regulator in RNA splicing, was detected at high levels in anagen but not telogen during wool follicle growth cycle induced by prolactin (Rufaut et al, 1999). Expression of the cellular retinoic acid-binding protein (CRABP) was also shown to correlate with the dynamics of the hair cycle (Bazzano et al, 1993). NCAM, an adhesion molecule expressed in normal hair follicles but absent in hair matrix cells in the hairless (hr/hr) mouse, was also localized to the rc region on chromosome 9 (Ahmad et al, 1999). Further positional and functional approaches will identify the gene mutated in the rc mice in order to understand the function of its encoded protein and its relevance to regulation of tissue specific loxl expression.

### Materials and Methods

All animal procedures were approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC). The C57BL/6J-rc/rc mice, control C57BL/6J mice, and BALB/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) or through in-house breeding. Unless otherwise specified, the rc mice used in this study were bred in the C57BL/6J background.

### Linkage analysis

Mouse backcrosses were used for linkage analysis to refine the map position of the rc locus. C57BL/6J-rc/rc mice were outcrossed with BALB/cJ mice, and F1 females (heterozygous for both the rc trait and microsatellite markers of interest) were crossed to male C57BL/6J-rc/rc mice. Backcross offspring were scored for recombination events that segregate the microsatellite markers contributed by the two parental chromosomes and the rc phenotypic marker. Murine Map Pairs, purchased from Research Genetics (Huntsville, Alabama), were used for microsatellite typing. In backcrosses to refine the map position of the loxl gene, segregation of loxl was monitored by an intron length polymorphism within intron 3 of the loxl gene that distinguished between the gene from the BALB/cJ and C57BL/6J strains. For this analysis, PCR primers that correspond to sequences at the 3’ end of exon 3 and the 5’ end of exon 4 of the loxl gene were used. This primer pair amplified a 700 bp fragment containing intron 3 in strain C57BL/6J, and a 500 bp fragment in strain BALB/cJ.

### Pathology and histology

Five male and five female adult rc mice between 12 and 20 mo of age and two male and two female age-matched normal control C57BL/6J mice were shipped on dry ice within 24 h of euthanasia for necropsy to a veterinary pathologist at IDEXX Veterinary Services (West Sacramento, California). Blood calcium levels were determined with colorimetric assay using o-cresolphthalein complex. For histology examinations, tissue specimens were obtained from three adult C57BL/6J homozygous rc male mice and three normal adult male mice. The dorsal skin, aorta, heart, kidney, liver, spleen, lung, and femur bone were excised and immediately fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. After fixation, tissues were washed in 0.1 M phosphate buffer containing 7% sucrose, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin or Elastic fiber van Giessen stain (Sigma Diagnostic #HT25, Sigma-Aldrich, St Louis, Missouri).

### Immunohistochemistry

Preparation and properties of the LOXL polyclonal antibody, generated in rabbit and used in this study, have been reported previously (Decitre et al, 1998). Procedures for immunostaining using the unlabeled antibody peroxidase–anti-peroxidase technique (Sternberger, 1979) were followed as described previously (Hayashi et al, 1987). Tissue sections were treated with 1% hydrogen peroxide in methanol for 30 min, washed and blocked with 5% normal goat serum in 0.1 M phosphate buffered saline, pH 7.4, for 30 min. Sections were reacted with the primary antibody in 1 mg per mL BSA in PBS overnight at 4°C, then with goat anti-rabbit IgG (Sigma Chemical) at 50 μg per mL dilution for 30 min and rabbit peroxidase–antiperoxidase (Sigma Chemical) diluted to 50 μg per mL for 30 min at room temperature. Sites of antigen binding were visualized by immersing the slides in 0.05% diaminobenzidine (Sigma Chemical) in 0.05 M Tris-HCl buffer, pH 7.6, and 0.01% hydrogen peroxide for 5 min. Sections were counterstained with Harris hematoxylin. Negative controls were incubated either with preimmune serum or in media containing no primary antibody and processed in the same manner as above.

### RNA extraction

Organs from rc and normal C57BL/6J mice were immediately frozen in liquid N₂ upon harvest. The organs were ground, and homogenized in TriReagent (Molecular Research Center, Cincinnati, Ohio) of 10 times the volume of the organs to extract total RNA. The homogenate was kept at room temperature for 5 min. Chloroform equal to 0.2 times the volume of TriReagent was added, and the samples were vortexed vigorously for 15 s. Samples were left at room temperature for 15 min before a centrifugation at 12,000 × g for 15 min at 4°C. Isopropanol equal to 0.5 times the volume of TriReagent was mixed with the aqueous phase, and the samples were centrifuged at 12,000 × g for 8 min
at 25°C after incubation at room temperature for 10 min. The RNA pellets were washed in 75% ethanol, and dissolved in RNase-free H2O.

Quantitative RT-PCR and northern blot analysis For the real-time RT-PCR experiments, total RNA was extracted from strips of dorsal skin from three wild-type C57BL/6J mice, 2 rc/rc mice, and 4 rc/− mice in the C57BL/6J strain (10–12 mo of age) using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) following the manufacturer's instructions. Samples were normalized by GAPDH amplification and quantitation. The quality of the RNA extract was verified by agarose gel electrophoresis with ethidium bromide staining showing sharp 28S and 18S rRNA bands (Fig 5B). Five micrograms of each RNA sample was reverse transcribed using SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. One microcortil (of 20) of the first-strand cDNA was used as template for PCR amplification. The loxl- and lox-specific primers were synthesized at IDT (Corvalle, Iowa) and the sequences are available upon request. Real-time PCR was performed using DyNAQo HS SYBR Green qPCR kit and an Opticon 2 DNA engine (MJ Research, Waltham, Massachusetts). The PCR conditions were optimized so that a single fragment was amplified. The copy number of loxl and lox cDNA molecules in a sample was measured by comparing its amplification with that of standard samples that contained 10 to 10⁶ copies of the respective cDNA. Each cDNA sample was amplified in triplicates or duplicates, and statistical significance was evaluated using an unpaired t test. RNA or cDNA from different mice were individually analyzed and never pooled.

For northern blot analysis, total RNA from an rc/rc and a +/+ C57BL/6J mouse (2 y old) was separated according to size on 1% agarose gel containing 4% formaldehyde. The gel was washed in 0.05 N NaOH and 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate) before the RNA was transferred onto HyBond-N- nylon membrane (Amersham Biosciences, Piscataway, New Jersey). After the RNA was UV cross-linked to the membrane, it was incubated in Ultra-Hyb buffer (Ambion, Austin, Texas) for 2 h at 42°C before a 3²P-labelled human loxl cDNA probe was added at 1 × 10⁶ cpm per mL. After overnight hybridization, the membrane was washed in 0.1 × SSC/0.1% SDS at 50°C before the radioactivity was visualized by exposure to an X-ray film. The experiment was repeated using RNA from another pair of mice with the same result (data not shown).

Catalytic activity assay Skin samples from three rc/rc and three normal C57BL/6J mice were excised and frozen-fresh in liquid nitrogen. LOX enzymes were extracted according to previously published protocols for bovine aorta extractions (Kagan et al, 1979; Borel et al, 2001). Briefly, frozen skin samples from the different genotypes were homogenized in 0.1 M K₂HPO₄ (pH 7.7), 0.15 M NaCl, and 1 × Pefabloc (Roche). After mixing at 4°C for 30 min, samples were centrifuged at 4°C for 20 min at 24,000 × g. The pellets were then resuspended in 0.016 M K₂HPO₄ (pH 7.8), 4 M Urea, and 1 × Pefabloc. After vortexing, the samples were left mixing at 4°C overnight. After centrifuging the urea-insoluble proteins into a pellet (4°C for 20 min at 24,000 × g), the urea-soluble proteins were transferred to a fresh tube and tested for BAPN-inhibitable amine oxidase activity. The protein concentrations of the samples were measured using the Bradford reagent (Biorad) and 30 μg of urea-fraction proteins were used in each activity assay. The activity assays were performed as previously detailed using Cadaverine as a substrate and Ampalex Red to detect released H₂O₂ (Li et al, 1997; Palamakumbura and Trackman, 2002).

RT-PCR and DNA sequencing The total RNA extracted from the kidney and skin of rc mice using TriReagent was reverse transcribed using SuperScript II Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's manual. The first-strand cDNA was used as template for PCR amplification. Primers were synthesized at IDT to amplify overlapping cDNA fragments and to sequence the coding region of loxl. The primer sequences are available upon request. PCR products were sequenced using one of the PCR primers and reagents and sequencer from Applied Biosystems (Foster City, California).

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Address correspondence to: Katalin Csiszar, PhD, Pacific Biomedical Research Center, University of Hawaii at Manoa, 1960 East-West Road, Biomed T-311, Honolulu, HI 96822, USA. Email: KCsiszar@ao.com

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