Alms1-disrupted mice recapitulate human Alström syndrome

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Mutations in the human ALMS1 gene cause Alström syndrome (AS), a progressive disease characterized by neurosensory deficits and by metabolic defects including childhood obesity, hyperinsulinemia and Type 2 diabetes. Other features that are more variable in expressivity include dilated cardiomyopathy, hypertriglyceridemia, hypercholesterolemia, scoliosis, developmental delay and pulmonary and urological dysfunctions. ALMS1 encodes a ubiquitously expressed protein of unknown function. To obtain an animal model in which the etiology of the observed pathologies could be further studied, we generated a mouse model using an Alms1 gene-trapped ES cell line. Alms12/2 mice develop features similar to patients with AS, including obesity, hypogonadism, hyperinsulinemia, retinal dysfunction and late-onset hearing loss. Insulin resistance and increased body weight are apparent between 8 and 12 weeks of age, with hyperglycemia manifesting at ~16 weeks of age. In addition, Alms12/2 mice have normal hearing until 8 months of age, after which they display abnormal auditory brainstem responses. Diminished cone ERG b-wave response is observed early, followed by the degeneration of photoreceptor cells. Electron microscopy revealed accumulation of intracellular vesicles in the inner segments of photoreceptors, whereas immunohistochemical analysis showed mislocalization of rhodopsin to the outer nuclear layer. These findings suggest that ALMS1 has a role in intracellular trafficking.

INTRODUCTION

Alström syndrome (AS) [ALMS, Online Mendelian Inheritance in Man (OMIM) no. 203800] is a genetically homogeneous and progressive disorder characterized by cardinal features including cone–rod dystrophy, neurosensory hearing loss, early childhood obesity and insulin resistance leading to Type 2 diabetes (1–3). Other features such as dilated cardiomyopathy, hepatic and urinary dysfunctions and developmental delay are also commonly observed in patients with AS (4).

Recently, mutations in human ALMS1, a novel gene of unknown function on chr 2p13, were identified as the basis of AS (5,6). The mutations reported so far have been nonsense and/or frameshift mutations found in exons 8, 10 and 16 (5–9). ALMS1 has an open reading frame of 4169 amino acids with no sequence homology to other genes. It ubiquitously expressed, and several splice variants have been identified (5). An interesting structural feature is a large 5 kb exon composed of 34 imperfect repeats of a 47 amino acid motif, which does not bear resemblance to other reported motifs, and its functional significance is not known.

Phenotypically, ALMS1 strongly resembles another genetically heterogeneous group of disorders, the Bardet–Biedl syndrome (BBS) (OMIM no. 209900). However, the presence of polydactyly and mental retardation in most forms of BBS distinguishes BBS from ALMS. Considerable progress has been made in elucidation of the molecular defects in BBS (10). Eight BBS loci have been identified, and several BBS proteins have recently been linked to basal body function and transport in ciliated cells (11–15). The phenotypic similarities between the two syndromes and the fact that the ALMS protein was identified as a component of the human centrosome (16) make it likely that ALMS acts in the same or related pathways as the BBS proteins.

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Here, we report a gene-trapped mouse model for AS, Alms1\textsuperscript{GT(pGT1Lxf)/Pjn}, which exhibits phenotypic features resembling those of ALMS patients, including obesity, hyperinsulinemia, testicular atrophy and cochlear and retinal degeneration.

**RESULTS**

Genomic characterization of the gene-trapped Alms1 locus

DNA prepared from the BayGenomics ES cell line, XH152 (17), was used to determine the gene-trap insertion site. Analysis of 5'-RACE product sequence obtained with a primer specific to lacZ showed that the gene was trapped downstream of exon 13. Sequence analysis revealed the insertion site to be in intron 13, 1.97 kb downstream of exon 13. Oligonucleotides for diagnostic PCR were designed to distinguish the Alms1\textsuperscript{+/+} wild-type versus Alms1\textsuperscript{−/−} trapped alleles (Fig. 1A). The genotypic ratio of F2 progeny, i.e. homozygous (Alms1\textsuperscript{−/−}) or heterozygous (Alms1\textsuperscript{+/−}) for the gene trap and homozygous wild-type (Alms1\textsuperscript{+/+}), was 110:229:139, not significantly different from the expected 1:2:1 Mendelian ratio (P = 0.11).

The mutation in this model, which would result in a prematurely terminated protein, mimics the most commonly found mutations in the human AS, i.e. premature terminations in the 3' half of the gene (5–7,9). Such truncations of the messages originating upstream of the last exon typically result in...
loss of mRNA due to nonsense mediated decay (18). To assess whether normal Alms1 splicing of exon 13 into exon 14 was still occurring despite the gene-trap insertion, we isolated total RNA from eye and brain tissue of wild-type and mutant mice. RT–PCR using Alms1 primers upstream of the gene-trap cassette (exons 8–10) showed strong expression in both Alms1 \( ^{-/-} \) and Alms1 \( ^{+/+} \) mice, whereas downstream expression (using exon 13 and exon 16-specific primers) was not detectable in homozygotes by RT–PCR (Fig. 1B). However, slight traces of normally spliced exon 13–14 mRNA species containing exons upstream and downstream of the gene trap, ALMS1 isoforms, which have been previously described (5), may exist and the mutation described here may represent a hypomorphic allele.

**ALMS1 is present during early development**

The Alms1 gene-trapped allele, Alms1\( ^{GT(pGT1Lx8)Pn} \), expresses a beta-galactosidase (\( \beta \)-gal) reporter gene. As some patients with AS display retinal and cardiac pathologies soon after birth, we determined the temporal and spatial localization of ALMS1 during early development using \( \beta \)-gal as a marker for Alms1 expression. Uniform \( \beta \)-gal expression was detected as early as embryonic day 7.5 throughout the embryo proper but was absent from the extra-embryonic lineages. At E8.0, \( \beta \)-gal expression was found ubiquitously in mesodermal- and ectodermal-derived layers. By E10.5, Alms1 expression becomes predominant in the mid- and hindbrain and in the fore- and hind limbs (Fig. 1D–F). Expression remains consistent throughout embryonic stages E15.5–E18.5 (data not shown). No obvious developmental abnormalities were observed in the embryos examined, and homozygous mutant offspring are obtained from heterozygote matings in the expected Mendelian ratio.

**Alms1 \( ^{-/-} \) mice are obese and males develop Type 2 diabetes**

In human patients with AS, one of the earliest clinical manifestations is rapid weight gain in infancy, which progresses to childhood obesity. In Alms1 \( ^{-/-} \) mice (Fig. 2A), body weight begins to increase between 8 and 12 weeks of age when compared with Alms1 \( ^{+/+} \) or Alms1 \( ^{+/+} \) controls. Both female and male Alms1 \( ^{-/-} \) mice have significantly higher body weights than their gender-matched littermates (Fig. 2B and C). The weight of white adipose tissue in individual fat pads was significantly greater in Alms1 \( ^{-/-} \) mice compared with littermate controls (Table 1). The majority of adipose tissue was found in the subcutaneous and reproductive regions. The accumulation of adipose tissue was most notable in female mutants. Adiposity indices in female Alms1 \( ^{-/-} \) mice were 3-fold higher than in controls, whereas affected male mice had adiposity indices that were only 2-fold higher than their lean littermate controls.

Plasma glucose (PG) levels measured between 4 and 24 weeks of age showed that mean PG levels were slightly elevated in both Alms1 \( ^{-/-} \) males and females when compared with control mice. However, overt diabetes (PG > 250 mg/dl) was observed only in affected males after ~16 weeks of age (Fig. 2D and E). All male and female Alms1 \( ^{-/-} \) mice were hyperinsulinemic when tested at 20 weeks of age (Fig. 3A). The hyperinsulinemia in Alms1 \( ^{-/-} \) mutants develops between 8 and 12 weeks of age (data not shown). In addition, although Alms1 \( ^{-/-} \) mice had moderately elevated levels of plasma total cholesterol, plasma triglyceride levels were comparable to controls at 20 weeks of age (Fig. 3B and C).

**Multiple organ pathologies in Alms1 \( ^{-/-} \) mice**

Livers of both male and female Alms1 \( ^{-/-} \) mice were significantly heavier than those of their heterozygous and wild-type littermates (Table 1). Histologic evaluation of hematoxylin and eosin (H&E) stained Bouin’s fixed tissue from 24–30-week-old mutant mice (Fig. 4A) revealed macro- and microvesicular lipid deposits in the liver. The islets in the pancreas were severely enlarged and hyperplastic. Aldehyde fuchsin staining of islets showed granulated islet beta cells but partial degranulation compared with wild-type (Fig. 4B).

Hypogonadism was observed in all Alms1 \( ^{-/-} \) mice com pared with the control littermates. Atrophic seminiferous tubules were observed in male testes. Although the degree varied among animals; some testis had severe vacuolization with no secondary spermatocytes (Fig. 4C,D), whereas other testes had normal meiotic I cells with no mature sperms (data not shown).

Kidneys of Alms1 \( ^{-/-} \) mice were enlarged and weighed nearly twice as much as in their wild-type littermates [mean average; 0.37 g per kidney in mutant (n = 5) and 0.22 g per kidney in littermates (n = 9)] at 6 months of age. Histopathological analysis of H&E stained sections showed dilation of the renal proximal tubules, which contained an eosin-positive, flocculent material of unknown origin (Fig. 4E). Interstitial inflammation and vacuolization, suggestive of degenerating tubules, were observed in a 24-week-old mutant (data not shown). Urinalysis revealed the presence of albumin in male, but not female mutants. Fifty percent of the males had abnormal creatinine:albumin ratios (>30), levels indicative of micro- and macro-albuminuria, whereas the females had normal ratios. By light microscopy, we more frequently observed large pyramidal-shaped calcium oxalate diphosphate crystals in the urine sediments of Alms1 \( ^{-/-} \) mice when compared with controls.

**Alms1 \( ^{-/-} \) have retinal and cochlear defects**

Light stimuli of increasing intensity on a rod-desensitizing background revealed reduced b-wave responses in 24-week-old Alms1 \( ^{-/-} \) mice when compared with their wild-type littermates (Fig. 5A and B). Reduced cone b-wave responses were observed as early as 9 weeks of age. At 24 weeks, the majority of mutant mice show normal dark-adapted ERGs, whereas 33% of mutants show a significantly reduced b-wave amplitude. Retinas from 24-week-old Alms1 \( ^{-/-} \) mice showed loss of cell bodies in the outer nuclear layer (ONL) from ten to six layers and shortening of the inner and outer segments in the majority of the mutants examined (Fig. 5C and D). Additionally, in retinas from 7 and
Table 1. Necropsy results of F2 progeny

<table>
<thead>
<tr>
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<th>Females</th>
<th>Males</th>
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<tr>
<td></td>
<td>Alms1^+/−</td>
<td>Alms1^+/+</td>
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<tr>
<td>Body weight</td>
<td>60.0 ± 4.9</td>
<td>27.5 ± 1.4</td>
</tr>
<tr>
<td>Total fat</td>
<td>17.6 ± 2.5</td>
<td>2.9 ± 0.8</td>
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<tr>
<td>Subcutaneous</td>
<td>6.5 ± 0.4</td>
<td>1.3 ± 0.3</td>
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<tr>
<td>Reproductive</td>
<td>5.9 ± 1.8</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>2.2 ± 0.6</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>2.2 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Pericardial</td>
<td>0.4 ± 0.2</td>
<td>Neg</td>
</tr>
<tr>
<td>Adiposity index</td>
<td>29.0 ± 2.1</td>
<td>10.2 ± 2.3</td>
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<tr>
<td>Liver weight</td>
<td>3.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
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Numbers represent averages (grams) ± SEM. Necropsies were done on four to five mice from each subgroup. *Adiposity is defined as the percentage of the sum of individual fat pad weights divided by the body weight. Neg, negligible adipose around the heart.
24-week-old mutant retinas, a mislocalization of rhodopsin to the ONL was observed (Fig. 5E–G). No evidence of mislocalization was observed with the cone opsins and ROM1 (data not shown), suggesting the defect may be specific to rhodopsin trafficking. Rhodopsin accounts for at least 90% of the protein content in rod photoreceptor discs (19) and thus for a large fraction of the transport across the photoreceptor cilium. Mislocalization of other transported proteins may, therefore, be more difficult to detect and can presently not be ruled out. By electron microscopy, intracellular membrane-bound vesicles were found to accumulate in the distal portion of the inner segments near the connecting cilium of Alms1<sup>−/−</sup> mice (Fig. 5H–J).
Figure 5. Retinal degeneration in Alms1<sup>−/−</sup> mice. (A and B) Plots of mean amplitudes (± SEM) versus intensity of dark and light adapted electroretinograms of 24-week-old wild-type and mutant mice (Alms1<sup>−/−</sup>, n = 7; littermate controls, n = 7). Rod and cone b-wave amplitudes are reduced significantly in Alms1<sup>−/−</sup> mice. H&E stained retinal sections of (C) 24 week wild-type retina and (D) 24-week-old Alms1<sup>−/−</sup> mutant retinas (10×). (E–G) Rhodopsin localization by fluorescent microscopy of retinas tagged with Cy-3 and anti-rhodopsin. (E) 7-week-old wild-type, (F) 7-week-old mutant and (G) 24-week-old mutant (20×). White arrows show the mislocalization of rhodopsin to the outer segments in Alms1<sup>−/−</sup> mice. (H–J) Ultrastructural analysis of the outer retina. (H) C57BL/6J wild-type adult mouse retina. Typical rough-surfaced endoplasmic reticulum (blue arrowhead) in the inner segments (IS) consists of small, flattened sacs. N indicates the nucleus of ONL. (I and J) Adult Alms1<sup>−/−</sup> mouse retina. Intracellular vesicles (red arrows) are observed throughout the inner segments, forming stacks resembling the Golgi apparatus. At higher magnification, orientation of the inner segments appears consistently disturbed, often yielding cross-sections (black arrowheads). The white arrows indicate the external limiting membrane and the edge of a photoreceptor nucleus provides orientation. Scale bars (H–J) = 500 nm.
To assess hearing, auditory brainstem response (ABR) analysis was performed at various time points between 1 and 12 months of age. Until 8 months of age, Alms1−/− mice have normal click ABR thresholds. In Alms1−/− mice, 8 months and older, thresholds are >55 db SPL (sound pressure level) and show abnormal ABR thresholds for all sound stimulations: click and 8, 16 and 32 kHz (Fig. 6). By light microscopy, the architecture and layering of the inner and outer hair cells in the middle turn of the organ of corti of 13-month-old hearing impaired mutant mice (n = 2) appear normal (data not shown).

Alms1−/− mice assemble cilia normally

To determine whether Alms1−/− assemble normal cilia, motile and primary cilia were examined by ultrastructural analyses. By transmission electron microscopy (TEM), ciliated cells from the nasal epithelium appear normal. Cross-sections of cilia revealed a typical ‘9+2’ microtubule arrangement (Fig. 7A). Connecting cilia in retina from 6-month-old homozygous mutant mice were present, and microtubules were arranged in a normal ‘9+0’ fashion (Fig. 7B). By scanning electron microscopy (SEM), primary cilia of renal collecting tubules appear normal in size and number (Fig. 7C).

DISCUSSION

In this study, we show that Alms1-disrupted mice recapitulate many of the clinical features of the human syndrome. Obesity, hyperinsulinemia, hypogonadism, retinal degeneration and renal dysfunction parallel those observed in the human syndrome. Similar to individuals with AS, the onset of hyperinsulinemia in mice occurs early; whereas only males become hyperglycemic. Gender bias for Type 2 diabetes is typically observed in mouse models (20). Pancreatic islets show marked hyperplasia associated with beta cell proliferation. This suggests that both insulin resistance and increased insulin secretion may contribute to the glucose intolerance observed.

When compared with the disease progression in human ALMS patients, some differences with respect to the onset and disease progression were observed in our mouse model. For instance, obesity is one of the earliest manifestations observed in human patients, usually presenting by the first year of life (1). In contrast, prepubertal Alms1−/− mice (4-week-old) tend to have lower body weights than their wild-type littermates, and the obesity manifests after puberty, between 8 and 12 weeks of age.

The deduced amino acid sequence of the ALMS1 protein has no similarity to other proteins with known function, nor does the protein sequence contain previously recognizable characterized domains. The first insight into ALMS1 function comes from the findings of Andersen et al. (16) who identified ALMS1 as a constituent of centrosomes, microtubule organizing centers that assemble the mitotic spindle and organize the microtubule scaffold, which guides organelle and vesicle trafficking (21). Additionally, in a recent study, ALMS1 was shown to localize to the basal bodies of ciliated cells as well as to the centrosomes (22). Basal bodies anchor the microtubular axoneme of cilia and flagella and help organize intraflagellar transport (23). In recent years, a role for these structures in human disease has become more evident (24). Particularly, the proteins associated with diseases that are phenotypically similar to ALMS, such as BBS, also localize to the centrosomal regions and within the basal bodies of ciliated cells, and studies of mouse models for BBS (Bbs1−/−, Bbs2−/−, Bbs4−/− and MKKS−/−) have implicated several BBS
proteins in ciliary function and intracellular trafficking (12,15,25,26).

Ultrastructural analyses of ciliated cells in Alms1−/− mice reveal that cilia undergo normal assembly. However, disruption of the Alms1 gene in the retina results in the accumulation of large membrane-bound vesicles within photoreceptor inner segments and mislocalization of rhodopsin to the outer segments. Normally, rhodopsin-containing vesicles fuse with the cell membrane at the base of the connecting cilium of photoreceptor cells, and rhodopsin is transported to the photoreceptor outer segments along the cell membrane by an intraflagellar transport system (27). The abnormal accumulation of vesicles in the photoreceptor inner segments in Alms1−/− mice indicates a potential role of ALMS1 in ciliary function, perhaps in the process that brings rhodopsin vesicles to the base of the cilium, the docking of the vesicles to the membrane or the attachment of cargo to the intraflagellar transport machinery. A similar retinal phenotype is also observed in null mice deficient in the BBS4 protein, which accumulates in the inner segments (12,28–31). Phenotypically, Alms1−/− and tubby (tub/tub) mice are strikingly similar. Tubby mice exhibit adult-onset obesity and progressive retinal and cochlear degeneration. On specific genetic backgrounds, tubby mice become hyperinsulinemic and males are susceptible to Type 2 diabetes (32). The similarities in clinical features lead us to speculate that ALMS1 and TUB proteins may function in similar biological pathways. Interestingly, the tubby superfamily protein has recently been identified in an analysis of the flagellar and basal body proteome (13), and Tubp2 was found to be induced during flagellar regeneration in Chlamydomonas (33), further suggesting a role for both TULPs and ALMS1 in cilia related functions.

In conclusion, the Alms1−/− mice described here recapitulate the phenotypes observed in patients with AS, including obesity, hyperinsulinemia leading to Type 2 diabetes, hypogonadism and neurosensory deficits. This new model for AS will allow detailed investigation of the disease pathology as well as testing hypotheses about ALMS1 function. Elucidation of the biochemical function of ALMS1 is crucial for a better understanding of the pathogenesis involved in AS as well as in the more common forms of obesity, diabetes and retinal and cochlear degeneration.

MATERIALS AND METHODS

Generation and husbandry of ALMS1−/− mice

A 129-derived ES cell line (XH152) (17) containing a gene-trap cassette in the Alms1 gene was obtained through the Mutant Mouse Regional Resource Center (MMRRC). Alms1+/− ES cells were microinjected into blastocysts of C57BL/6J mice. Chimeric males were backcrossed to C57BL/6J and the agouti pups from the F1 progeny were genotyped as described subsequently. Alms1+/− heterozygous mice were intercrossed to produce a total of 478 progeny.

Mice, in this study, were bred in the Research Animal Facility at The Jackson Laboratory. Mice were ad libitum fed a NIH31 diet with 6% fat (PMI Feeds Inc., St. Louis, MO, USA) and provided unlimited access to water (HCl acidified, pH 2.8–3.2) in a temperature and humidity controlled setting with a 12 h light/dark cycle.

Analysis of gene-trap insertion site

Genomic DNA from ES cells XH152 was PCR amplified with an Alms1 exon 13-specific primer (5′-GATTATAGCAA-GAGGGCTGC-3′) and a gene-trap cassette-specific primer (5′-GGACTAACAGAAGACCCGT-3′) using the Expand Template System (Roche). The resulting product of 1.97 kb was subcloned into TOPO XL (Invitrogen) and the plasmid was electrophorated into TOPO electrocompetent cells. Plasmids were purified using the Qiaprep spin columns (Qiagen) and subsequently sequenced with M13F and M13R primers. Sequence alignments were performed using MacVector 6.0 to determine the position of the gene-trap insertion site.

Analysis of mutant cDNA by RT–PCR

Total RNA from a mutant (Alms1−/−) and wild-type (Alms1+/+) adult brain and eye was isolated using the Trizol method (GIBCO), followed by DnaseI digestion of residual DNA (Ambion). cDNA was made from RNA by RT–PCR (Superscript). PCR was performed on the cDNA using the Expand Template System (Roche) according to the manufacturer’s instructions. 5′-RACE of XH152 revealed that the slice donor site of intron 13 was splicing into the splice acceptor site of the gene trap (BayGenomics). Oligonucleotides were designed to amplify regions upstream (exon 8F, 5′-TCCCTATGATGACAATCAT-3′ and exon 10R, 5′-GTGCATTTCTGATTCCTCTGG-3′) and downstream of the insertion site (exon13F, 5′-CAGTGAACAATTAAGCAC-3′ and exon 16R, 5′-GAACAGGCCCTTGGCCCAA-3′). Amplification products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining under UV illumination.

Real-time PCR with Taqman gene expression assays (Applied Biosystems) was performed according to manufacturer’s protocol with an ABI7900HT system. Primer/probes for Alms1 coding regions spanning the gene trap (exons 13−14) were manufactured by Applied Biosystems’ Assay By Design technology. Absolute quantitation expression analysis (34) was performed using eye and brain from three Alms1−/− and three Alms1+/+ biological replicates. For technical validation, all reactions were performed in triplicates. Real-time PCR reactions using known concentrations (10−5, 10−4, 10−3, 10−2, 10−1 and 1 ng) of PCR-amplified Alms1 cDNA products were used as standards. A ‘no-template control’ was performed as a negative control. Standard curves were generated and absolute expression values were determined. In the figure, the ‘no-template control’ values were subtracted from the absolute values to generate a ‘no expression’ baseline. All standard curves were linear.
Genotyping
A forward primer specific to Alms1 intron 13 upstream of the gene trap (GT-F) and a reverse primer specific to the gene-trap cassette (GT-R) were designed to detect the mutant Alms1 allele. A forward primer specific to Alms1 intron 13 upstream of gene trap (WT-F) and a reverse primer specific to Alms1 intron 13 downstream of the gene trap (WT-R) were used to detect the wild-type allele. The sequences of primers are shown in Figure 1. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 45 cycles of 94°C for 20 s, 54°C for 30 s and 72°C for 30 s and a final extension of 72°C for 7 min. As the genotypes were based on the presence or absence of bands, the annealing temperature was reduced to allow for non-specific binding such that we could visualize bands in the gene-trap animal (to control for DNA content).

X-gal staining of embryos
Timed matings were performed using Alms1+/− female bred to Alms1+/+ male mice. At embryonic days E7.5, E8.0, E10.5, E15.5 and E18.5, pregnant females were sacrificed and embryos were harvested. The yolk sac from each embryo was collected for genotypic analysis. Embryos were fixed in 2% PFA or glutaraldehyde at room temperature for 2 h, rinsed with PBS and then placed in X-gal solution (www.baygenomics.ucsf.edu/protocols/) and incubated at 37°C overnight. Protein expression of whole embryos was visualized by stereomicroscopy for the presence of a blue color.

Biochemical measurements
Mice (N1F2) were weighed monthly until termination at 24 weeks of age. Whole blood was collected via the orbital sinus bi-weekly using EDTA coated capillary tubes. Samples were centrifuged and plasma was used for all biochemical assays. PG, cholesterol and triglyceride levels were measured using a Beckman Coulter Synchron CX5 Delta chemistry analyzer. Plasma insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (ALPCO Diagnostics). PG levels were monitored bi-weekly, whereas lipid and insulin levels were determined at 20 weeks of age and at termination.

Histological and immunohistochemical analysis
Mice (N1F2) were sacrificed by CO₂ asphyxiation and tissues were dissected and placed in Bouin’s fixative overnight. Eyes were oriented by cautery and placed in cold acetic acid/methanol (1:3 ratio) overnight. Tissues embedded in paraffin, using standard procedures, were cut into 6–8 µm sections, stained with H&E or aldehyde fuchsin (pancreas only) and visualized by light microscopy.

For immunohistochemical analysis, retinal sections were de-paraffinized and incubated with 1:500 anti-rhodopsin (Leinco Technologies), 1:500 anti-ROM1, 1:500 anti-blue opsin (Chemicon) and/or 1:500 anti-red/green opsin overnight. After several washes in PBS, sections were incubated with a Cy3-conjugated mouse secondary antibody (Jax Immunoresearch) and stained with DAPI (Sigma) for nuclear staining. Localization of rhodopsin, ROM1, and cone opsins was visualized by fluorescence microscopy.

Electron microscopy
TEM on retina. Mice were sacrificed by CO₂ euthanasia and eyes were immediately removed and fixed in phosphate-buffered gluteraldehyde/paraformaldehyde solution for 3 h at 4°C. Following fixation, the anterior segment was removed and the posterior segment was cut into transverse blocks (1 x 2 mm²), including retina, choroid and sclera, and placed in fresh fixative for an additional 2–8 h. Tissues were then post-fixed in 1% osmium tetroxide, dehydrated and embedded in plastic. Tissue sections were cut and stained in uranyl acetate and lead citrate and examined under a TEM.

SEM on kidneys. Kidneys cut longitudinally were fixed in 2.5% gluteraldehyde in cacodylate buffer overnight. Tissues were rinsed twice in cacodylate buffer, dehydrated and freeze fractured with liquid nitrogen. Tissues were then rehydrated, treated with osmium tetroxide, dehydrated and sputter coated. Specimens were mounted and examined under a SEM.

Fundus evaluation and electroretinograms
Mice were dark-adapted and their pupils dilated with atropine prior to examination by indirect ophthalmoscopy with a 60 or 78 diopter aspheric lens. Fundus photographs were taken with a Kowa fundus camera using a Volk superfield lens held 2 in. from the eye. The highest flash intensity was used with Kodak black and white Tmax 400 ASA professional film for the photodocumentation. The protocol for full field ERGs has been previously described (35). Briefly, a gold loop electrode was placed on the corneal surface at the limbus and referenced to a gold wire placed in the mouth of mice anesthetized with a mixture of xylene/ketamine (7 and 15 mg/gm, respectively). A needle electrode inserted in the tail served as ground. Mice were placed on a 38°C heating pad throughout the procedure. Signals were amplified (×10 000, CP511 AC amplifier, Grass Instruments), sampled every 0.8 ms and averaged. Rod-mediated ERGs were recorded from mice adapted to the dark overnight and exposed to short-wavelength flashes in a Ganzfeld dome, which varied over a 4.0 log unit range of intensities up to the maximum allowable by the photopic stimulator (PS33 Plus, Grass Instruments). Cone-mediated responses were obtained with white flashes on a rod-saturating background after 10 min of light adaptation. Responses were computer averaged at all intensities with up to 50 records averaged for the weakest signals. A signal rejection window was used to eliminate electrical artifacts produced by blinking and eye movements.

Assessment of hearing by ABR
ABRs were conducted as previously described (36). Briefly, mice were anesthetized and their body temperature was maintained at 37–38°C by placing them on a heating pad in the soundproof chamber during testing. Subdermal needle
electrodes were inserted at the vertex (active) and ventrally to the right ear (reference) and the left ear (ground). Specific auditory stimuli (broadband click and pure-tone pips of 8, 16 and 32 kHz) from high-frequency transducers were delivered binaurally through plastic tubes to the ear canals. Evoked brain-stem responses were amplified and averaged and their wave patterns were displayed on a computer screen. Auditory threshold was obtained for each stimulus by varying the SPL at 10 dB steps and finally at 5 dB step up and down to identify the lowest level at which an ABR pattern could be recognized.

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