A Hypomorphic Mutation in Lpin1 Induces Progressively Improving Neuropathy and Lipodystrophy in the Rat*

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The Lpin1 gene encodes the phosphatidate phosphatase (PAP1) enzyme Lipin 1, which plays a critical role in lipid metabolism. In this study we describe the identification and characterization of a rat model with a mutated Lpin1 gene (Lpin1HHubr), generated by N-ethyl-N-nitrosourea mutagenesis. Lpin1HHubr rats are characterized by hindlimb paralysis and mild lipodystrophy that are detectable from the second postnatal week. Sequencing of Lpin1 identified a point mutation in the 5’-end splice site of intron 18 resulting in mis-splicing, a reading frameshift, and a premature stop codon. As this mutation does not induce nonsense-mediated decay, it allows the production of a truncated Lipin 1 protein lacking PAP1 activity. Lpin1HHubr rats developed hypomyelination and mild lipodystrophy rather than the pronounced demyelination and adipocyte defects characteristic of Lpin1fldfld mice, which carry a null allele for Lpin1. Furthermore, biochemical, histological, and molecular analyses revealed that these lesions improve in older Lpin1HHubr rats as compared with young Lpin1HHubr rats and Lpin1fld fld mice. We observed activation of compensatory biochemical pathways substituting for missing PAP1 activity that, in combination with a possible non-enzymatic Lipin 1 function residing outside of its PAP1 domain, may contribute to the less severe phenotypes observed in Lpin1HHubr rats as compared with Lpin1fld fld mice. Although we are cautious in making a direct parallel between the presented rodent model and human disease, our data may provide new insight into the pathogenicity of recently identified human LPIN1 mutations.

Lipin 1 plays a crucial role in lipid metabolism in multiple cell types including glia, adipocytes, myocytes, and hepatocytes (1–4). Previous studies have shown that Lipin 1 is a Mg2+-dependent phosphatidate phosphatase (PAP1)5 enzyme catalyzing the dephosphorylation of phosphatidic acid (PA), yielding inorganic phosphate and diacylglycerol needed for the synthesis of triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine (5–8). The mammalian Lipin protein family consists of three members, Lipin 1, Lipin 2, and Lipin 3 (9), which are encoded by transcripts with specific tissue expression patterns (5). Lpin1 is expressed at high levels in white adipose tissue (WAT) and muscle (5), and is also present in other tissues including peripheral nerve (2). Alternative splicing of Lpin1 generates two Lipin 1 isoforms that play distinct, but complementary, roles in adipogenesis: Lipin 1α, which affects adipocyte differentiation, and Lipin 1β, which induces lipogenic gene expression (10). The amino- and carboxyl-terminal regions of Lipin 1 (NLIP and CLIP, respectively), and a predicted nuclear localization signal are highly conserved among the three mammalian Lipin family members and among species (9). The CLIP domain contains multiple key protein functional domains: four halocacid dehalogenase motifs and a transcription factor-binding motif (LXXIL) (1, 6, 11). Moreover, Lipin 1 and Lipin 2 have been predicted to possess the same structural organization as previously characterized HAD protein family members (11). In addition to its enzymatic function, Lipin 1 also has the ability to regulate gene expression, and it is likely that this ability is shared by Lipin 2 and Lipin 3 (1, 12, 13).

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5 The abbreviations used are: PAP1/2, phosphatidate phosphatase 1/2; WAT, white adipose tissue; SC, Schwann cell; HAD, halocacid dehalogenase; PND, postnatal day; ENLI, N-ethyl-N-nitrosourea; BN, Brown Norway; PPARγ, peroxisome proliferator-activated receptor-γ; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; Cds1, CDP-diacylglycerol synthase gene; NMD, nonsense-mediated decay; MSE, myelinating Schwann cell enhancer; FABP, fatty acid-binding protein.

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Hypomorphic Mutation in Rat Lpin1

Overexpression of Lpin1 in either adipose tissue or skeletal muscle promotes obesity when mice are fed a high-fat (adipose and muscle tissue) or chow diet (muscle tissue) (4). On the other hand, a spontaneous null mutation of Lpin1 present in fatty liver dystrophy (Lpin1fl/fl) mice results in lipodystrophy, a progressive demyelinating neuropathy affecting peripheral nerves, and a neonatal fatty liver that resolves at weaning (9, 14). Recently, mice with a Schwann cell (SC)-specific deletion of Lpin1 were created resulting in SC abnormalities and a neuropathy similar to the full Lpin1-knock-out Lpin1fl/fl mice (2). This indicated that the neuropathy present in Lpin1fl/fl mice is a direct consequence of the absence of Lipin 1 within SCs. Moreover, SC-specific deletion of Lpin1 also revealed an interaction between PA and the MEK-ERK signaling pathway mediating SC de-differentiation and proliferation (2). In addition, mutant mice (Lpin1fl/flΔ20884) bearing a tyrosine to asparagine missense mutation (Y873N) were recently described (15). Lpin1fl/flΔ20884 mice develop adult-onset transitory hindlimb paralysis, a floppy gait, and a tendency to clench the hind limbs in toward the body when suspended by the tail. These phenotypes are, however, less severe as compared with Lpin1fl/fl mice, probably due to partial retention of PAP1 activity in Lpin1fl/flΔ20884 mice.

Here we describe a Lpin1 mutant allele in the rat (hereafter Lpin1H11Hube) characterized by early-onset transitory peripheral neuropathy, muscle wasting, and lipodystrophy. The Lpin1H11Hube mutation resulted in out-of-frame transcription leading to disruption of the Lipin 1 HAD domain IV. This resulted in complete inactivation of PAP1 activity, whereas preserving the function of the remainder of the Lipin 1 protein. Our data suggest that the age-related improvement of the Lpin1H11Hube phenotypes may relate to Lipin 1 function that is independent of its PAP1 activity and/or to the activation of compensatory biochemical pathways partially substituting for the lack of PAP1 activity. In addition to its importance for the understanding of the role of Lipin 1 in lipid metabolism, the generated Lpin1H11Hube rat model may provide an interesting model to study the pathophysiology of recently described human LPIN1 mutations.

EXPERIMENTAL PROCEDURES

Animal Housing—The Animal Care Committee of the Royal Dutch Academy of Science and the University of Lausanne approved all experiments according to Dutch and Swiss legal ethical guidelines. Lpin1H11Hube pups were obtained at the expected Mendelian frequency, and the phenotype was not gender specific. If more than 8 littersmates were present and survival of Lpin1H11Hube pups could be compromised, wild-type/heterozygous pups were removed as soon as the Lpin1H11Hube phenotype could be observed, until a total of 8 pups remained. Chow pellets were also added to the maternal cage floor to increase feeding chances of Lpin1H11Hube pups. After weaning (around postnatal day (PND) 21), two rats were housed together, unless noted otherwise, under controlled experimental conditions (12 h light/dark cycle, light period 0600–1800, 21 ± 1 °C, ~60% relative humidity). The standard fed diet in our animal facility (semi-high protein chow: RM3, 26.9% protein, 11.5% fat, and 61.6% carbohydrates; 3.33 kcal/g of AFE; SDS, Witham, United Kingdom) was provided ad libitum together with water.

Identification of Lpin1H11Hube Rats—Following an N-ethyl-N-nitrosourea (ENU) mutagenesis screen (16), an accidental brother-sister mating between two F2 animals (Wistar/Crl background; backcrossed twice to wild-type Wistar background) revealed offspring that could be identified approximately between PND 7 and 14 by hindlimb paralysis and hindquarter muscle wasting.

Forward Screen, Sequencing, and Animals—For the mapping cross, the two initial F2 carriers of the Lpin1H11Hube mutation were outcrossed with Brown Norway (BN/Crl) rats. F2 Wistar/BN rats were intercrossed to identify them as carriers of the mutation and to generate offspring homozygous for the mutation. After onset of the phenotype, DNA was isolated from F2 wild-type/heterozygous and homozygous Wistar/BN offspring. DNA was processed as described before (17), and a previous described Wistar/BN SNP panel (17) was used to map the phenotype in F2 Wistar/BN rats. In total, the SNP distribution pattern for 67 mutant Wistar/BN F2 rats was determined. Primers were designed to cover each exon with partial intron overhang, and sequencing of Lpin1 in 4 wild-type/heterozygous and 4 mutant pups revealed a point mutation (Thr > Ala) in the conserved 5’-end splice site of intron 18. In addition to the cross of BN/Crl animals, the initial F2 carriers of the Lpin1H11Hube mutation were also backcrossed to wild-type Wistar/Crl animals for 4 additional generations to eliminate confounding effects from background mutations induced by ENU. F2 Wistar/Crl rats were used in all described experiments except for the mapping screen.

Genotyping— Genotyping was done using the KASP SNP Genotyping System (KBiosciences, Hoddesdon, United Kingdom; as described by Ref. 18) using gene-specific primers (forward common, GAGCC CTTTT ATG TCTT TGGGA A; reverse wild-type, GAAGG TGACC AAGTT CATGC TCCAC TGACC TCTCC GGGTA; reverse homozygous, GAAGG TCGGA GTCAA CGGAT TCTCC ACTGA CTCAC CTCGG TT). All pups were genotyped around PND 21. Genotypes were reconfirmed when experimental procedures were completed.

Quantitative RT-PCR— For all analyses, total RNA from complete sciatic nerve, endoneurium, and WAT was isolated using the Qiagen RNeasy lipid tissue kit (Qiagen) following the manufacturer’s instructions. Total RNA was subjected to reverse transcription using the SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer’s instructions. The resulting cDNA was used as a template for relative quantitative RT-PCR as described previously (19). Results were normalized using the reference gene Ubiquitin. See supplemental Table S1 for a complete list of oligonucleotides used for relative gene expression.

Immunohistochemistry— The sciatic nerves were dissected and embedded in OCT medium (Sakura) and longitudinal or
cross-sections were prepared. Prior to specific staining, the slides were post-fixed in 4% paraformaldehyde for 10 min. For immunohistochemistry, slides were briefly (30 s) washed in PBS and blocked for 1 h using 10% normal donkey or goat serum (DAKO) and 0.3% Triton X-100 (Merck) in PBS (PBS−T). Slides were incubated overnight at 4 °C with primary antibodies diluted in PBS−T. The following primary antibodies were used: Lipin 1 (rabbit; 1:100; described below), Krox20 IgG (rabbit, 1:100; Covance), and Oct6 IgG (goat, 1:100; Abcam). The slides were then washed in PBS−T and hybridized with the appropriate secondary fluorescent antibodies (Alexa Fluor 594 or 488 conjugated anti-rabbit and mouse; all at a dilution of 1:200; Invitrogen) for 2 h at room temperature. Slides were washed in PBS−T and mounted with Vectashield mounting medium containing DAPI to counterstain cell nuclei (Vector Laboratories). Sections were visualized as described above.

BrdU Incorporation Assay—At PND 21, rats were injected intraperitoneally with 100 μg of BrdU/g of body weight. Six hours later, the sciatic nerves were dissected, fixed in 4% paraformaldehyde for 24 h, and embedded in OCT medium. Longitudinal cross-sections were prepared, post-fixed in 4% paraformaldehyde (10 min), denatured with 2 M HCl for 20 min at 37 °C, and neutralized in 0.1 M sodium borate (pH 8.5) for 10 min. Sections were incubated with rat anti-BrdU (at a 1:200 dilution; Abcam) in 0.3% Triton X-100 overnight at 4 °C. The next day, the sections were incubated with anti-rat secondary antibody conjugated to Alexa Fluor 594 (at a 1:200 dilution; Invitrogen) and visualized by fluorescence microscopy. The nuclei were counterstained with DAPI.

WAT Morphology—Rats of both genders were sacrificed at PND 4 or 10, and male rats were sacrificed at PND 21 or 90 by decapitation after measuring body weight. The left and right dorsal subcutaneous WAT fat pads were subsequently harvested and weighed. WAT samples were then collected in 4% formaldehyde, rotated overnight at 4 °C, rinsed twice with 100% ethanol for 2 h, left in xylene overnight at RT, and embedded in paraffin. The tissue was then cut into 5-μm sections and stained with hematoxylin and eosin. Average adipocyte cell diameter was measured using NIH ImageJ freeware.

Western Blotting—Cells were lysed in ice-cold lysis buffer (20 mM Na2H2PO4, 250 mM NaCl, Trition X-100 1%, SDS 0.1%) supplemented with Complete protease inhibitors (Roche Applied Science). Protein levels were quantified using the Bio-Rad protein assay with BSA as a standard. Equal amounts of protein extracts were resolved by 10% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Blots were blocked in Tris-buffered saline containing 0.1% Tween (TBS−T) supplemented with 4% milk powder and subsequently incubated overnight at 4 °C in the same buffer supplemented with antibodies against the HA tag (Cell Signaling) and Tubulin (Sigma). After washing in TBS−T, blots were exposed to the appropriate horseradish peroxidase-conjugated secondary antibodies (DAKO) in TBS−T for 1 h at room temperature. Finally, the blots were developed using ECL reagents (Pierce) and Kodak Scientific Imaging Films (Kodak).

Microdissection of Sciatic Nerve—Sciatic nerves were harvested at PND 16, 21, 90, or 200–300 and placed in ice-cold PBS (pH 7.4). The perineurium and epineurium were gently dissected away from the endoneurium along the whole length of the nerve as previously described (3).

PAP Activity Measurement—PAP activity measurement was performed as previously described (2). Tissue samples were disrupted using a Dounce homogenizer at 4 °C in 50 mM Tris-HCl (pH 7.5) buffer containing 0.25 m sucrose, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM benzamidine, 0.5 mM PMSF, 5 μg/ml of aproatin, leupeptin, and pepstatin. The lysed cells were centrifuged at 1,000 × g for 10 min at 4 °C, and the supernatant was used as cell extract. Total PA phosphatase activity (Mg2+-dependent and Mg2+-independent) was measured at 37 °C for 20 min in the reaction mixture (total volume of 100 μl) containing 50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 10 mM β-mercaptoethanol, 0.2 mM [32P]PA (5,000 cpm/nmol), 2 mM Triton X-100, and enzyme protein. The Mg2+-independent PA phosphatase activity was measured in the same reaction mixture except that 2 mM EDTA was substituted for 1 mM MgCl2. The Mg2+-dependent PA phosphatase activity was calculated by subtracting the Mg2+-independent enzyme activity from total enzyme activity. One unit (U) of PA phosphatase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min. Specific activity was defined as units/mg protein.

Phosphatidic Acid Quantitation—PA was quantified as previously described (2). Tissues (the endoneurium from two nerves and about 300 mg for adipose tissue) were homogenized in 1.5 ml of PBS containing 0.5 mM sodium orthovanadate (Sigma) and extracted twice with 1 volume of butanol. After evaporation, phospholipids were solubilized in 1 ml of PBS containing 1% BSA and 0.5 mM sodium orthovanadate. An aliquot of the solution was incubated for 90 min at 37 °C with or without bovine pancreatic phospholipase A2 (3.8 units/ml, from Sigma). At the end of the incubation, phospholipids were extracted with butanol, dried, and LPA was quantified. The amount of PA corresponds to the amount of LPA detected after treatment with phospholipase A2 after subtraction of the amount of LPA detected without phospholipase A2 treatment. The assay was performed in triplicate for each sample.

Lipid Analysis—Lipids were extracted from sciatic nerve endoneurium derived from rats at PND 16 or PND 200–300 rats according to the method of Bligh and Dyer (20). Phospholipids were separated on silica gel high performance TLC plates (Merck, Darmstadt, Germany) using a solvent system consisting of chloroform, methanol, acetic acid, formic acid, and water (75:32:12.5:4:1, v/v) (21). Lipid spots were visualized by exposure to iodine vapor, and phosphorus was determined according to Rouser et al. (22). Because of incomplete resolution of phosphatidylinositol (PI) and phosphatidylserine (PS), the phosphorus content of the combined spots was measured and the contribution of each of the two lipid classes was determined using negative mode precursor scanning of m/z 241 atomic mass units (PI) and neutral loss scanning of 87 atomic mass units (PS), respectively, on an AB Sciex (Foster City, CA) 4000 Qtrap MS instrument. A mixture of authentic PI and PS standards was used to correct for ionization and fragmentation efficiencies (23). Cholesterol was quantified as described previously, using deuterated cholesterol (Avanti Polar Lipids) as an internal standard (24).
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Toluidine Blue Staining and Morphometric Analysis—Semi-thin nerve cross-sections were stained with 1% toluidine blue and digitalized using AxioVision release 4.5 software (Zeiss). For each myelinated axon present, both an axonal area (defined by the inner limit of the myelin sheath) and a total fiber area (defined by the outer limit of the myelin sheath) were automatically measured using image analysis software (G-ratio calculator 1.0, Image J plug-in, Yannick Krempp, Cellular Imaging Facility, Lausanne, Switzerland). The g-ratio was calculated by dividing the axon area by the total fiber area. Each experimental group consisted of two rats.

Nile Red Staining—Sciatic nerve cross-sections were mounted in Nile Red (Sigma) solution (0.5 mg/ml in acetone) diluted 1,000 times in 75% glycerol, and visualized using a Zeiss Axioplan 2 microscope with an AxioCam MRc camera and AxioVision release 4.5 software (Zeiss).

Lipin 1 Antibody—A rabbit anti-Lipin 1β antibody was generated with a peptide (PGSPRPSTPKDSDEL) specific for the murine Lipin 1β isofrom (Eurogentec) and used at 1:100 for immunohistochemistry.

cDNA Constructs—Constructs used in this study were generated using murine and rat Lipin 1α and 1β cDNAs with an N-terminal hemagglutinin (HA) epitope tag. The original mouse constructs were provided by Dr. Thurl Harris (University of Virginia, Charlottesville, VA) and the rat constructs (wild-type and truncated forms) were purchased as mini-genes in pcDNA3.1 from Integrated DNA Technologies (IDT, Coralville, IA).

Cell Culture and Transfections—HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM L-glutamine (all reagents were from Invitrogen) cultured in a humidified 37 °C incubator with 5% CO₂. Plasmid transfections were performed using Effectene (Qiagen) and used at the manufacturer’s instructions.

For the luciferase assay, rat Schwann cells were seeded in 6-well PRIMARIA (BD Biosciences) dishes. At 70 – 80% confluence, the cells were transfected in triplicate using FuGENE (Roche Applied Science) in the presence of 3% FCS, 5% Neudifferentiation factor β-conditioned medium and 2 µM forskolin. The next day, the cells were switched to serum-free medium (DMEM/F-12, 1× N2, 1× PS, 10 ng/ml of NGF, 1× BSA, 5% Neudifferentiation factor β conditioned medium) and incubated for 18 h. The next day, the cells were induced to differentiate for 36 h in induction medium (100 µM cAMP, DMEM/F-12, 1× N2, 1× PS, 10 ng/ml of NGF, 1× BSA, 5% Neudifferentiation factor β-conditioned medium) and used for luciferase assays. HeLa cells were plated in 12-well dishes (Greiner-bio one) containing DMEM, 10% FCS, and 1% PS. At 90% confluence they were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. DNA quantity was kept constant using PBS empty vector. 36 h post-transfection, the cells were washed and lysed in 1× reporter lysis buffer (Promega). The extracts were measured for β-galactosidase (β-gal) activity using 2-nitro-phenyl-galactopyranoside as substrate. Luciferase activity was measured on a luminometer (PerkinElmer Life Sciences) using the Steady Glo luciferase assay substrate (Promega) and values were normalized to β-gal activity.

Immunofluorescence—HEK293 cells were grown as described above and transfected with plasmids for expression of rat Lipin 1 proteins (wild-type and truncated forms). One day after transfection, the cells were fixed with 4% formaldehyde for 30 min and permeabilized for 15 min with 0.1% Triton X-100. The cells were blocked with PBS containing 10% normal goat serum for 1 h before incubation with a primary antibody against the HA tag (Cell Signaling, 1:200) overnight at 4 °C. After washing in PBS-T, the cells were incubated with the appropriate secondary fluorescent antibody (Alexa Fluor 594) for 1 h in the dark followed by washing with PBS-T. The cells were mounted with Vectashield mounting medium containing DAPI to counterstain cell nuclei (Vector Laboratories) and visualized by confocal microscopy (Leica TCS SP5).

Data Analysis—All data are shown as mean ± S.E. All data were analyzed using a commercially available statistical program (SPSS for Macintosh, version 16.0) and were controlled for normality and homogeneity. Differences in longitudinal body weight measurements were assessed using repeated measure analysis, followed by Bonferroni post hoc analyses if significant overall interactions were observed. The statistical analysis for the lipid analysis was performed using the Prism GraphPad version 5.00 (GraphPad Software, San Diego, CA), and lipid analysis was assessed by two-way analysis of variance, using age and mutation as factors, followed by a Bonferroni post hoc analysis if a significant overall interaction was observed. The null hypothesis was rejected at the 0.05 level.

RESULTS

The Identification of the Lpin1Hubr Mutation in Rat—After an ENU mutagenesis screen on a Wistar/Crl background (16), a brother-sister mating (F₃ generation) revealed offspring that could be identified approximately between PND 7 and 14 by difficulty walking, muscular atrophy, and a reduced amount of body fat (supplemental Fig. S1, A and B, and supplemental Video S1). Further evaluation of older rats (PND 28) revealed, in addition to the above mentioned phenotypes, presence of loss of hindlimb joint mobility, the absence of characteristic retraction of the hindlimbs when picked up by the neck, and the inability to splay hindlimbs when suspended by the tail (supplemental Fig. S1, C and D). The Wistar/Crl F₂ rats heterozygous for the mutation were outcrossed with BN/Crl rats to generate F₃ Wistar/BN population for mapping purposes. On PND 21, DNA was collected from wild-type/heterozygous and mutant Wistar/BN F₂ rats, and a forward screen was performed using a Wistar/BN-specific SNP marker panel (17). This resulted in the identification of a 3.6 Mb region on chromosome 6 between 37.1 and 40.7 Mb, resulting in the identification of Lpin1 (Chr.6; 40.3 Mb) as a prime candidate gene (supplemental Fig. S2). Sequencing of Lpin1 revealed a mutation (T>A) in the conserved 5’-end splice site of intron 18 potentially leading to the use of an alternative splice site 16 bp downstream of the original site. The new splice site was predicted to result in a change in reading frame introducing a premature stop codon in exon 19 (Fig. 1A, panel I). We have named this mutated allele of the rat Lpin1 gene Lpin1Hubr.
Consequences of Lipin1\textsuperscript{Hubr} Mutation on Lipin 1 Expression and Function—To verify these predictions, we evaluated the correct splicing of intron 18 using primers spanning the wild-type exon 18–19 boundary (Lpin1\textsuperscript{Ex18–19}; Fig. 1A, panel II). The expression of correctly spliced Lipin1\textsuperscript{Ex18–19} was almost undetectable in muscle, WAT, liver, and brain samples of Lipin1\textsuperscript{Hubr} rats at PND 21 or 90 (Fig. 1B). RT-PCR using primers spanning the exon 18–19 border (primers shown in Fig. 1A, panel III) failed to show any residual wild-type expression in sciatic nerve endoneurium (Fig. 1C). Furthermore, expression of the Lipin1\textalpha and Lipin1\textbeta isoforms (10) was investigated in sciatic nerve endoneurium (Fig. 1D, primers shown in Fig. 1A, panel IV). The expression of both Lipin1 isoforms was increased in Lipin1\textsuperscript{Hubr} rats as compared with wild-type rats at PND 21. Later in development, at PND 90, only Lipin1\textbeta expression was increased in Lipin1\textsuperscript{Hubr} rats as compared with wild-type rats (Fig. 1D). Taken together these data therefore suggested the absence of nonsense-mediated decay (NMD) of the mutated Lipin1\textsuperscript{Hubr} transcript. We further validated the absence of NMD by immunofluorescence analysis of the localization of Lipin 1 in sciatic nerves of wild-type and Lipin1\textsuperscript{Hubr} rats. Using a Lipin 1 antibody we detected the presence of Lipin 1 in sciatic nerve endoneurium of both adult (PND 90) wild-type and Lipin1\textsuperscript{Hubr} rats (Fig. 2A). Importantly, this staining was lost in Lipin1\textsuperscript{ fld/fld} mice in which the Lipin 1 protein is absent (Fig. 2B). In silico analysis suggested that the Lipin1\textsuperscript{Hubr} mutation disrupts the conserved amino acid in the predicted HAD motif IV (wild-type, GNRPAD; Lipin1\textsuperscript{Hubr}, GNRPAV, Fig. 2A). Importantly, the terminal acidic residue, aspartic acid, of motif IV, along with those in motif I, are required for positioning of the Mg\textsuperscript{2+} ion into the active site (reviewed in Ref. 25). The disruption of the Mg\textsuperscript{2+} binding site therefore potentially leads to inactivation of Lipin 1 PAP activity. We have investigated PAP activity in Lipin1\textsuperscript{Hubr} rats and observed that although we can detect the presence of mutated Lipin 1 protein in Lipin1\textsuperscript{Hubr} rats, its PAP1 activity was completely lost in both sciatic nerve endoneurium and WAT (Fig. 2C). Interestingly, the level of PAP2, which has a similar enzymatic activity (26), was up-regulated in endoneurial samples from Lipin1\textsuperscript{Hubr} rats. We then assessed the level of the substrate of the PAP1 enzyme, PA, in sciatic nerve and WAT isolated from Lipin1\textsuperscript{Hubr} rats as compared with wild-type rats. Unlike in Lipin1\textsuperscript{ fld/fld} sciatic nerve (2), we found no significant differences in the concentration of PA in Lipin1\textsuperscript{Hubr} sciatic nerves. However, the amount of PA in the WAT from Lipin1\textsuperscript{Hubr} was three times higher as compared with wild-type rats (Fig. 2D).

Sciatic Nerve Abnormalities in Lipin1\textsuperscript{Hubr} Rats—Null deletions of Lipin1 in mice result in severe sciatic nerve myelin abnormalities (2, 14), whereas Lipin1\textsuperscript{20884} mutant mice show a less severe demyelination phenotype (15). We therefore investigated sciatic nerve morphology using toluidine blue-stained semi-thin nerve sections at PND 4, 10, 21, and 90. At PND 4 and 10, no clear sciatic nerve myelin abnormalities were observed between genotypes (Fig. 3A). This observation was strength-
FIGURE 2. The Lpin1 PAP1 activity is disrupted in Lpin1<sup>1<sub>Hubr</sub></sup> rats. A, amino acid sequence showing the four predicted HAD motifs, the conserved amino acids from the HAD family of proteins are indicated in red letters, the transcription coactivator motif LXXIL in yellow letters, green ellipses indicate predicted α-helices (11). The partial intron retention disrupts the conserved amino acid in the predicted HAD motif IV (WT, GNRPAD; Lpin1<sup>1<sub>Hubr</sub></sup>, GNRPAV). Moreover, the out-of-frame translation (indicated in blue) disrupts the predicted α-helix and the second predicted β-strand of the predicted HAD motif IV. B, immunolabeling of Lipin 1 (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue) in cross-sections of sciatic nerve derived from wild-type (mWT; B, panel I) or Lpin1<sup>1<sub>Hubr</sub></sup> mice at PND 56 (B, panel II), and derived from wild-type (WT; B, panel III), or Lpin1<sup>1<sub>Hubr</sub></sup> rats (8, B, panel IV) at PND 90. The endoneurial part of the control nerves shows the expression of Lipin 1 in myelinating Schwann cells (red-stained croissant-shaped cells indicated by white arrows). This labeling is completely absent in Lpin1<sup>1<sub>Hubr</sub></sup> mice but partially preserved in Lpin1<sup>1<sub>Hubr</sub></sup> rats. C, PAP1 activity is substantially decreased in sciatic nerve endoneurium and WAT of Lpin1<sup>1<sub>Hubr</sub></sup> rats as compared with wild-type rats at PND 21. PAP2 activity is increased in sciatic nerve endoneurium of Lpin1<sup>1<sub>Hubr</sub></sup> rats as compared with wild-type rats, whereas no significant difference in WAT PAP2 activity was observed between genotypes (*, p = 0.005; **, p < 0.001; n = 5–6 per group). D, at PND 21, PA levels were unchanged in sciatic nerve and increased in WAT tissue of Lpin1<sup>1<sub>Hubr</sub></sup> rats as compared with wild-type rats (*, p < 0.01; n = 6 per group). Data are expressed as mean ± S.E.

Phosphatidic acid in mammalian cells is metabolized via two pathways: the cytidine diphosphate-diacylglycerol pathway and the Kennedy pathway (Fig. 4A). But in contrast to observations in Lpin1<sup>1<sub> fld/fld</sub></sup> mice, Lpin1<sup>1<sub>Hubr</sub></sup> rats exhibited age-dependent increases in PS levels (time × genotype interaction: F<sub>(1,12)</sub> = 7.0; p < 0.05), age-dependent decreases in PI levels (time × genotype interaction: F<sub>(1,12)</sub> = 61.0; p < 0.001), and a tendency for cholesterol levels to increase over time (time × genotype interaction: F<sub>(1,12)</sub> = 2.3; p = 0.16). This suggested that the sciatic nerve myelin lipid composition of Lpin1<sup>1<sub>Hubr</sub></sup> rats improved with age.

Next, we examined the amplitude of myelin perturbation in Lpin1<sup>1<sub>Hubr</sub></sup> rats as compared with Lpin1<sup>1<sub> fld/fld</sub></sup> mice, which completely lack Lipin 1 function. Similar to previously reported changes in Lpin1<sup>1<sub> fld/fld</sub></sup> sciatic nerves (14), thin-layer chromatography analysis performed on lipids extracted from sciatic nerves isolated from young (PND 16) and adult (between PND 200 and 300) Lpin1<sup>1<sub>Hubr</sub></sup> rats revealed major changes in PS, PI, phosphatidylcholine, sphingomyelin, and cholesterol (Fig. 4A). In the cytidine diphosphate-diacylglycerol pathway, CDS1 catalyzes the synthesis of cytidine diphosphate-diacylglycerol, which serves as a precursor of PI. Consistent with the observed accumulation of PI especially at PND 16, we observed elevated levels of Cds1 mRNA in sciatic nerves derived from Lpin1<sup>1<sub>Hubr</sub></sup> rats between PND 4 and 16, whereas Cds1 expression was unchanged between wild-type
and Lpin1<sup> fld/fld </sup> mice at PND 56 or even decreased in adult Lpin1<sup> Hubr </sup> rats (PND 90) as compared with adult wild-type rats (Fig. 4, C and D).

Nile Red staining, emitting a green fluorescence in the presence of neutral lipids (cholesterol, lipoproteins, and triglycerides) and a red fluorescence in the presence of polar lipids (phospholipids) (28) allowed us to determine that demyelinated sciatic nerves from Lpin1<sup> fld/fld </sup> mice accumulated neutral lipids in their perineurial and endoneurial compartments, which was rarely observed at PND 21 and not observed at PND 90 in sciatic nerves from Lpin1<sup> Hubr </sup> rats (Fig. 5A). In addition, the Nile Red staining confirmed the presence of hypomyelination at PND 21 in Lpin1<sup> Hubr </sup> rats, a phenotype that substantially improved in Lpin1<sup> Hubr </sup> rats at PND 90 (Fig. 5A).

We further evaluated the level of myelin gene expression using a set of molecular markers. Mpz (also designated P0) and Pmp22, two transcripts encoding key myelin structural proteins, were expressed at equal levels between genotypes at PND 4, but were decreased in Lpin1<sup> Hubr </sup> rats as compared with wild-type rats at PND 10, 21, and 90 (Fig. 5B). These results were in agreement with the observed myelin phenotypes at PND 21 and 90 (Figs. 3 and 5A), and confirmed a relatively early onset (~PND 10) of the myelin phenotype in Lpin1<sup> Hubr </sup> rats on a molecular level. Also, the relative increase in expression of both Mpz and Pmp22 at PND 90 as compared with PND 21 further confirmed partial recovery of the myelin phenotype in Lpin1<sup> Hubr </sup> rats. These observations led us to investigate expression of the POU domain transcription factor Pou3f1/Oct6/Scip/Tst-1 (hereafter Oct6), a marker of immature promyelinating SCs (29, 30), and the zinc finger transcription factor Krox20/Egr2 (hereafter Krox20), a marker of mature SCs (29), and an important controller of myelination. The level of expression of both of these markers was not significantly different between genotypes at PND 4 (Fig. 6A). Starting at PND 10, deregulation of the level of relative Krox20 expression became visible and was followed by an up-regulation of relative Oct6 expression starting at PND 21. At PND 90, both relative Oct6 and Krox20 expression was increased in Lpin1<sup> Hubr </sup> rats as compared with wild-type rats (Fig. 6A). This suggested the presence of a mixed SC population in PND 90 nerves. Immunostaining of wild-type and Lpin1<sup> Hubr </sup> sciatic nerves indeed revealed that wild-type and mutant nerves contained SCs at different stages.
Hypomorphic Mutation in Rat Lpin1

At PND 21, wild-type nerves contained a high number of myelinated (Krox20⁺, Oct6⁺) SCs in addition to early myelinating (Krox20⁺, Oct6⁻) and immature (Krox20⁻, Oct6⁺) SCs, whereas the large proportion of SCs in Lpin1⁺/⁻ nerves were immature (Krox20⁻, Oct6⁺) SCs. At PND 90, a substantial increase in the number of myelinated (Krox20⁺, Oct6⁺) cells was observed in wild-type nerves, whereas most SCs in Lpin1⁺/⁻ nerves were early myelinating (Krox20⁺, Oct6⁻) SCs (Fig. 6, B and C). Additionally, we observed an increased cellularity and increased number of replicating cells in sciatic nerve of Lpin1⁺/⁻ rats as compared with wild-type sciatic nerve at PND 90 (supplemental Fig. S3A). This, together with a maintained elevated level of Cyclin D1 expression in Lpin1⁺/⁻ rats as compared with wild-type rats at PND 21 and 90 (supplemental Fig. S3B), further confirmed that even at PND 90, Lpin1+++ nerve endoneurium contains immature and pro-myelinating SCs.

Lipin 1 was previously described to have a transcriptional coactivator activity (1), which could potentially participate in the observed increase of Krox20 gene expression in Lpin1+++ nerves at PND 90. We first examined the subcellular localization of wild-type and mutated forms of rat Lipin 1α and β in HEK293 cells (Figs. 7, A and B). Although rat Lipin 1α and Lipin 1β wild-type forms localized to the cytoplasm and the nucleus, the mutated rat Lipin 1α and Δβ forms preferentially localized to the cytoplasm (Fig. 7B). Using luciferase gene reporter assays, we found that both wild-type and mutated forms of mouse Lipin 1α and Lipin 1β did not influence Krox20 myelinating Schwann cell enhancer (MSE) (31) mediated activation of the hsp68 promoter in differentiating Schwann cells (Fig. 7C). In addition, whereas overexpression of Oct6 and Sox10 strongly activated the Krox20 MSE in HeLa cells, wild-type and mutated forms of Lipin 1α and/or Lipin 1β showed no or minimal effect (Fig. 7D). These data indicate that Lipin 1α or Lipin 1β (either alone or through Sox10 or Oct6) does not activate Krox20 transcription.

WAT Morphology in Lpin1+++ Rats—Lipodystrophy is one of the major phenotypes observed in models with null deletions of Lipin1 (9, 14). We have, therefore, evaluated the WAT phenotype in Lpin1+++ rats. The body weight of Lpin1+++ and wild-type rats was not significantly different until PND 10, but at PND 21 and onward, Lpin1+++ rats showed a reduction in body weight as compared with wild-type rats (supplemental Fig. S4, A–F). WAT tissue analysis revealed a decreased adipocyte size, both at PND 21 and 90, whereas adipocyte cell size was equal between genotypes at PND 4 or 10 (Fig. 8, A and B). The decreased ability to store lipids at a later age was reflected by a reduction in adipose mass at PND 21 and 90 (Fig. 8C). To evaluate the WAT phenotype on a molecular level, we measured the relative expression of peroxisome proliferator-activated receptor-γ1 and 2 (Ppara1 and Ppara2), key markers of adipocyte differentiation (32), and fatty acid-binding protein 4

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Fabp4, also designated aP2), which reversibly bind fatty acids and other lipids (33). In WAT derived from Lpin1

markers was not significantly different between genotypes (Fig. 8D). However, relative expression of Pparγ1, Pparγ2, and Fabp4 was decreased in Lpin1

in WAT of Lpin1

samples was dramatically reduced (Fig. 8D). Together, these data demonstrated an aberrant WAT phe-

FIGURE 6. Myelination status of Schwann cells in Lpin1

A, relative gene expression analysis of Oct6 and Krox20 (SC markers) in sciatic nerve tissue of Lpin1

rats as compared with wild-type rats at PND 4, 10, 21, and 90 (n = 3, n = 3, n = 3, and n = 2 per group, respectively; *, p < 0.05; **, p < 0.001). B, sciatic nerve cross-sections of wild-type (WT) and Lpin1

rats at PND 21 and 90 were immunostained with antibodies against transcription factors Krox20 (red) and Oct6 (green). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). C, the percentage of DAPI

and only Oct6

cells is shown (n = 2 per group). Data are expressed as mean ± S.E.
notype in Lipin1<sub>1Hubr</sub> rats, consistent with the finding that Lipin 1 is strongly expressed in adipose tissues (9). However, similar to the above described phenotype in peripheral nerves, the WAT phenotype in Lipin1<sub>1Hubr</sub> rats seems to attenuate over time, as reflected by higher expression of adipogenic factors at PND 90 as compared with PND 21 (Fig. 8D), and relatively increased adipose mass at PND 90 (51% at PND 21 and 69% at PND 90 in Lipin1<sub>1Hubr</sub> rats as compared with wild-type rats).

**DISCUSSION**

Here we describe a new Lipin1<sub>1Hubr</sub> rat model that is characterized by a peripheral neuropathy, muscle wasting, and lipo-dystrophy. Importantly, the Lipin1<sub>1Hubr</sub> mutation does not prevent the production of a truncated protein that lacks PAP1 enzymatic activity. Moreover, in contrast to the models with complete loss of Lipin 1 function, the deficits present in Lipin1<sub>1Hubr</sub> rats become attenuated during adulthood, resulting in improvement of its phenotypes.

The Lipin1<sub>1Hubr</sub> phenotype was initially discovered in F<sub>3</sub> rats that were already outcrossed twice to wild-type Wistar/Crl rats. This highlights the importance of outcrossing ENU-mutagenized rats to clear the background of unwanted mutations. The F<sub>3</sub> Wistar/Crl Lipin1<sub>1Hubr</sub> mutation was backcrossed to the Wistar background for an additional 4 generations (6 outcrosses in total), which should theoretically decrease the total number of random background mutations to 1 (34). However, we cannot formally exclude the presence of tightly linked confounding mutations in Lipin1<sub>1Hubr</sub> rats.

The Lipin1<sub>1Hubr</sub> mutation disrupted the 5'/H11032-3'-end splice site of intron 18. Although potentially deleterious for correct splicing, this mutation led to a small amount (1–6%) of Lipin1 transcript with a correctly spliced exon 18–19 boundary (Lipin1<sub>1Ex18–19</sub>) in various tissues isolated from Lipin1<sub>1Hubr</sub> rats at either PND 21 or 90. This is probably a consequence of the capacity of the splicing machinery to occasionally use the mutated splicing site (see for example, Ref. 35). Our data, however, clearly demonstrate that the attenuation of the different Lipin1<sub>1Hubr</sub> phenotypes is not a consequence of an age-related increase of the presence of correctly spliced exon 18–19 boundary in Lipin1<sub>1Hubr</sub> rats. Importantly, by using three different sets of
primers (covering the boundary region between exons 18 and 19, and the region around exons 4–6, which can distinguish Lpin1α and Lpin1β isoforms), we detected the presence of mutated Lpin1 transcript in Lpin1<sup>1<sub>Hubr</sub></sup> rats. We even observed increased expression of both Lpin1α and Lpin1β isoforms in Lpin1<sup>1<sub>Hubr</sub></sup> rats at PND 21, suggesting a functional feedback loop affecting their expression. These data, together with our observation that the stop codon is induced in the 19th exon of Cds1, which results in partial reduction of PAP1 activity underlying a milder transient peripheral neuropathy (15). In addition, the mutant mouse line 20884 also harbors a mutation in Ncam, which is not the case for Lpin1<sup>1<sub>Hubr</sub></sup> rats (data not shown).

In our previous study (2), we have demonstrated that a decrease in PAP1 activity was followed by significant accumulation of PA in the endoneurium of both Lpin1<sup>fld/fld</sup> and Lpin1-SC conditional (MPZ<sup>Cre/+</sup>/Lp<sub>E2–3</sub>/E2–3<sup>)<sub>Hubr</sub></sup>) mutant mice. Surprisingly, the Lpin1<sup>1<sub>Hubr</sub></sup> mutation does not provoke the same effect. We found no significant differences in the levels of PA in the sciatic nerve tissue derived from Lpin1<sup>1<sub>Hubr</sub></sup> mice. However, similar to the observation in Lpin1<sup>fld/fld</sup> mice the amount of PA in the Lpin1<sup>1<sub>Hubr</sub></sup> WAT was three times higher as compared with wild-type rats. This difference between Lpin1 null mice (Lpin1<sup>fld/fld</sup>, MPZ<sup>Cre/+</sup>/Lp<sub>E2–3</sub>/E2–3<sup>)<sub>Hubr</sub></sup>) and the Lpin1<sup>1<sub>Hubr</sub></sup> rat model could be explained by a process of compensation that is tissue- and species-dependent. SCs may compensate the loss of PAP1 activity by PAP2 (26). Indeed, we observed that PAP2 activity and increased production of PI may underlie the phenotype in Lpin1<sup>1<sub>Hubr</sub></sup> rats as compared with Lpin1<sup>fld/fld</sup>.
mice. In line with this hypothesis, we observed partial restoration (e.g., relatively elevated or increased levels of cholesterol and phosphatidylserine, respectively) of lipid levels in peripheral nerves of adult Lpin1Habr rats, which is in parallel with the observed improvement in myelination in adult Lpin1Habr rats.

In the PNS, partially recovered Mpz and Pmp22 expression levels accompanied the progression of myelination in Lpin1Habr sciatic nerves at PND 90. In contrast to Lpin1fld/fld and MPZCre+/+ /LpE2–3/fE2–3 mice, expression of Krox20 was decreased but not abolished at PND 21, whereas it was increased at PND 90. Krox20 is an important controller of myelination and a marker of mature SCs (29), thus indicating that myelination processes are down-regulated in Lpin1Habr sciatic nerve tissue at PND 21 but up-regulated at PND 90, resulting in a relative increase in myelination at PND 90, a process that was never observed in Lpin1fld/fld and MPZCre+/+ /LpE2–3/fE2–3 mice (2). Oct6, a marker of immature and promyelinating SCs (29, 30), is up-regulated at PND 21, thus indicative of an increased number of non-differentiated SCs. Interestingly, at PND 90, Oct6 levels are even further increased, which correlates with the observed elevated level of cell proliferation at this stage. Indeed, we observed the presence of a mixed population of three different SC lineages in Lpin1Habr PND 90 nerves: immature promyelinating (Krox20+, Oct6+), early myelinating (Krox20+, Oct6+), and mature myelinated (Krox20+, Oct6−) SCs. In contrast, the main SC population observed in adult Lpin1fld/fld and MPZCre+/+ /LpE2–3/fE2–3 mice were immature (Krox20−, Oct6+) SCs. These observations, which are further strengthened by our Nile Red staining data, reveal that Lpin1Habr animals predominantly develop characteristics of hypomyelination rather than characteristics of demyelination, which was observed in Lpin1fld/fld and MPZCre+/+ /LpE2–3/fE2−3 mice.

Lipin 1 was previously reported to play a role as a transcriptional coactivator (1). However, our observations that both Lipin 1α and 1Δβ forms preferentially localized to the cytoplasm and that both wild-type and mutated forms of Lipin 1α and 1β failed to activate the Krox20 MSE do not support the idea that the truncated form of Lipin 1 in Lpin1Habr rats acts as a transcriptional regulator involved in PNS myelination and suggest that Lipin 1 may have additional yet to be discovered function(s).

The transitory lipodystrophy phenotype in Lpin1Habr rats might be the result of immobility-induced hypophagia and a subsequent decreased lipid accumulation at a young age, whereas at an older age rats become more able to feed correctly due to increased mobility. This explanation is, however, unlikely, as most Lpin1Habr pups retained partial ability to move around after the onset of the Lpin1Habr phenotype. In addition, chow pellets were provided to the maternal cage floor to enable feeding under competitive conditions. An alternative explanation could come from the previously observed regulatory role of Lipin 1 during initial phases of adipogenesis, for the induction of adipogenic factors including PPARγ and CCAAT enhancer-binding protein (C/EBP) α (9, 37). Our observation that relative expression levels of Fabp4, PPARγ1, and PPARγ2 are increased in WAT derived from Lpin1Habr rats at PND 90 as compared with PND 21, mirrored by a relative increase in WAT amount in Lpin1Habr rats, potentially indicates that a regulatory function of Lipin 1 in Lpin1Habr rats remained functional in WAT, thus enabling partial stimulation of adipogenesis. Additional studies are required to corroborate the suggested Lipin 1 involvement in regulation of nerve myelination and WAT function.

In humans, mutations in LPIN1 were recently described to result in recurrent myoglobinuria (i.e., the presence of myoglobin in urine, which is usually linked to destruction of skeletal muscle fibers, the latter also known as rhabdomyolysis) or statin-induced myopathy (38). An additional study reported severe early childhood myoglobinuria due to an in-frame intragenic Lpin1 deletion, removing a large part of the Lipin 1 CLIP domain (exons 18 and 19) but preserving its transcriptional LXXIIL motif (39). Unfortunately, the PNS status of these patients was not evaluated and these patients may be too young to clearly detect a potentially mild WAT-related phenotype. The cause of the difference between so far described human phenotypes and the phenotypes of rodents with mutated Lipin 1 forms remains largely unknown.

Interestingly, a combination of episodic rhabdomyolysis and peripheral neuropathy occurred in patients with mitochondrial trifunctional protein deficiency (40). Thus, it remains possible that so far undetected peripheral neuropathies might indeed affect patients carrying a Lpin1 mutation.

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