LRP1 Regulates Peroxisome Biogenesis and Cholesterol Homeostasis in Oligodendrocytes and is Required in CNS Myelin Development and Repair

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Abstract

The low-density lipoprotein related-receptor-1 (LRP1) is a large endocytic and signaling receptor. We show that Lrp1 is required for proper CNS myelinogenesis in vivo. Either global inducible or oligodendrocyte (OL)-lineage specific ablation of Lrp1 impairs myelin development and adult white matter repair. In primary oligodendrocyte progenitor cells (OPCs), Lrp1 deficiency reduces cholesterol levels and attenuates differentiation into mature OLs. Despite a strong increase in the sterol-regulatory element-binding protein-2, Lrp1\(-/-\) OPCs are not able to maintain normal cholesterol levels, suggesting more global metabolic deficits. Mechanistic studies identified a decrease in peroxisomal biogenesis factor-2 and a reduction in peroxisomes localized to OL processes. Treatment of Lrp1\(-/-\) OPCs with cholesterol or pharmacological activation of peroxisome proliferator-activated receptor-\(\gamma\) with pioglitazone is not sufficient to promote differentiation; however when combined, cholesterol and pioglitazone treatment enhance OL production. Collectively, our studies identify a novel link between LRP1, peroxisomes, and OPC differentiation during white matter development and repair.
**Introduction**

In the central nervous system (CNS), the myelin-producing cell is the oligodendrocyte (OL). Mature OLs arise from oligodendrocyte progenitor cells (OPCs), a highly migratory pluripotent cell type (Rowitch and Kriegstein, 2010, Zuchero and Barres, 2013). OPCs that commit to differentiate along the OL-lineage undergo a tightly regulated process of maturation, membrane expansion, and axon myelination (Emery et al., 2009, Li and Yao, 2012, Simons and Lyons, 2013, Hernandez and Casaccia, 2015). Even after developmental myelination is completed, many OPCs persist as stable CNS resident cells that participate in normal myelin turnover and white matter repair following injury or disease (Franklin and Ffrench-Constant, 2008, Fancy et al., 2011).

LRP1 is a member of the LDL receptor family with prominent functions in endocytosis, lipid metabolism, energy homeostasis, and signal transduction (Boucher and Herz, 2011). Lrp1 is broadly expressed in the CNS and abundantly found in OPCs (Zhang et al., 2014, Auderset et al., 2016). Global deletion of Lrp1 is embryonically lethal (Herz et al., 1992) and conditional deletion revealed numerous tissue specific functions in neural and non-neural cell types (Lillis et al., 2008). In the PNS, Lrp1 regulates Schwann cell survival, myelin thickness, and morphology of Remak bundles (Campana et al., 2006, Mantuano et al., 2010, Orita et al., 2013). In the CNS, Lrp1 influences neural stem cell proliferation (Auderset et al., 2016), synaptic strength (Nakajima et al., 2013, Gan et al., 2014), axonal regeneration (Stiles et al., 2013, Yoon et al., 2013, Landowski et al., 2016), and clearance of amyloid beta (Liu et al., 2010, Zlokovic et al., 2010, Kanekiyo and Bu, 2014, Kim et al., 2014). Recent evidence shows that neurospheres deficient for Lrp1 produce more GFAP+ astrocytes at the expense of O4+ OLs and TuJ1+ neurons (Hennen et al., 2013, Safina et al., 2016). Whether LRP1 is required for proper CNS myelogenesis, nerve conduction, or repair of damaged adult CNS white matter, however, has not yet been examined. Moreover, the molecular basis of how LRP1 influences OPC differentiation remains poorly understood.

LRP1 is a large type 1 membrane protein comprised of a ligand binding 515-kDa α chain non-covalently linked to an 85-kDa β chain that contains the transmembrane domain and cytoplasmic portion. Through its α chain, LRP1 binds over 40 different ligands with diverse biological functions (Lillis et al., 2008, Fernandez-Castaneda et al., 2013). LRP1 mediates endocytotic clearance of a multitude of extracellular ligands (May et al., 2003, Tao et al., 2016) and participates in cell signaling, including activation of the Ras/MAPK and AKT pathways (Martin et al., 2008, Fuentealba et al., 2009, Muratoglu et al., 2010). The LRP1β chain can be processed by γ-secretase and translocate to the nucleus where it associates with transcription factors to regulate gene expression (May et al., 2002, Carter, 2007).

Here we combine conditional Lrp1 gene ablation with ultrastructural and electrophysiological approaches to show that Lrp1 is important for myelin development, nerve conduction, and adult CNS white matter repair. Gene expression analysis in Lrp1 deficient OPCs identified a reduction in peroxisomal gene products. We show that Lrp1 deficiency decreases production of peroxisomal proteins and disrupts cholesterol homeostasis. Mechanistic studies uncover a novel role for Lrp1 in PPARγ mediated OPC differentiation, peroxisome biogenesis, and CNS myelination.
Results

**Lrp1 is required for proper CNS myelin development.** In the early postnatal brain, Lrp1 is broadly expressed (Zhang et al., 2014). In the OL-lineage, LRP1 protein is highly enriched in OPCs and absent in mature OLs (Auderset et al., 2016). To study the role of Lrp1 in CNS myelination, we pursued a mouse genetic approach in vivo. To circumvent the early lethality of Lrp1 global knockout mice (Herz et al., 1992), we generated Lrp1<sup>floxed/lox</sup>;CAG-creERT<sup>TM</sup> mice (Lrp1 iKO) that allow tamoxifen (TM)-inducible gene ablation under the CMV immediate enhancer/β-actin promoter (Figure 1-figure supplement 1). To assay whether LRPI is required for proper CNS myelinogenesis, neonatal Lrp1 control and iKO mice were subjected to TM injection at P5 and analyzed at P21 (Figure 1a). Western blot analysis of whole brain lysates revealed a ~40% reduction for LRP1 in Lrp1 iKO brain homogenate, indicating that mice are hypomorph rather than null for LRPI (Figure 1b). The decrease in neural LRP1 leads to a simultaneous reduction of OL-lineage markers, of which 2’,3’-cyclic-nucleotide 3’-phosphodiesterase (CNP), proteolipid protein (PLP), and myelin basic protein (MBP) reach significance (Figure 1c). Ultrastructural analysis of P21 optic nerve cross-sections revealed hypomyelination in Lrp1 iKO mice (Figure 1d). Only 49.7± 4.2% of axons are myelinated in Lrp1 iKO mice, whereas 74.7± 2.4% of axons are myelinated in littermate controls (Figure 1e). Independent of Lrp1 genotype, the majority of large caliber axons (>1 µm in diameter) is myelinated, while small caliber axons (<0.2 µm) are not myelinated. However, intermediate-to-small sized axons, 0.3-0.9 µm in caliber, are vulnerable to Lrp1 deficiency and show hypomyelination (Figure 1-figure supplement 2a). To assess the insulating properties of myelin sheaths in the optic nerve, the ratio of the axonal diameter to the total fiber diameter (g-ratio) was calculated. The average g-ratio for myelinated axons in Lrp1 control and iKO mice is 0.76± 0.001 and 0.81± 0.001, respectively (Figure 1f and Figure 1-figure supplement 2b). Axon density in the optic nerve of Lrp1 iKO mice is similar to controls (Figure 1-figure supplement 2c). However, intermediate sized axons (0.4-0.8 µm) are less frequent and small axons (<0.4 µm) are more abundant in Lrp1 iKO optic nerves (Figure 1-figure supplement 2d). Together, these studies show that global inducible ablation of Lrp1 at P5 leads to a partial loss of LRPI and CNS hypomyelination at P21.

**Figure 1-figure supplement 1:** Tamoxifen induced global Lrp1 ablation and genotyping. (a) Cartoon showing Lrp1 wildtype (wt) and targeted, LoxP flanked (floxed), alleles. The location of PCR primers used for genotyping neomycin cassette (Neo), and LoxP sites are shown. (b) For global inducible gene ablation, the CAG-CreER<sup>TM</sup> mouse line was used, in which the Cre recombinase is fused with a TM responsive estrogen receptor (Ers1) and expressed under the control of a ubiquitous chicken β-actin-CMV hybrid (CAG) promoter. (c) Following tamoxifen (TM) administration there are two possible outcomes: deletion of the Neo cassette only or deletion of the Neo cassette and exon 1. (d) Analysis of PCR products amplified from genomic brain DNA of Lrp1<sup>+/−</sup> mice with (+) or without (-) the cre allele; Lrp1<sup>floxed/−</sup> mice ±cre and ±TM treatment; Lrp1<sup>floxed/−</sup> mice ±cre allele and ±TM treatment. The F1/R1 primer pair amplifies a ~300 bp PCR product from wt Lrp1 allele and a ~400 bp PCR product if the Neo cassette is deleted. The F2/R2 primer pair amplifies a 291 bp PCR product from wt Lrp1 allele and a 350 bp PCR product from Lrp1 flox allele. The F1/R2 primer pair amplifies a ~500 bp PCR product if exon 1 in deleted. The IL-2pF/IL-2pR primer pair amplifies a 324 bp fragment and served as internal PCR quality control. The CreF/CreR primer pair amplifies a ~200 bp PCR product from Cre allele.
**Figure 1**: Inducible global ablation of *Lrp1* leads to CNS hypomyelination and reduced remyelination of a chemically induced white matter lesion. (a) Timeline in days showing when *Lrp1* ablation was induced and when mice were sacrificed. (b) Immunoblotting of whole brain lysates prepared from *Lrp1* control (Ctrl) and *Lrp1* inducible knockout (*Lrp1<sup>lox<sub>lox</sub>,CAG-CreERT<sub>2</sub>, Lrp1 iKO) mice. Representative blots probed with anti-LRP1β, anti-CNP, anti-PLP, anti-MAG, anti-MBP, anti-β-III tubulin, anti-GAPDH, anti-βiFP, and anti-β-actin are shown. (c) Quantification of protein levels detected in brain lysates of *Lrp1* control (n = 3) and iKO (n = 3) mice. (d) Ultrastructural images of optic nerve cross-sections from P21 *Lrp1* control and iKO mice. Scale bar= 1 μm. (e) Quantification of myelinated axons in *Lrp1* control (n=3) and iKO (n=3) mice. (f) Averaged g-ratio of optic nerve fibers of *Lrp1* control (n=1932 axons, 3 mice) and iKO (n=2461 axons, 3 mice). Scale bar= 1 μm. (g) Timeline in weeks indicating when *Lrp1* ablation was induced, lysolecithin (LPC) injected, and when animals were sacrificed. (h) Cartoon showing unilateral injection of LPC in the corpus callosum (CC) and injection of PBS on the contralateral side. Coronal brain sections (series of 6, each 120 μm apart) probed for *Mbp* by *in situ* hybridization (ISH). Brain sections containing the lesion center were subjected to quantification. (i) Coronal brain sections through the CC 21 days post LPC injection (21 DPI). The outer rim of the lesion area (lesion<sup>out</sup>) is demarcated by the elevated *Mbp* signal (white dashed line). The non-myelinated area of the lesion is defined by the inner rim of elevated *Mbp* signal (lesion<sup>in</sup>) and delineated by a solid yellow line. Scale bar= 200μm. (j) Quantification of the initial lesion size (lesion<sup>out</sup>) in *Lrp1* control (n=8) and iKO (n=6) mice. (k) Quantification of the remyelinated area in *Lrp1* control (n=8) and iKO (n=6) mice. The extent of remyelination was calculated as the percentile of (lesion<sup>out</sup> - lesion<sup>in</sup>)/(lesion<sup>out</sup>). Results are shown as mean ± SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure1-supplement data1.

**Inducible ablation of *Lrp1* in adulthood attenuates white matter repair.** To study white matter repair, 8-week-old *Lrp1* control and iKO mice were treated with i.p. TM. One month later, mice were subjected to unilateral injection of 1% lysophosphatidylcholine (LPC) into the corpus callosum and sacrificed 10 or 21 days later (Figure 1g). LRP1 protein levels in brain lysates of *Lrp1* iKO mice were assessed 31 and 52 days after TM injection by Western blot analysis and revealed a reduction compared to control brains (Figure 1-figure supplement 3a). Fluoromyelin-Green (FM-G) labeling and *in situ* hybridization (ISH) for *Mbp* transcripts of *Lrp1* control and iKO forebrain 31 days post TM injection showed comparable...
staining (Figure 1-figure supplement 3b). To examine whether Lrp1 participates in the remyelination process following LPC-induced axon demyelination, adult mice were subjected to unilateral and focal injection of LPC into the corpus callosum. The contralateral side was injected with saline (PBS) and served as an internal control (Figure 1h). At 10 and 21 days post injection (DPI), mice were killed, brains extracted, and serially sectioned through the lesion area. Sections were stained with FM-G, anti-GFAP, and the nuclear dye Hoechst 33342 (Figure 1-figure supplement 3c and 3d). Intracranial injection of PBS led to a transient increase in GFAP, but not a reduction in FM-G staining (Figure 1-figure supplement 3c). Independent of Lrp1 genotype, at 10 days following LPC injection, similar-sized white matter lesions (area devoid of FM-G labeling) and comparable astrogliosis, as assessed by GFAP staining, were noted (Figure 1-figure supplement 3d). At 21 DPI however, astrogliosis was reduced and the lesion area was significantly smaller in LPC injected Lrp1 control mice compared to iKO mice (Figure 1-figure supplement 3d). For quantification of lesion repair, serial sections were stained with cRNA probes specific for the OPC/OL markers Pdgfra, Mag, Plp1, and Mbp. The LPC lesion was readily detected by the upregulation of Pdgfra, Mag, Plp1, and Mbp transcripts (Figure 1-figure supplement 3e and 3f). No changes for any of these transcripts were observed on the PBS injected side (Figure 1-figure supplement 3e). Because Mbp mRNA is strongly upregulated in myelin producing OLs and transported into internodes(Ainger et al., 1993), we used Mbp in situ hybridization on serial sections to find the center of the white matter lesion. The center was defined as the section with the largest circumference of the intensely labeled Mbp+ area (Figure 1h). The extent of initial white matter lesion, the outer rim of elevated Mbp labeling (white dotted line), was comparable between Lrp1 control and iKO mice (Figure 1l). However, the area that failed to undergo repair, the inner rim of elevated Mbp labeling (yellow solid line), was larger in Lrp1 iKO mice (Figure 1l). Quantification of lesion repair revealed a significant decrease in Lrp1 iKO mice compared to controls (Figure 1k). As an independent assessment, serial sections through the lesion were stained for Pdgfra, Plp1, and Mag transcripts and revealed fewer labeled cells within the lesion (Figure 1-figure supplement 3g). Together these findings indicate that in adult mice, Lrp1 is required for the timely repair of a chemically induced white matter lesion.

**Figure 1-figure supplement 2: Global inducible ablation of Lrp1 in neonatal mice leads to CNS hypomyelination and reduced axon caliber.** (a) Graph showing the percentage of myelinated axons in the optic nerve of P21 mice as a function of axon caliber. Axon calibers of Lrp1 control (n=3) and iKO (n=3) mice were binned into 9 groups of 0.2 μm intervals, ranging from 0.1 to 1.7 μm. The percentile of myelination is not significantly different for axons <0.3μm, significantly different for axons between 0.3-0.9μm, and not significantly different for axons between 1.0-1.7μm. (b) Scatter plot showing the distribution of g-ratios for individual fibers in the optic nerve of P21 Lrp1 control (n=2461 axons, 3 mice) and iKO (n=1934 axons, 3 mice). (c) Quantification of axon density per μm² in P21 optic nerve cross sections of Lrp1 control (n=3) and iKO (n=3) mice. (d) Morphometric assessment of axon caliber distribution in P21 optic nerves of Lrp1 control (n=3) and iKO (n=3) mice. Axon diameters were measured on electron microscopy images and quantification revealed a shift toward smaller-sized axons in Lrp1 iKO mice. Results are presented as the mean ± SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure1-source data1.
Figure 1-figure supplement 3: Lrp1 is important for white matter repair (a) Immunoblots of whole brain lysates prepared from Lrp1<sup>flox/flox</sup>;CAG-cre<i>ERTM</i> mice 31 and 52 days after TM (+) or vehicle (-) treatment. Representative blots probed with anti-LRP1β, anti-GFAP, anti-β-actin, anti-β-III tubulin, and anti-GAPDH. (b) Coronal sections of adult Lrp1 control and iKO mice 31 days after i.p. TM administration. Sections were stained with FM Green or probed for Mbp mRNA by in situ hybridization. Scale bar= 1mm. (c) Coronal forebrain sections of Lrp1 control mice without injection (naïve) or PBS injection in the corpus callosum. At 10 days post injection (10 DPI) and 21 DPI of PBS brains were sectioned and stained with FM Green, anti-GFAP and Hoechst dye33342. The white dotted lines demarcate the corpus callosum. (d) Coronal forebrain sections of Lrp1 control and iKO mice at 10 DPI and 21 DPI of LPC stained with FM Green, anti-GFAP, and Hoechst dye33342. White dotted lines demarcate the corpus callosum. Scale bar= 200µm. (e) Serial coronal-sections of adult brain after PBS and LPC injection in the corpus callosum were probed for <i>Pdgfra</i>, <i>Plp1</i>, <i>Mag</i>, and <i>Mbp</i> mRNA to identify the lesion area and to examine gene expression changes in the OL lineage. The PBS injection site is marked by an arrowhead and the LPC injection site is marked by an arrow. Scale bar= 200µm. (f) Serial brain sections of adult Lrp1 control and iKO mice injected with LPC at 21 DPI. Sections that contain lesion area (120 µm apart) were probed for <i>Pdgfra</i>, <i>Plp1</i>, and <i>Mag</i> mRNA expression. Scale bar= 200µm. (g) Coronal sections through the corpus callosum of Lrp1 control and Lrp1 iKO mice injected with LPC. Serial sections were probed for <i>Pdgfra</i>, <i>Plp1</i>, and <i>Mag</i> mRNA expression. Scale bar= 200µm.

Ablation of Lrp1 in the OL-lineage causes hypomyelination. To demonstrate an OL-lineage specific function for Lrp1 during CNS myelination, we generated Lrp1<sup>flox/flox</sup>;Olig2-cre mice (Lrp1 cKO<sup>Ol</sup>) (Figure 2-figure supplement 1a). Lrp1 cKO<sup>Ol</sup> pups are born at the expected Mendelian frequency and show no obvious abnormalities at the gross anatomical level. LRP1 protein levels in the brains of P10, P21, and P56 Lrp1 control and cKO<sup>Ol</sup> mice were analyzed by Western blot analysis and revealed a partial loss of LRP1β (Figure 2-figure supplement 1b). The partial loss of LRP1β in brain lysates of OL-lineage specific Lrp1 cKO<sup>Ol</sup> mice is due to Lrp1 expression in several other neural cell types (Zhang et al., 2014).
To examine whether Lrp1 cKO<sub>OL</sub> mice exhibit defects in myelination, optic nerves were isolated at P10, the onset of myelination; at P21, near completion of myelination; and at P56, when myelination is thought to be largely completed. Ultrastructural analysis at P10 revealed no significant difference in myelinated axons between Lrp1 control (17± 6%) and cKO<sub>OL</sub> (7± 2%) optic nerves (Figure 2a and 2b). At P21 and P56, the percentile of myelinated axons in the optic nerve of cKO<sub>OL</sub> mice (49± 4% and 66± 5%, respectively) is significantly reduced compared to controls (70± 2% and 88± 1%, respectively) (Figure 2a and 2b). Similarly to Lrp1 iKO mice (Figure 1-figure supplement 2a), in Lrp1 cKO<sub>OL</sub> mice intermediate to small sized axons, 0.3-0.9µm in caliber, are more vulnerable to hypomyelination (Figure 2-figure supplement 1c, 1f, and 1i). As an independent assessment of fiber structure, the g-ratio was determined. At P10, P21, and P56 the average g-ratio of Lrp1 cKO<sub>OL</sub> optic fibers is significantly larger than in age-matched Lrp1 control mice (Figure 2c and 2-figure supplement 1d, 1g, and 1j). While axon density in the optic nerve of Lrp1 cKO<sub>OL</sub> mice is similar to littermate controls at all three time points examined, there is a shift in axon caliber, similar to Lrp1 iKO mice (Figure 2-figure supplement 1e, 1h, 1k, and Figure 1-figure supplement 2d). Comparable to P21 Lrp1 iKO mice (Figure 1b and 1c), Western blot analysis of adult Lrp1 cKO<sub>OL</sub> brain lysates revealed a significant reduction in CNP, MAG, and MBP (Figure 2-figure supplement 1l and 1m). Together, these studies show that in the OL lineage Lrp1 functions in a cell-autonomous manner and is required for proper CNS myelinogenesis.

Figure 2: Lrp1 ablation in the oligodendrocyte (OL)-lineage leads to hypomyelination, nodal defects, and reduced myelin repair. (a) Ultrastructural images of optic nerve cross-sections from P10, P21, and P56 control and Lrp1<sup>lox/lox</sup>/Olig2<sup>Cre</sup> conditional knockout mice (Lrp1<sup>cKO</sup>). Scale bar= 1µm. (b) Quantification of myelinated axons in the optic nerve of Lrp1 control (n= 4 mice per time point) and cKO<sub>OL</sub> (n= 4 mice per time point) mice. (c) Averaged g-ratio of Lrp1 control and cKO<sub>OL</sub> optic nerve fibers from 4 mice per time points in each group. At P10, n= 488 axons for control and n= 261 axons for cKO<sub>OL</sub>; at P21, n= 1015 axons for control and n= 997 axons for cKO<sub>OL</sub>; at P56, n= 1481 axons for control and n= 1020 axons for cKO<sub>OL</sub>-were quantified. (d) Nodes of Ranvier in P21 optic nerves of Lrp1 control and cKO<sub>OL</sub> mice were labeled by anti-PanNaCh (green, node) and anti-Caspr (red, paranode) staining. Scale bar= 1µm. (e) Nodal defects detected include elongated node, heminode, and missing node (Na<sup>+</sup> channels absent). (f) Representative node staining categorized by axon diameter. (g) Quantification of nodal density in Lrp1 control (n= 6) and cKO<sub>OL</sub> (n= 5) optic nerves. (h) Quantification of abnormal nodes of Ranvier in Lrp1 control (n= 6) and cKO<sub>OL</sub> (n= 5) optic nerves. (i) Quantification of nodal frequency in axons with large, intermediate, and small caliber for Lrp1 control (n= 6) and cKO<sub>OL</sub> (n= 5) optic nerves. (j) Timeline in weeks showing when OL-lineage specific Lrp1 ablation (Lrp1<sup>lox/lox</sup>/Pdgfra-CreER<sup>TM</sup>, Lrp1 iKO<sub>OL</sub>) was induced, LPC injected, and when animals were sacrificed. (k) Coronal brain sections through the CC at 21 days post LPC injection of Lrp1 control and iKO<sub>OL</sub> mice. The initial
lesion area is demarcated by a white dashed line. A solid yellow line delineates the non-myelinated area. Scale bar= 200 µm. (l) Quantification showing the initial lesion size in Lrp1 control (n= 4) and iKOOL (n= 4) mice. (m) Quantification of the remyelinated area in Lrp1 control (n= 4) and iKOOL (n= 4) mice. Results are shown as mean ± SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure2-source data1.

Figure 2-figure supplement 1: Lrp1 ablation in the OL lineage leads to CNS hypomyelination. (a) Lrp1floflo mice were crossed with Olig2-cre mice to conditionally ablate Lrp1 in OL lineage (cKOOL). (b) Immunoblotting of whole brain lysates prepared from P10, P21, and P56 of Lrp1 control (Ctrl) and cKOOL mice. Representative blots probed with anti-LRP1β and anti-β-Actin. (c, f, and i) Graphs show the percentage of myelinated axons at P10, P21 and P56 as a function of axon caliber for Lrp1 control (n= 4 for each time point) and cKOOL (n= 4 for each time point) mice. Axon calibers were binned into 9 groups of 0.2 µm intervals, ranging from 0.1 to 1.7 µm. (d, g, and j) Scatter plot showing the distribution of g-ratios for individual fibers in the optic nerve at P10, P21 and P56 of Lrp1 control and cKOOL mice. P10, n= 488 axons from Lrp1 control mice and n= 261 axons from cKOOL mice; P21, n= 1015 axons from Lrp1 control mice and n= 997 axons from 4 cKOOL mice; P56, n= 1481 axons from Lrp1 control mice and n= 1020 axons from 4 cKOOL mice. (e, h, and k) Morphometric assessment of axon caliber distribution in P10, P21 and P56 optic nerves of Lrp1 control (n= 4) and cKOOL (n= 4) mice. Measurements of axon diameter were made from electron microscopy images. In Lrp1 cKOOL optic nerves, the population at smaller-sized (0.2-0.4 µm) axons is reduced at P21 and P56. In addition, there is shift towards larger sized axons at P21 in Lrp1 cKOOL optic nerves (h). (l) Immunoblotting of whole brain lysates prepared from Lrp1 control (Ctrl) and cKOOL mice. Representative blots probed with anti-LRP1β, anti-CNP, anti-MAG, anti-MBP, anti-β-III tubulin, anti-GFAP, and anti-β-actin. (m) Quantification of protein levels detected by Western blotting of Lrp1 control (n= 3) and cKOOL (n= 3) brain lysates. (n) Electron microscopy images of optic nerve cross- and longitudinal-sections acquired from P21 Lrp1 control and cKOOL mice. Axons that are >1 µm in diameter are colored in light blue. Scale bar= 1 µm. (o) Quantification of axon density in the P21 optic nerve for Lrp1 control (n= 4) and cKOOL (n= 4) mice. (p) Quantification of axons in the optic nerve that are smaller than < 0.5 µm, between 0.5-1.0 µm and larger than 1 µm for Lrp1 control (n= 4) and cKOOL (n= 4) mice. Results are presented as the mean ± SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure2-source data1.
Loss of Lrp1 in the OL-lineage causes defects in nodes of Ranvier and faulty nerve conduction. To examine whether Lrp1 in OLs is required for nodal or paranodal organization, optic nerve sections of P21 Lrp1 control and cKO\textsubscript{OL} mice were immunostained for sodium channels (PanNaCh) and the paranodal axonal protein (Caspr). Nodal density, the number of PanNaCh\textsuperscript{+} clusters in longitudinal optic nerve sections is significantly reduced in Lrp1 cKO\textsubscript{OL} mice (Figure 2d and 2g). In addition, an increase in nodal structural defects, including elongated nodes, heminodes, and nodes in which sodium channel staining is missing, was observed in mutant nerves (Figure 2e). Quantification of nodal structural defects revealed an increase from 13.7±1.3% in Lrp1 control mice to 33.4±2.9% in cKO\textsubscript{OL} optic nerves (Figure 2h). Of the total number of PanNaCh\textsuperscript{+} nodes quantified (including elongated nodes and heminodes), a greater fraction is associated with large (>1 μm) and intermediate (0.5-1 μm) caliber axons in Lrp1 cKO\textsubscript{OL} mice, while the number of nodes associated with small (<0.5 μm) caliber axons is significantly reduced in mutants (Figure 2i). The total number of optic nerve axons does not change between Lrp1 control and cKO\textsubscript{OL} mice, but the fraction of large diameter axons is significantly increased in mutants (Figure 2-figure supplement 1n-1p).

To assess whether structural defects observed in optic nerve myelin, nodes of Ranvier, and axon caliber distribution in Lrp1 cKO\textsubscript{OL} mice are associated with impaired nerve conduction, we used electrophysiological methods to measure compound action potentials (CAPs) in acutely isolated nerves (Figure 2-figure supplement 2a). Analysis of CAP recordings identified 4 peaks with distinct latencies in P21 optic nerves. Representative traces of Lrp1 control and cKO\textsubscript{OL} nerve recordings were fitted as the sum of four Gaussians (Figure 2-figure supplement 2b). Peaks 1, 2, and 3 are likely obtained from myelinated axons and Peak 4 represents a population of slow conducting, possibly non-myelinated axons known to be present in the P21 optic nerve (Mironova et al., 2016). A shift in conduction to the right, which reflects an increase in the relative contribution of unmyelinated axons to the CAP and a reduction in amplitudes were observed in Lrp1 cKO\textsubscript{OL} nerves (Figure 2-figure supplement 2c-2f). Changes in electrophysiological properties in Lrp1 cKO\textsubscript{OL} nerves fit well with defects at the ultrastructural level and aberrant node assembly. Taken together, OL-lineage specific ablation of Lrp1 leads to impaired nerve conduction.

Figure 2-figure supplement 2: Loss of Lrp1 in the OL lineage leads to faulty nerve conduction. (a) Scheme depicting the orientation of an optic nerve prepared for compound action potential (CAP) recording. Positions of the stimulating electrode, the recording electrode, and artifact subtraction electrode are shown. (b) Left: representative raw CAP traces of P21 optic nerves. Right: For each recording, traces were fitted with 4 Gaussians representing peak 1 (red), peak 2 (green), peak 3 (blue), peak 4 (cyan), and the sum of the four peaks (magenta). (c) The distribution of peak populations in Lrp1 control and cKO\textsubscript{OL} mice. (d) Quantification of amplitudes (mV) of peaks 1, 2, 3 and 4 in Lrp1 control and cKO\textsubscript{OL} optic nerves. (e) Quantification of conduction velocities (m/sec) of peaks 1, 2, 3 and 4 in Lrp1 control and cKO\textsubscript{OL} optic nerves. (f) Reconstituted averaged peak1-4 amplitude as a function of time. For Lrp1 control mice n= 21 nerves from 14 mice and for cKO\textsubscript{OL} n= 9 nerves from 7 mice. Results are presented as the mean ±SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure2-source data1.
OL-lineage specific ablation of Lrp1 impairs timely repair of damaged white matter. To determine the cell autonomy of Lrp1 in adult white matter repair, we generated mice that allow inducible Lrp1 ablation selectively in OPCs in adult mice. Inducible gene ablation was necessary to rule out potential confounding effects on repair, originating from developmental white matter defects observed in adult Lrp1 cKOOL mice (Figure 2a). For repair studies, Lrp1^floxed/floxed;PDGFRα-/-creERT2 (Lrp1 iKOOL) mice were generated and gene ablation was induced by TM administration at 8 weeks of age (Figure 2j). One month later, mice were subjected to stereotaxic injection of LPC into the corpus callosum. The contralateral side was injected with PBS and served as a negative control. Twenty-one days post injection (21 DPI) brains were collected and analyzed. Detection of the initial white matter lesion and quantification of the extent of axon re-myelination was carried out as shown above (Figure 1h and 1i). The initial size of the LPC inflicted white matter lesion is comparable between Lrp1 control and iKOOL mice (Figure 2k and 2l). The extent of lesion repair was significantly decreased in Lrp1 iKOOL mice (Figure 2m), demonstrating a cell-autonomous role for Lrp1 in the OL-lineage for the timely repair of a white matter lesion.

Figure 3: Loss of Lrp1 in the OL-lineage attenuates OL differentiation. (a) Cross-sections of Lrp1 control and cKOOL mice stained with anti-PDGFRα (OPC marker), anti-Olig2 (pan-OL marker), anti-CC1 (mature OL marker), and Hoechst dye33342. Scale bar= 100µm. (b) Cross- and -longitudinal sections of Lrp1 control and cKOOL optic nerves probed for Pdgfra, Mag, and Plp mRNA expression. Scale bar= 100µm. (c) Quantification of labeled cells per nerve cross-section. Anti-PDGFRα, n= 8 for controls and n= 6 for cKOOL mice; anti-Olig2 and anti-CC1, n=11 for control and n=12 for cKOOL mice. (d) Quantification of labeled cells per nerve cross-section. Pdgfra, n= 8 for control and n= 6 for cKOOL mice; Mag, n= 11 for controls and n= 11 for cKOOL mice; Plp, n= 11 for controls and n= 10 for cKOOL mice. (e) Workflow for OPC isolation with timeline when growth medium (GM) or differentiation medium (DM) was added and cells were harvested. (f) OPC/OL culture after 3 days in DM stained with anti-NG2 (premyelinating marker), anti-CNP (differentiating OL marker), and Hoechst dye33342. Scale bar= 100µm.
Immunoblot of OL lysates prepared from Lrp1 control and cKO<sup>OL</sup> cultures after 3 days in DM probed with anti-LRP1β and anti-β-actin. (h) Quantification of NG2<sup>+</sup> (n = 3 per condition) and CNP<sup>+</sup> (n = 3 per condition) cells in Lrp1 control and cKO<sup>OL</sup> cultures. (i) OL cultures after 5 days in DM stained with anti-MAG, anti-PLP, and anti-MBP. Scale bar=100μm. (j) Immunoblotting of OL lysates prepared from Lrp1 control and cKO<sup>OL</sup> cultures after 5 days in DM probed with anti-LRP1β, anti-CNp, anti-MAG, anti-PLP, anti-MBP, and anti-β-actin. (k) Quantification of MAG<sup>+</sup> (n = 3 per condition), PLP<sup>+</sup> (n = 3 per condition), and MBP<sup>+</sup> (n = 5 per condition) cells in Lrp1 control and cKO<sup>OL</sup> cultures. (l) Quantification of protein levels in OL lysates detected by immunoblotting. Anti-LRP1, CNP, and PLP, n=3 per condition; anti-MAG, n=4 per condition; anti-MBP, n=5 per condition. Results are shown as mean values ±SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure-3-source data1.

Conditional ablation of Lrp1 in the OL-lineage attenuates OPC differentiation. CNS hypomyelination in Lrp1 cKO<sup>OL</sup> mice may be the result of reduced OPC production or impaired OPC differentiation into myelin producing OLs. To distinguish between these two possibilities, optic nerve cross-sections were stained with anti-PDGFRα, a marker for OPCs, anti-Olig2 to account for all OL lineage cells in the culture, and anti-CC1, a marker for mature OLs. No change in OPC density was observed, but the number of mature OLs was significantly reduced in Lrp1 cKO<sup>OL</sup> mice (Figure 3a and 3c). Optic nerve ISH for Pdgfra<sup>+</sup> revealed no reduction in labeled cells in Lrp1 cKO<sup>OL</sup> mice, a finding consistent with anti-PDGFRα immunostaining. The density of Plp and Mag expressing cells, however, is significantly reduced in the optic nerve cross-sections and longitudinal-sections of Lrp1 cKO<sup>OL</sup> mice (Figure 3b and 3d). These studies reveal that OPCs are present at normal density and tissue distribution in Lrp1 cKO<sup>OL</sup> mice, but apparently fail to generate sufficient numbers of mature, myelin-producing OLs.

**Figure 3-figure supplement 1: Loss of Lrp1 does not alter OPC proliferation.** (a) Timeline in days indicating when growth medium (GM) was added to cells and when cells were harvested (H) to assess proliferation. (b) OPC/OL culture after 1 or 2 days in GM stained with anti-Ki67 (proliferation marker) and Hoechst dye33342. Scale bar=100μm. (c) Quantification of cell proliferation in OPC/OL cultures prepared from Lrp1 control and cKO<sup>OL</sup> mice. The percentile of Ki67<sup>+</sup>/Hoechst<sup>+</sup> cells was calculated on day 1 for Lrp1 control (n=5) and cKO<sup>OL</sup> (n=5) cultures and on day 2 for Lrp1 control (n=4) and cKO<sup>OL</sup> (n=4) cultures. (d) Immunoblotting of cell lysates prepared from Lrp1 control and cKO<sup>OL</sup>OPC/OL cultures after 3 days in differentiation medium (DM). Blots were probed with anti-LRP1β, anti-CNp, anti-MAG, anti-PLP, and anti-β-actin. (e) Quantification of protein levels detected by immunoblotting per OPC/OL culture from Lrp1 control and cKO<sup>OL</sup> mice. Anti-LRP1 and anti-MAG, n=3 for per condition; anti-CNp and anti-PLP, n=4 per condition. (f) Immunoblotting of lysates prepared from Lrp1 control and cKO<sup>OL</sup>OPC/OL cultures after 3 days in DM. Representative blots were probed with anti-p-AKT (S473), anti-p-TAKT, anti-pERK (1/2), anti-tERK (1/2), and anti-GAPDH. (g) Quantification of protein levels detected by immunoblotting per OPC/OL culture from Lrp1 control and cKO<sup>OL</sup> mice. pAKT/AKT, n=5 for Lrp1 control and cKO<sup>OL</sup> mice; pERK/tERK, n=3 for Lrp1 control and cKO<sup>OL</sup> mice. Results are presented as the mean ± SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure 3-source data1.

Loss of Lrp1 attenuates OPC differentiation in vitro. To independently assess the role of Lrp1 in OL differentiation, we isolated OPCs from brains of Lrp1 control and cKO<sup>OL</sup> pups (Figure 3e). OPCs were kept in PDGF-AA containing growth medium (GM), allowing them to proliferate or switched to differentiation medium (DM) containing triiodothyronine (T3). Staining of cells for the proliferation marker Ki67 did not reveal any change in OPC proliferation in Lrp1 cKO<sup>OL</sup> cultures after 1 or 2 days in GM (Figure 3-figure supplement 1a-1c). After 3 days in DM, the number of NG2<sup>+</sup> and CNP<sup>+</sup> OLs was comparable between Lrp1 control and cKO<sup>OL</sup> cultures (Figure 3f and 3h). An abundant signal for LRP1β was detected in
Lrp1 control lysate, but LRP1 was not detectable in Lrp1 cKOOL cell lysate, demonstrating efficient gene deletion in the OL lineage (Figure 3g). Moreover, a significant reduction in CNP, MAG, and PLP was detected in Lrp1 cKOOL cell lysates (Figure 3-figure supplement 1d and 1e). As LRPI signaling is known to regulate Erk1/2 and AKT activity (Yoon et al., 2013), immunoblots were probed for pAKT (S473) and pErk1/2. When normalized to total AKT, levels of pAKT are reduced in Lrp1 cKOOL lysate, while pErk1/2 levels are comparable between Lrp1 control and cKOOL lysates (Figure 3-figure supplement 1f and 1g). Extended culture of Lrp1 deficient OLs in DM for 5 days is not sufficient to restore myelin protein levels. Compared to Lrp1 control cultures, mutants show significantly fewer MAG+, PLP+, and MBP+ cells (Figure 3i and 3k) and immunoblotting of cell lysates revealed a reduction in total CNP, MAG, PLP, and MBP (Figure 3j and 3l). Collectively, our studies demonstrate a cell-autonomous function for Lrp1 in the OL lineage, important for cell differentiation into myelin sheet producing OLs.

Figure 4: Lrp1 deficient OPCs show reduced levels of free cholesterol. (a) OPCs were isolated from P10 brains by anti-PDGFRα immunopanning, sonicated and subjected to measurement of free and esterified cholesterol. (b and c) Quantification of free cholesterol (Chol) (b) and total cholesterol (Chol & Chol ester) (c) from Lrp1 control (n = 5) and cKOOL (n = 5) OPCs. (d) Lrp1 control and cKOOL OLs after 5 days in DM stained with filipin and anti-MBP. Scale bar=10µm. (e-g) Quantification of OL size in µm² (e), the intensity of filipin and MBP labeling per cell (f), and the intensity of filipin and MBP staining per µm² (g). For Lrp1 control and cKOOL OLs, n = 29 cells from 3 mice in each group. (h) Timeline in days showing when growth medium (GM) or differentiation medium (DM) with cholesterol was added and when cells were harvested. (i and k) Immunoblotting of OL lysates prepared from Lrp1 control and cKOOL cultures after 3 days in DM. Representative blots were probed with anti-LRP1β, anti-SREBP2, anti-β-actin, anti-PLP, anti-MAG, anti-CNP, and anti-GAPDH. (j, l-n) Quantification of SREBP2 (j), PLP (l), MAG (m), and CNP (n) in Lrp1 control and cKOOL cultures with (+) or without (-) bath applied cholesterol. Number of independent immunoblots: anti-PLP, and MAG, n = 3 per condition; anti-SREBP2 and anti-CNP, n = 4 per condition. (o) Immunoblotting of OL lysates prepared from Lrp1 control and cKOOL cultures after 5 days in DM with (+) or without (-) bath applied cholesterol. Representative blots were probed with anti-LRP1β, anti-PLP/DM20, and anti-β-actin. (p) Quantification of PLP (n=4 per condition) in Lrp1 control and cKOOL cultures with (+) or without (-) bath applied cholesterol (q) Immunostaining of OLs after 5 days in DM with (+) or without (-) bath applied cholesterol. Primary OLs stained with anti-MBP and Hoechst dye33342. Scale bar=100µm. (r) Quantification showing relative number of MBP+ cells in Lrp1 control and cKOOL cultures (n= 3 per condition). Results are shown as mean values ± SEM, *p<0.05, **p<0.01, and ***p<0.001, 2-way ANOVA, post hoc t-test. For a detailed statistical report, see Figure 4-source data1.
Lrp1 deficiency in OPC and OLs causes a reduction in free cholesterol. While LRP1 has been implicated in cholesterol uptake and homeostasis in non-neural cell types (van de Sluis et al., 2017), a role in cholesterol homeostasis in the OL-lineage has not yet been investigated. We find that OPCs deficient for Lrp1 (Lrp1−/−) have reduced levels of free cholesterol compared to Lrp1 control OPCs (Figure 4a, b). Levels of cholester-yl-ester are very low in the CNS (Björkhem and Meaney, 2004) and near the detection limit in the Lrp1 control and Lrp1−/− OPCs (Figure 4c). Morphological studies with MBP+ OLs revealed a significant reduction in myelin-like membrane sheet expansion in Lrp1−/− OLs (Figure 4d and 4e), reminiscent of wildtype OLs cultures treated with statins to inhibit HMG-CoA reductase, the rate limiting enzyme in the cholesterol biosynthetic pathway (Maier et al., 2009, Paintlia et al., 2010, Smolders et al., 2010). To assess cholesterol distribution in primary OLs, cultures were stained with filipin. In Lrp1 control OLs, staining was observed on myelin sheets and was particularly strong near the cell soma. In Lrp1−/− OLs, filipin and MBP staining was significantly reduced (Figure 4f). Reduced filipin staining is not simply a reflection of smaller cell size, as staining intensity was decreased when normalized to myelin sheet surface area (Figure 4g).

Thus, independent measurements revealed a dysregulation of cholesterol homeostasis in Lrp1−/− OPCs/OLs.

Figure 4-figure supplement 1: Lrp1 deficient OLs are sensitive to statin treatment but not to exogenously supplied mevalonate. (a) Cholesterol biosynthetic pathway and site of action of statin (simvastatin). (b) Timeline in days showing when growth medium (GM) and differentiation medium (DM) supplemented with cholesterol pathway supplements (CPS) either simvastatin or mevalonate (Mev) were supplied, and when cells were harvested (H). (c) Immunostaining of OL cultures after 5 days in DM ± statin. Representative cell culture probed with anti-MBP and Hoechst dye33342. Scale bar= 50µm. (d) Quantification of MBP+ cells in Lrp1 control cultures + vehicle (n= 4), Lrp1 control cultures + statin (n= 3), Lrp1 cKOOL cultures + vehicle (n= 4), and Lrp1 cKOOL cultures + statin (n= 3). (e) Immunostaining of OL cultures after 5 days in DM ± mevalonate. Representative cell culture probed with anti-MBP and Hoechst dye33342. Scale bar= 50µm. (f) Quantification of MBP+ cells in Lrp1 control culture + vehicle (n= 3), Lrp1 control culture + mevalonate (n= 3), Lrp1 cKOOL culture + vehicle (n= 3), and Lrp1 cKOOL culture + mevalonate (n= 3). Results are shown as mean values ±SEM, *p<0.05, **p<0.01, and ***p<0.001, 2-way ANOVA, post hoc t-test. For a detailed statistical report, see Figure4-source data1.

Cellular lipid homeostasis is regulated by a family of membrane-bound basic helix-loop-helix transcription factors, called sterol-regulatory element-binding proteins (SREBPs). Precursors of SREBPs are localized to the ER and activated if cholesterol levels drop below a certain threshold (Goldstein et al., 2006, Faust and Kovacs, 2014). To assess whether Lrp1 deficiency leads to an increase in SREBP2, OLs were cultured for 3 days in DM and analyzed by immunoblotting. Compared to Lrp1 control OLs, we observed a strong upregulation of SREBP2 in Lrp1−/− OLs. Elevated SREBP2 can be reversed to normal levels by exogenous cholesterol directly added to the culture medium (Figure 4i and 4j). This shows the existence of LRP1 independent cholesterol uptake mechanisms in Lrp1−/− OLs and a normal
physiological response to elevated levels of cellular cholesterol. In Lrp1 control cultures, bath application of cholesterol leads to a small, yet significant decrease in SREBP2 (Figure 4j).

Given the importance of cholesterol in OL maturation (Sahe et al., 2005, Kramer-Albers et al., 2006, Mathews et al., 2014), we examined whether the differentiation block of Lrp1−/− OLs can be rescued by bath-applied cholesterol. Remarkably, treatment of Lrp1−/− OLs for 3 days with cholesterol failed to elevate PLP, MAG or CNP anywhere near the levels observed in Lrp1 control OLs (Figure 4k-n). Cholesterol treated Lrp1−/− OLs showed a modest increase in PLP but levels remained below Lrp1 control OLs. To ask whether prolonged treatment with exogenous cholesterol promotes OL differentiation in Lrp1−/− cultures, cells were kept for 5 days in DM, either with or without cholesterol. Similar to the 3 day treatment, the 5 day treatment failed to increase PLP levels (Figure 4o and 4p) or the number of MBP+ OLs (Figure 4q and 4r). While differentiation of Lrp1−/− OLs cannot be “rescued” by bath applied cholesterol, cell are highly sensitive to a further reduction in cholesterol, as blocking of cholesterol synthesis with simvastatin leads to a further reduction in MBP+ OLs (Figure 4-figure supplement 1c and 1d). As cholesterol is only one of many lipid derivatives produced by the cholesterol biosynthetic pathway (Figure 4-figure supplement 1a), we tested whether mevalonate, an upstream metabolite in the cholesterol biosynthetic pathway improves OPC differentiation. However, similar to cholesterol, exogenously supplied mevalonate fails to increase differentiation of Lrp1−/− OPCs into MBP+ OLs (Figure 4-figure supplement 1e and 1f). Taken together, Lrp1 deficiency in the OL-lineage leads to a drop in cellular cholesterol, and bath application of cholesterol or mevalonate is not sufficient to drive differentiation into mature OLs. Our data suggest that in addition to cholesterol homeostasis, Lrp1 regulates other biological processes important for OPC differentiation.

Figure 5-figure supplement 1: Gene ontology (GO) analysis of Lrp1 deficient OPCs revealed enrichment of peroxisomal genes. Acutely isolated OPCs from Lrp1−/− and Lrp1fl/fl/Olig2-cre mouse pups were subjected to microarray analysis. (a) GO structure of biological process module related to peroxisome function. Each box shows the GO term ID, p-value, GO term, and the genes from the input list associated with the GO term. The color of each box shows the level of enrichment for each GO term. Specific GO terms were queried with the Mouse Genome Informatics (MGI) GO browser. P-values were calculated by Fisher’s exact test. The fold enrichment was calculated by dividing the ratio of genes that are associated with each GO term from the input list by the ratio of genes that are expected in the database. (b) Quantification showing relative expression level of gene products that are associated with specific GO terms listed in (a). Gene products were prepared from acutely isolated OPCs for Lrp1 controls (n= 4) and cKOOL (n=4) and analyze by Affymetrix mouse gene 2.1 ST array. Differentially regulated gene products include Pex2 (peroxisomal biogenesis factor 2), Pex5l (peroxisomal biogenesis factor 5 like), Hrasls (hRas-like suppressor), Ptgis (prostaglandin 12 synthase), Mavs (Mitochondrial antiviral signaling), and Stard10 (StAR-related lipid transfer protein 10). (c) Immunoblotting of lysates prepared from Lrp1 control and cKOOL cultures after 5 days in DM. Representative blots were probed with anti-LRP1β, anti-Pex2, and anti-β-actin. (d) Quantification of Pex2 in Lrp1 control (n=3) and cKOOL (n=3) cultures. Results are shown as mean values ±SEM, *p<0.05 and **p<0.01, Student’s t-test. For a detailed statistical report, see Figure 5-source data1.
**Lrp1 deficiency impairs peroxisome biogenesis.** To further investigate what type of biological processes might be dysregulated by Lrp1 deficiency, we performed transcriptomic analyses of OPCs acutely isolated from Lrp1 control and cKO<sub>OL</sub> pups. Gene ontology (GO) analysis identified differences in “peroxisome organization” and “peroxisome proliferation-associated receptor (PPAR) signaling pathway” (**Figure 5-figure supplement 1a**). Six gene products regulated by Lrp1 belong to peroxisome and PPAR GO terms, including Pex2, Pex5l, Hrasl, Ptgis, Mavs, and Stard10 (**Figure 5-figure supplement 1b**). Western blot analysis of Lrp<sup>1−/−</sup> OLs further revealed a significant reduction in PEX2 after 5 days in DM (**Figure 5-figure supplement 1c and 1d**). Because PEX2 has been implicated in peroxisome biogenesis (Gootjes et al., 2004), and peroxisome biogenesis disorders (PBDs) are typically associated with impaired lipid metabolism and CNS myelin defects (Krause et al., 2006), this prompted us to further explore a potential link between LRP1 and peroxisomes. To assess whether the observed reduction in PEX2 impacts peroxisome density in primary OLs after 5 days in DM, MBP<sup>+</sup> OLs were stained with anti-PMP70, an ATP-binding cassette transporter enriched in peroxisomes (**Figure 5a**). In Lrp<sup>1−/−</sup> OLs, we observed reduced PMP70 staining (**Figure 5b**) and a decrease in the total number of peroxisomes (**Figure 5c**). Normalization of peroxisome counts to cell size revealed that the reduction in Lrp<sup>1−/−</sup> OLs is not simply a reflection of smaller cells (**Figure 5d**).

**Figure 5: In the OL-lineage loss of Lrp1 leads to a reduction in peroxisomes** (a) Primary OLs prepared from Lrp1 control and cKO<sub>OL</sub> OL pups, cultured for 5 days in DM were stained with anti-MBP and anti-PMP70. Scale bar= 10µm. (b-d) Quantification of PMP70 labeling intensity per cell, PMP<sup>70+</sup> puncta per cell, and scatter plot showing the number of PMP70<sup>+</sup> peroxisomes as a function of cell size for MBP<sup>+</sup> OLs of Lrp1 control and Lrp1 cKO<sub>OL</sub> cultures. (d). For Lrp1 control OLs, n= 112 cells from 3 mice. For Lrp1 cKO<sub>OL</sub> OLs, n= 60 cells from 3 mice. (e) Representative distribution of the PMP70<sup>+</sup> puncta of Lrp1 control and cKO<sub>OL</sub> cells. The center of the cell is marked with a red cross. Puncta within a 25µm radius from the center (dashed circle) were subjected to quantification. (f) Quantification of peroxisome number plotted against the distance from the center of Lrp1 control (n= 113 cells, 3 mice) and cKO<sub>OL</sub> (n= 63 cells, 3 mice) OLs. (g and h) Representative high magnification views of PMP<sup>70+</sup> puncta from white dashed box in (a) aligned with (f). Scale bar= 1 µm. Results are shown as mean values ± SEM, *p<0.05, **p<0.01, and ***p<0.001, Student’s t-test. For a detailed statistical report, see Figure5-source data1.

The peroxisome is a highly dynamic organelle, comprised of over 50 enzymes, many of which participate in lipid metabolism, including the pre-squalene sequence of the cholesterol biosynthetic pathway (Faust and Kovacs, 2014). The subcellular localization of peroxisomes is thought to be important for ensuring a timely response to metabolic demands (Berger et al., 2016). This prompted us to analyze the subcellular distribution of peroxisomes in primary OLs. Interestingly, while the number of PMP70 positive puncta near the cell soma is comparable between Lrp1 control and Lrp<sup>1−/−</sup> OLs, we observed a significant drop in peroxisomes along radial processes of MBP<sup>+</sup> OLs (**Figure 5e-5h**).
Figure 6: Peroxisome proliferator and cholesterol combined treatment rescues OL differentiation in Lrp1 deficient OLs. (a) Timeline in days showing when growth medium (GM) or differentiation medium (DM) with pioglitazone (Pio) were supplied and cells were harvested for analysis. (b) Immunoblots of OL lysates prepared from Lrp1 control and cKOOL cultures after 5 days in DM, probed with anti-LRP1 and anti-β-actin. (c) Immunostaining of Lrp1 control and cKOOL cultures after 5 days in DM. Representative cell culture probed with anti-MBP and Hoechst. Scale bar= 50µm. (d) Quantification of MBP+ cells in Lrp1 control + vehicle (n= 6), Lrp1 control + pioglitazone (n= 6), cKOOL + vehicle (n= 4), and cKOOL + pioglitazone (n= 4) treated cultures. (e–f) Primary OLs probed with anti-MBP and anti-PMP70. Scale bar= 10µm. (g–i) Quantification of OL size in µm² (g), the number of PMP70+ puncta and (h), the intensity of MBP staining per cell (i). (j) Distribution of peroxisomes as a function of distance from the cell center in Lrp1 control and cKOOL cultures with (+) or without (-) pioglitazone. The number of PMP70+ peroxisomes between 6-15µm in Lrp1 control and cKOOL cultures was subjected to statistical analysis in (k). Lrp1 control (n= 112 cells, 3 mice), Lrp1 control + pio (n= 180 cells, 3 mice), cKOOL (n= 60 cells, 3 mice), and cKOOL + Pio. (n= 110 cells, 3 mice) OLs (k). (l) Immunostaining of OLs after 5 days in DM with (+) or without (-) GW9662, probed with anti-MBP and Hoechst dye33342. Scale bar= 50µm. (m) Quantification of MBP+ cells under each of the 4 different conditions (n=3 per condition). (n) Immunostaining of Lrp1 control and cKOOL cultures after 5 days in DM, probed with anti-MBP and Hoechst dye33342. Scale bar= 50µm. (o) Quantification of MBP+ cells in Lrp1 control + vehicle (n= 4), Lrp1 control + pioglitazone & cholesterol (n= 3), cKOOL + vehicle (n= 4), and cKOOL + pioglitazone & cholesterol (n= 3) treated cultures. Results are shown as mean values ± SEM, *p<0.05, **p<0.01, and ***p<0.001, 2-way ANOVA, post hoc t-test. For a detailed statistical report, see Figure6-source data1.

Combination treatment of cholesterol and PPARγ agonist rescues the differentiation block in Lrp1 deficient OPCs. In endothelial cells, the LRP1-ICD functions as a co-activator of PPARγ, a key regulator of lipid and glucose metabolism (Mao et al., 2017). Activated PPARγ...
moves into the nucleus to control gene expression by binding to PPAR-responsive elements (PPREs) on numerous target genes, including Lrp1 (Gauthier et al., 2003). In addition, PPREs are found in genes important for lipid and glucose metabolism, and peroxisome biogenesis (Fang et al., 2016, Hofer et al., 2017). *In vitro*, a 5 day treatment of Lrp1 control OPCs with pioglitazone, an agonist of PPARγ, results in elevated LRP1 (Figure 6a and 6b) and accelerated differentiation into MBP+ OLs (Figure 6c and 6d) (Bernardo et al., 2009). This stands in marked contrast to Lrp1−/− cultures, where pioglitazone treatment fails to accelerate OPC differentiation (Figure 6c and 6d). Moreover, pioglitazone does not regulate PMP70 staining intensity in Lrp1 control or Lrp1−/− OLs, nor does it have any effect on total peroxisome counts per cell (Figure 6e-6i). However, pioglitazone leads to a modest but significant increase in the number of peroxisomes located in cellular processes of Lrp1−/− OLs (Figure 6j and 6k). Treatment of Lrp1 control OPCs with the PPARγ antagonist GW9662 blocks differentiation into MBP+ OLs (Roth et al., 2003), but does not lead to a further reduction in MBP+ cells in Lrp1−/− OL cultures (Figure 6m). This suggests that in Lrp1−/− OLs PPARγ is not active. Given LRP1’s multifunctional receptor role, we asked whether simultaneous treatment with pioglitazone and exogenous cholesterol is sufficient to rescue the differentiation block of Lrp1−/− OPCs. This is indeed the case, as the number of MBP+ cells in Lrp1−/− cultures is significantly increased by the combination treatment, suggesting an additive effect toward OPC differentiation (Figure 6n and 6o). Together, these findings indicate that in OPCs LRP1 regulates multiple metabolic functions important for OL differentiation. In addition to its known role in cholesterol homeostasis, LRP1 regulates expression of PEX2 and thereby metabolic functions associated with peroxisomes.
Discussion

LRP1 function in the OL-lineage is necessary for proper CNS myelin development and the timely repair of a chemically induced focal white matter lesion in vivo. Optic nerves of Lrp1 cKOOL show fewer myelinated axons, thinning of myelin sheaths, and an increase in nodal defects. Morphological defects have a physiological correlate, as Lrp1 cKOOL mice exhibit faulty nerve conduction. Mechanistically, Lrp1 deficiency disrupts multiple signaling pathways implicated in OL differentiation, including AKT activation, cholesterol homeostasis, PPARγ activation, and peroxisome biogenesis. The pleiotropic roles of LRP1 in OPC differentiation are further underscored by the fact that restoring cholesterol homeostasis or activation of PPARγ alone is not sufficient to drive differentiation. Only when cholesterol supplementation is combined with PPARγ activation, differentiation of Lrp1−/− OPC into MBP+ OLs is significantly increased. Taken together, our studies identify a novel link between LRP1 and peroxisomes and suggest that broad metabolic dysregulation in Lrp1−/− OPCs attenuates differentiation into mature OLs (Figure 7).

Figure 7: Working model of LRP1 regulated pathways in the OL-lineage (a) LRP1 function in the OL-lineage is necessary for proper CNS myelin development and the timely repair of a chemically induced focal white matter lesion. In OPCs, Lrp1 deficiency leads to dysregulation of cholesterol homeostasis and impaired peroxisome biogenesis. (b) LRP1 is a key regulator of multiple pathways important for OPC differentiation into mature myelin producing OLs: I) LRP1 regulates cholesterol homeostasis; II) LRP1 regulates peroxisome biogenesis; and III) the combined treatment of Lrp1 deficient primary OPCs with cholesterol and pioglitazone is sufficient to drive maturation into MBP+ myelin sheet producing OLs.
In the embryonic neocortex, LRP1 is strongly expressed in the ventricular zone and partially overlaps with nestin+ cells, suggesting expression in undifferentiated neural stem and precursor cells (NSPCs) (Hennen et al., 2013). In Lrp1\textsuperscript{flx/flx} neurospheres, conditional ablation of Lrp1 reduces cell proliferation and survival, and also negatively impacts differentiation into neurons and O4+ OLS, while astrocyte production is significantly increased (Safina et al., 2016). In line with these observations, ablation of Lrp1 with the Olig2-cre driver attenuates OPC differentiation, but neither neurogenesis nor astrocyte production are significantly altered in these mice. A likely explanation for these discrepancies is the OL lineage restricted gene ablation in Lrp1 cKO\textsuperscript{OL} mice. Studies with highly purified OPCs in vitro and OL-lineage specific ablation of Lrp1 in vivo, both during development and adult white matter repair, suggest a cell-autonomous role for Lrp1 in OPC maturation and differentiation into myelin producing OLS. Global inducible ablation of Lrp1 at P5 or at P56 with the CAG-cre\textsubscript{ERTM} driver achieves a ~40% reduction of LRP1 protein in the brain. Despite multiple daily injections of TM via different routes at P5 or i.p. in adult mice, we were unable to achieve a greater than 40% decrease in LRP1, yet this partial ablation is sufficient to negatively impact myelin development and adult white matter repair. LRP1 is elevated in astrocytes and myeloid cells near multiple sclerosis (MS) lesions (Chuang et al., 2016). Deletion of Lrp1 in microglia worsens the course of experimental autoimmune encephalomyelitis (EAE) and it has been proposed that loss of Lrp1 in microglial leads to a proinflammatory phenotype and disease exacerbation (Chuang et al., 2016). Thus, reduced white matter repair in Lrp1 iKO mice injected with LPC may be due to loss of Lrp1 in OPCs, an increase in proinflammatory microglia or a combination thereof. However, studies with Lrp1 iKO\textsuperscript{OL} mice clearly show that in the adult brain, Lrp1 function in the OL lineage is necessary for the timely repair of a demyelination lesion. Additional studies, including cell type specific ablation in microglia, astrocytes, and neurons are needed to determine whether Lrp1 is required in other neural cell types for the repair of LPC inflicted white matter lesions.

Cholesterol does not cross the blood-brain-barrier (Saher and Stumpf, 2015) and CNS resident cells need to either synthesize their own cholesterol or acquire it through horizontal transfer from neighboring cell types, including astrocytes (Camargo et al., 2017). In the OL lineage cholesterol is essential for cell maturation, including myelin gene expression, myelin protein trafficking, and internode formation (Saher et al., 2005, Kramer-Albers et al., 2006, Mathews et al., 2014). Sterol biosynthesis is in part accomplished by peroxisomes. Specifically, the pre-squalene segment of the cholesterol biosynthetic pathway takes place in peroxisomes. However, cholesterol is only one of many lipid derivatives produced by this pathway (Faust and Kovacs, 2014). A drop in intracellular cholesterol leads to an increase in SREBPs, a family of transcription factors that regulate expression of gene products involved in cholesterol and fatty acid synthesis (Goldstein et al., 2006, Faust and Kovacs, 2014). In Schwann cells, SREBPs, and the SREBP activating protein SCAP, are required for AKT/mTOR dependent lipid biosynthesis, myelin membrane synthesis, and normal PNS myelination (Verheijen et al., 2009, Norrmen et al., 2014). In the OL lineage blockage of SREBP activation inhibits CNS myelination (Camargo et al., 2017, Monnerie et al., 2017). Blocking of SREBP processing in primary OLs leads to a drop in cellular cholesterol and inhibits cell differentiation and membrane expansion. This can be rescued by addition of cholesterol to the culture medium (Monnerie et al., 2017). In primary OLs, Lrp1 deficiency leads to activation of SREBP2, yet cells seem to be unable to maintain cholesterol homeostasis, suggesting more global metabolic deficits. The cholesterol sensing apparatus in Lrp1 deficient OPCs appears to be largely intact, as bath applied cholesterol leads to a reduction in SREBP2. As SREBP2 can be induced by ER stress (Faust and Kovacs, 2014), reversibility of elevated SREBP2 by bath applied cholesterol suggests that Lrp1 cKO\textsuperscript{OL} cultures upregulate SREBP2 due to cholesterol deficiency and not due to an elevated ER stress response (Faust and Kovacs, 2014).
Significantly, restoring cellular cholesterol homeostasis in Lrp1−/− OPC is not sufficient to overcome the differentiation block, suggesting more widespread functional deficits.

Members of the PPAR subfamily, including PPARα, PPARβ/δ, and PPARγ, are ligand-activated transcription factors that belong to the nuclear hormone receptor family (Michalik et al., 2006). PPARs regulate transcription through heterodimerization with the retinoid X receptor (RXR). When activated by a ligand, the dimer modulates transcription via binding to a PPRE motif in the promoter region of target genes (Michalik et al., 2006). A critical role for PPARγ in OL differentiation is supported by the observation that activation with pioglitazone or rosiglitazone accelerates OPC differentiation into mature OLs (Saluja et al., 2001, Roth et al., 2003, Bernardo et al., 2009, De Nuccio et al., 2011, Bernardo et al., 2013) and inhibition with GW9662 blocks OL differentiation. Deficiency for the PPARγ-coactivator-1 alpha (PGC1α) leads to impaired lipid metabolism, including an increase in very long chain fatty acids (VLCFAs) and disruption of cholesterol homeostasis (Xiang et al., 2011, Camacho et al., 2013). In addition, PGC1α deficiency results in defects of peroxisome-related gene function, suggesting the increase in VLCFAs and drop in cholesterol reflects impaired peroxisome function (Baes and Aubourg, 2009). Following γ-secretase dependent processing, the LRP1-ICD can translocate to the nucleus where it associates with transcriptional regulators (May et al., 2002, Carter, 2007). In endothelial cells, the LRP1-ICD binds directly to the nuclear receptor PPARγ to regulate gene products that function in lipid and glucose metabolism (Mao et al., 2017). Treatment of Lrp1−/− OPCs with pioglitazone leads to an increase in peroxisomes in OL processes but fails to promote differentiation into myelin sheet producing OLs. In the absence of the LRP1-ICD, pioglitazone may fail to fully activate PPARγ (Mao et al., 2017), but the observed increase in PMP70+ peroxisomes in OL processes of Lrp1−/− cultures suggests that mutant cells can respond to pioglitazone. Because Lrp1 cKOOL cultures are cholesterol deficient and the LRP1-ICD participates in PPARγ regulated gene expression, we examined whether a combination treatment of cholesterol and pioglitazone rescues the differentiation block in Lrp1−/− OPCs/OLs. This was indeed the case, suggesting that Lrp1 deficiency leads to dysregulation of multiple pathways important for OPC differentiation.

The importance of peroxisomes in the human nervous system is underscored by inherited disorders caused by complete or partial loss of peroxisome function, collectively described as Zellweger spectrum disorders (Berger et al., 2016, Waterham et al., 2016). PEX genes encode peroxins, the proteins required for normal peroxisome assembly and when mutated can cause peroxisome biogenesis disorder (PBD), characterized by a broad range of symptoms, including aberrant brain development, white matter abnormalities, and neurodegeneration (Berger et al., 2016). The genetic basis for PBD is a single mutation in one of the 14 PEX genes, typically leading to deficiencies in numerous metabolic functions carried out by peroxisomes (Steinberg et al., 1993). In developing OLs, Lrp1 deficiency leads to a decrease in peroxisomal gene products, most prominently a >50% reduction in PEX2, an integral membrane protein that functions in the import of peroxisomal matrix proteins. Mice deficient for Pex2 lack normal peroxisomes but do assemble empty peroxisome membrane ghosts (Faust and Hatten, 1997). Pex2 mutant mice show significantly lower plasma cholesterol levels and in the brain the rate of cholesterol synthesis is significantly reduced (Faust and Kovacs, 2014). Recent evidence shows that PBDs not only lead to defects in lipid metabolism, but may also lead to dysregulation of carbohydrate metabolism (Wangler et al., 2017). Mounting evidence points to a close interaction of peroxisomes with other organelles, mitochondria in particular, and disruption of these interactions may underlie the far reaching metabolic defects observed in PBD and genetically manipulated model organisms deficient for a single PEX (Fransen et al., 2017, Wangler et al., 2017). Our studies provide a potential new link between peroxisomes
and LRP1 and suggest a new mechanism for how \textit{Lrp1} deficiency may lead to impaired lipid and carbohydrate metabolism.
Methods

Mice: All animal handling and surgical procedures were performed in compliance with local and national animal care guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC). Lrp1\textsuperscript{floxed/floxed} mice were obtained from Steven Gonias (Stiles et al., 2013) and crossed with Olig2-Cre (Schuller et al., 2008), CAG-CreERT\textsuperscript{TM} (#004682, Jackson Laboratories), and Pdgfra-CreERT\textsuperscript{TM} (Kang et al., 2010) mice. For inducible gene ablation in adult male and female mice, 3 intraperitoneal (i.p.) injections of tamoxifen (75 mg/kg) were given every 24 hrs. Tamoxifen (10 mg/ml) was prepared in a mixture of 9% ethanol and 91% sunflower oil. For inducible gene ablation in juvenile mice, 2 injections in the stomach of 4-hydroxytamoxifen (4OH-TM, 15 µg/g) were given 24 hrs apart. A 10 mg/ml stock solution of 4OH-TM was prepared in 100% ethanol. Mice were kept on a mixed background of C57BL/6J and 129SV. Throughout the study, male and female littermate animals were used. Lrp1 “control” mice harbor at least one functional Lrp1 allele. Any of the following genotypes Lrp1\textsuperscript{+/+}, Lrp1\textsuperscript{floxed}, Lrp1\textsuperscript{floxed/floxed}, or Lrp1\textsuperscript{floxed+/+;Cre} served as Lrp1 controls.

Genotyping: To obtain genomic DNA (gDNA), tail biopsies were collected, boiled for 30 min in 100µl alkaline lysis buffer (25mM NaOH and 0.2mM EDTA in ddH\textsubscript{2}O) and neutralized by adding 100µl of 40mM Tris-HCl (pH 5.5). For PCR genotyping, 1-5µl of gDNA was mixed with 0.5µl of 10mM dNTP mix (Promega, C1141), 10µl of 25mM MgCl\textsubscript{2}, 5µl of 5X Green GoTaq\textsuperscript{®} Buffer (Promega, M791A), 0.2µl of GoTaq\textsuperscript{®} DNA polymerase (Promega, M3005), 0.15µl of each PCR primer stock (90µM), and ddH\textsubscript{2}O was added to a total volume of 25µl. The following cycling conditions were used: the DNA denaturing step (94°C for 3 min) 1X, amplification steps (94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min) 30X, followed by an elongation step (72°C for 10 min) then kept at 4°C for storage. The position of PCR primers used for genotyping is shown in Figure 1-figure supplement 1. Lrp1 WT and loxP-flanked (floxed) alleles were amplified with the forward primer [Lrp1f\textsuperscript{10290}, F2] 5'-CAT ACC CTC TTC AAA CCC CTT G-3' and the reverse primer [Lrp1r\textsuperscript{10291}, R2] 5'-GCA AGC TCT CCT GCT CAG ACC TGG A-3'. The WT allele yields a 291-bp product and the floxed allele yields a 350-bp product. The recombinant Lrp1 allele was amplified with the forward primer [Lrp1f\textsuperscript{R1}, F1] 5'-CCC AAG GAA ATC AGG CCT CGG C-3’ and the reverse primer [F2], resulting in a 400-bp product (Hennen et al., 2013). For detection of Cre, the forward primer [oIMR1084, CreF] 5'-GGG GTG TTC GGG CAG TAA AAA CTA TC-3' and reverse primer [oIMR1085, CreR] 5'-GTG AAA CAG CAT TGC TGT CAC TT-3' were used, resulting in a ~200-bp product. As a positive control, the forward primer [oIMR7338, Il-2pF] 5'-CTA GCC CAG ACA ATT GAA AGA-3' and the reverse primer [oIMR7339, Il-2pR] 5'-GTA GGT GGA AAT TCT AGC ATC-3’ were mixed with CreF and CreR primers in the same reaction, this reaction yields a 324-bp product (The Jackson laboratory).

Stereotaxic injection. Male and female mice at postnatal-day (P) 42-56 were used for stereotaxic injection of L-α-Lysophosphatidylcholine (LPC) (Sigma, L4129) into the corpus callosum. Mice were anesthetized with 4% isoflurane mixed with oxygen, mounted on a Stoeling stereotaxic instrument (51730D), and kept under 2% isoflurane anesthesia during surgery. A 5µl-hamilton syringe was loaded with 1% LPC in PBS (Gibco, 10010023), mounted on a motorized stereotaxic pump ( Quintessential Stereotaxic injector, 53311) and used for intracranial injection at the following coordinates, AP: 1.25mm, LR: ±1mm, D: 2.25mm. Over a duration of 1 min, 0.5µl of 1% LPC solution was injected on the ipsilateral site and 0.5µl PBS on the contralateral side. After the injection was completed, the needle was kept in place for 2 min before retraction. Following surgery, mice were treated with 3 doses of 70µl of buprenorphine (0.3 mg/ml) every 12 hours. Brains were collected at day 10, and 21 post injection.
**Histochemistry.** Animals were deeply anesthetized with a mixture of ketamine/xylazine (25mg/ml ketamine and 2.5mg/ml xylazine in PBS) and perfused trans-cardially with ice-cold PBS for 5 min, followed by ice-cold 4% paraformaldehyde in PBS (4%PFA/PBS) for 5 min. Brains were harvested and post-fixed for 2 hours in perfusion solution. Optic nerves were harvested separately and post-fixed for 20 min in perfusion solution. Brains and optic nerves were cryoprotected overnight in 30% sucrose/PBS at 4°C, embedded in OCT (Tissue-Tek, 4583), and flash frozen on dry ice. Serial sections were cut at 20µm (brain tissue) and 10µm (optic nerves) at -20°C using a Leica CM 3050S Cryostat. Serial sections were mounted onto Superfrost+ microscope slides (Fisherbrand, 12-550-15) and stored at -20°C.

**In Situ Hybridization.** Tissue sections mounted on microscope slides were post-fixed overnight in 4%PFA/PBS at 4°C. Sections were then rinsed 3 times for 5 min each in PBS and the edge of the microscope slides was demarcated with a DAKO pen (DAKO, S2002). Sections were subsequently incubated in a series of ethanol/water mixtures: 100% for 1 min, 100% for 1 min, 95% for 1 min, 70% for 1 min, and 50% for 1 min. Sections were then rinsed in 2x saline-sodium citrate (SSC, 150mM NaCl, and 77.5mM sodium citrate in ddH₂O, pH7.2) for 1 min, and incubated at 37°C for 30 min in proteinase K solution (10μg/ml proteinase K, 100mM Tris-HCl pH8.0, and 0.5mM EDTA in ddH₂O). Proteinase digestion was stopped by rinsing sections in ddH₂O and then in PBS for 5 min each. To quench RNase activity, slides were incubated in 1% triethanolamine (Sigma, 90278) and 0.4% acetic anhydride (Sigma, 320102) mixture in ddH₂O for 10 min at room temperature, rinsed once in PBS for 5 min and once in 2X SSC for another 5 min. To reduce non-specific binding of cRNA probes, sections were pre-incubated with 125µl hybridization buffer (10% Denhardt’s solution, 40mg/ml baker’s yeast tRNA, 5mg/ml sheared herring sperm DNA, 5X SSC, and 50% formamide in ddH₂O) for at least 2 hours at room temperature. Digoxigenin-labeled cRNA probes were generated by run-off in vitro transcription as described (Winters et al., 2011a). Anti-sense and sense cRNA probes were diluted in 125µl pre-hybridization buffer to ~200ng/ml, denatured for 5 min at 85°C, and rapidly cooled on ice for 2 min. Probes were applied to tissue sections, microscope slides covered with parafilm, and incubated at 55°C overnight in a humidified and sealed container. The next morning slides were rinsed in 5X SSC for 1 min at 55°C, 2X SSC for 5 mins at 55°C, and incubated in 0.2X SSC/50% formamide for 30min at 55°C. Sections were rinsed in 0.2X SSC at room temperature for 5min then rinsed with Buffer1 (100mM Tris-HCl pH7.5, and 1.5M NaCl in ddH₂O) for 5 min. A 1% blocking solution was prepared by dissolving 1g blocking powder (Roche, 11096176001) in Buffer1 at 55°C, cooled to room temperature (RT), and applied to slides for 1 hour at RT. Slides were rinsed in Buffer1 for 5 min and 125µl anti-Digoxigenin-AP antibody (Roche, 11093274910, 1:2500) in Buffer1 was applied to each slide for 1.5 hours at RT. Sections were rinsed in Buffer1 for 5 min, then rinsed in Buffer2 (100mM Tris-HCl pH9.5, 100mM NaCl, and 5mM MgCl₂ in ddH₂O) for 5 min, and incubated in alkaline phosphatase (AP) substrate (Roche, 11681451001, 1:50) in Buffer2. The color reaction was developed for 1-48 h and stopped by rinsing sections in PBS for 10 min. Sections were incubated in Hoechst dye 33342 (Life technology, H3570) for 5 min, air dried, mounted with Fluoromount-G® (SouthernBiotech, 0100-01), and dried overnight before imaging under bright-field. The following cRNA probes were used, Pdgfr and Plp (DNA templates were kindly provided by Richard Lu (Dai et al., 2014)), Mag (Winters et al., 2011a), and Mbp (a 650-bp probe based on template provided in the Allen Brain Atlas).

**Quantification of lesion size and myelin repair.** Serial sections of the corpus callosum, containing the LPC and PBS injection sites were mounted onto glass coverslips and stained by ISH with digoxigenin-labeled cRNA probes specific for Mbp, Mag, Plp and Pdgfra. For quantification of the white matter lesion area, the same intensity cutoff was set by Image J threshold for all brain sections and used to measure the size of the lesion. The outer rim of the...
strongly $Mbp^+$ region (lesion$^{out}$) was traced with the ImageJ freehand drawing tool. The inner rim facing the $Mbp^+$ region (lesion$^{in}$) was traced as well. For each animal examined, the size of the initial lesion area (lesion$^{out}$) in $\mu$m$^2$ and remyelinated area (lesion$^{out}$-lesion$^{in}$) in $\mu$m$^2$ was calculated by averaging the measurement from two sections at the lesion core. The lesion core was defined as the section with the largest lesion area (lesion$^{out}$). To determine remyelination, the ratio of (lesion$^{out}$-lesion$^{in}$)/(lesion$^{out}$) in % was calculated. As an initial lesion depth control, criteria of lesion$^{out}$ area must cover the center of the corpus callosum in each serial section set. If a lesion$^{out}$ area was not located within the corpus callosum, the animal and corresponding brain sections were excluded from the analysis.

**Immunostaining.** Tissue sections mounted onto microscope slides were rehydrated in PBS for 5 min, permeabilized in 0.1% TritonX-100, and blocked in PHT (1% horse serum and 0.1% TritonX-100 in PBS) for 1 hour at RT. Primary antibodies were diluted in PHT and applied overnight at 4°C. Sections were rinsed in PBS 3 times for 5 min each and appropriate secondary antibodies were applied (Life technologies, Alexa-fluorophore 405, 488, 555, 594, or 647nm, 1:1000). Slides were rinsed in PBS 3 times for 5 min each and mounted with ProLong® Gold antifade reagent (Life technologies, P36930). For quantification of nodal structures, randomly selected fields of view in each nerve were imaged at 96X magnification with an Olympus IX71 microscope, a maximum projection of 6 Z-stacked images of each region was generated, and the stacked images were used for quantification. As axons run in and out of the plane within longitudinal sections, criteria were set to exclude structures in which Caspr staining was unpaired to reduce “false positive” as nodal defect. The following primary antibodies were used: rabbit anti-Olig2 (Millipore, AB9610, 1:500), rat anti-PDGFRα (BD Pharmingen, 558774, 1:500), rabbit anti-GFAP (Dako, Nr. A 0334, 1:2000), mouse anti-APC (Calbiochem, OP80, Clone CC1, 1:500), rabbit anti-Caspr (1:1000, (Peles et al., 1997)), mouse anti-Na Channel (1:75, (Rasband et al., 1999)). For myelin staining, sections were incubated in Fluoromyelin-Green (Life technologies, F34651 1:200) reagent for 15min.

**Transmission electron microscopy (TEM).** Tissue preparation and image acquisition were carried out as described by Winters et al., 2011. Briefly, mice at P10, P21, and P56 were perfused trans-cardially with ice cold PBS for 1 min, followed by a 10 min perfusion with a mixture of 3% PFA and 2.5% glutaraldehyde in 0.1M Sorensen’s buffer. Brains and optic nerves were dissected and post-fixed in perfusion solution overnight at 4°C. Post-fixed brain tissue and optic nerves were rinsed and transferred to 0.1M Sorensen’s buffer and embedded in resin by the University of Michigan Imaging Laboratory Core. Semi-thin (0.5µm) sections were cut and stained with toluidine blue and imaged by light microscopy. Ultra-thin (75nm) sections were cut and imaged at the ultrastructural level with a Philips CM-100 or a JEOL 100CX electron microscope. For each genotype and age at least 3 animals were processed and analyzed. For each animal over 1000 axons in the optic nerve were measured and quantified by ImageJ. For each optic nerve, 10 images at 13,500x magnification were randomly taken and quantified to calculate the g-ratio and the fraction of myelinated axons. The inner (area$^{in}$) and outer (area$^{out}$) rim of each myelin sheath was traced with the ImageJ freehand drawing tool and the area within was calculated. We then derived axon caliber and fiber caliber (2r) by the following: area$^{in}$ = r$^2$π. The g-ratios were calculated as such: $\frac{\sqrt{\text{area}^{in}}}{\sqrt{\text{area}^{out}}}$. The g-ratio is only accurate if the compact myelin and axon outline can clearly be traced, individual fibers with not clearly define features, i.e. detached myelin were excluded from the quantification.

**Optic nerve CAP recordings** were carried out as described elsewhere(Winters et al., 2011b, Carbajal et al., 2015). Briefly, optic nerves were acutely isolated from P21 mice and transferred into oxygenated ACSF buffer (125 mM NaCl, 1.25mM NaH$_2$PO$_4$, 25mM glucose,
25mM NaHCO₃, 2.5mM CaCl₂, 1.3mM MgCl₂, 2.5mM KCl) for 45 min at RT before transferring into a recording chamber at 37± 0.4°C. Suction pipette electrodes were used for stimulation and recording of the nerve. A computer-driven (Axon pAlamp10.3 software) stimulus isolation unit (WPI, FL) was used to stimulate the optic nerve with 2 mA/50 µs pulses. The recording electrode was connected to a differential AC amplifier (custom-made). A stimulus artifact-subtracting pipette was placed near the recording pipette. A data acquisition system (Axon digitata 1440A, Axon pClamp 10.3, Molecular Devices, CA) was used to digitize the signals. Conduction velocity was calculated from the length of the nerve and the time to peak of each component of the CAP. Amplitudes were normalized to a resistance ratio of 1.7, as described (Fernandes et al., 2014). Raw traces were fitted with 4 Gaussian curves with Origin9.1 software for analysis of individual components of the CAP. Due to limitations in the resolution of individual peaks in short nerves, CAP recordings from nerves that were shorter than 1mm in length were excluded from the analysis.

OPC/OL primary cultures and drug treatment. OPCs were isolated from P6-P9 mouse pups by anti-PDGFRα (BD Pharmingen, 558774) immunopanning as previously described (Mironova et al., 2016). For plating of cells, 5-7.5 x 10³ cells (for 12mm cover glass) or 3-5 x 10⁴ (12-well plastic plate) were seeded onto PDL pre-coated surface. Primary OPCs were kept in a 10% CO₂ incubator at 37°C. To maintain OPCs in a proliferative state, growth medium (20ng/ml PDGF-AA (Peprotech, 100-13A), 4.2µg/ml Forskolin (Sigma, F6886), 10ng/ml CNTF (Peprotech, 450-02), and 1ng/ml NT-3 (Peprotech, 450-03) in SATO) were added to the culture. To induce OPC differentiation, differentiation medium was constituted by adding (4.2µg/ml Forskolin, 10ng/ml CNTF, and 4ng/ml T3 (Sigma, T6397)) in SATO to the culture. For drug treatment, all compounds were mixed with differentiation medium at the desired concentration, and the compound-containing medium was replaced every other day. Stock and working solutions including 20mg/ml cholesterol (Sigma, C8667) in 100% EtOH were kept at RT and warmed up to 37°C before use, then diluted in differentiation medium to 5µg/ml; 10mM pioglitazone (Sigma, E6910) in DMSO was kept at -20°C and diluted in differentiation medium to 1µM; 10mM simvastatin (Sigma, S6196) in DMSO was kept at -20°C and diluted in differentiation medium to 0.5µM; 10mM GW9662 (Sigma, M6191) in DMSO was kept at -20°C and diluted in differentiation medium to 1µM.

OPC staining and quantification. At different stages of development, OPC/OL cultures were fixed for 15 min in 4%PFA/PBS. Cells were rinsed three times in PBS and permeabilized with 0.1% Triton/PBS solution for 3 mins. Cells were then rinsed in PBS and incubated in blocking solution (3% BSA/PBS) for 1 hour at RT. Primary antibodies were prepared in blocking solution. For immunostaining, 35µl were dropped onto a sheet of parafilm, the coverslips were inverted onto the primary antibody drop, and incubated overnight at 4°C. The following day, coverslips were transferred back to a 24 well-plate and rinsed with PBS 3 times for 5 min each. Secondary antibody ± filipin (Sigma, F9765, 0.1mg/ml) was prepared in blocking solution, 350 µl were added to each well, and the coverslips were incubated for 2 hours at RT. Coverslips then were rinsed in PBS 3 times for 5 min each and stained with Hoechst (1:50,000) for 10s. Coverslips then were rinsed in ddH₂O and mounted in ProLong® Gold antifade reagent. For quantification in Figure 3, the % of OL markers+/Hoechst+ cells was calculated from 10 images that were taken from randomly selected areas in each coverslip at 20X magnification with an Olympus IX71 microscope. For quantification in Figure 4 and after, the % of OL markers+/Hoechst+ cells was calculated from 25 images that were taken from randomly selected areas in each coverslip at 10X magnification with a Zeiss Axio-Observer microscope. For single cell intensity and size measurement in Figures 4-7, individual cell images were taken at 40X magnification with a Zeiss Axio-Observer microscope with Apotom.2. For quantification, the same intensity cutoff was set by Image J threshold to all cells.
and binary images were generated to define each cell outline. The individual cell outline was applied to original images to measure the intensity of filipin, MBP, or PMP70 staining per cell.

For PMP70 puncta distribution analysis, the coordinates of each PMP70+ center were acquired by the process> find maxima function in ImageJ, the cell center coordinate was defined by point selection function, and the distance of each PMP70+ dot to the cell center was then calculated. The data were then binned from 1 – 25 mm at 1 mm divisions, and plotted.

Primary antibodies included: rat-anti PDGFβ (BD Pharmingen, 558774, 1:500), rabbit anti-CNPlase (Aves, 27490 R12-2096, 1:500), mouse anti-MAG (Millipore, MAB1567, 1:500), rat anti-MBP (Millipore, MAB386, 1:1000), chicken anti-PLP (Aves, 27592, 1:500), mouse anti-GFAP (Sigma, G3893, 1:1000), chicken anti-GFAP (Aves, GFAP, 1:500), rabbit anti-NG2 (Millipore, AB5320, 1:500), rabbit anti-LRP1β (Abcam, ab92544, 1:500), rabbit anti-PMP70 (Thermo, PA1-650, 1:1000).

**Western blot analysis.** Protein lysates were separated by SDS-PAGE and transferred onto PVDF membranes for immunoblotting. Depending on the application, 2 to 10µg of total protein were loaded per well. 2% Blotting-Grade Blocker (Bio-Rad, #170-6404) or 2% BSA fraction V (Fisher, BP1600-100) in 0.1%TBST buffer (0.1% Tween-20, 3M NaCl, 200mM Tris-HCl pH7.4) were used as blocking solutions and membranes were incubated for 1 hour at RT. Primary antibodies were diluted in blocking buffer and used for incubation at 4°C overnight.

For protein detection and densitometric analysis, membranes were incubated in Super Signal® West Pico substrate (Thermo, 34080), WesternSure® PREMIUM Chemiluminescent Substrate (LI-COR Biosciences, 926-95000), or Super Signal® West Femto substrate (Thermo, 34095) followed by scanning on a C-Digit® blot scanner (LI-COR®, P/N 3600-00). Images were quantified with Image Studio Lite Western Blot Analysis Software, relative to loading controls. Blots were used for quantification only when the loading control signals were comparable between groups and signals between technical repeats were similar. Primary antibodies included: rabbit anti-LRP1β 85kDa (Abcam, ab92544, 1:2000), mouse anti-βIII tubulin (Promega, G7121, 1:5000), mouse anti-β-actin (Sigma, AC-15 A5441, 1:5000), rat anti-MBP (Millipore, MAB386, 1:1000), rabbit anti-MAG (homemade serum, 1:1000), rabbit anti-PLP (Abcam, ab28486, 1:1000), rat anti-PLP/DM20 (Wendy Macklin AA3 hybridoma, 1:500) rabbit anti-Olig2 (Millipore, AB9610, 1:1000), mouse anti-GFAP (Sigma, G3893, 1:1000), mouse anti-CNPlase (Abcam, ab6319, 1:1000), rabbit anti-PXMP3 (PE2) (One world lab, AP9179c, 1:250), and rabbit anti-SREBP2 (One world lab, 7855, 1:500).

**Cholesterol measurement.** OPCs were isolated by immunopanning as described above. OPCs bound to panning plates were collected by scraping with a Scraper (TPP, TP99002) in 250µl of ice-cold PBS and sonicated in an ice-cold water bath (Sonic Dismembrator, Fisher Scientific, Model 500) at 50% amplitude 3 times for 5 sec with a 5 sec interval. The sonicated cell suspensions were immediately used for cholesterol measurement following the manufacturer’s instructions (Chemicon, 428901). For colorimetric detection and quantification of cholesterol, absorbance was measured at 570nm with a Multimode Plate Reader (Molecular Devices, SpectraMax® M5®). Results were normalized to total protein concentration measured by DCTM Protein Assay according to the manufacturer’s manual (Bio-Rad, 5000112).

**Microarray and gene ontology analysis.** OPCs were isolated by immunopanning as described above and RNA was isolated with the RNasy Micro Kit (Qiagen, 74004). To compare Lrp1 control and cKOOL RNA expression profiles, the Mouse Gene ST2.1 Affymetrix array was used. Differentially expressed genes, with a p-value <0.05 set as cutoff, were subjected to gene ontology (GO) analysis. Go terms were quarried from Mouse Genome Informatics (MGI) GO browser. The fold enrichment was calculated by dividing the number of
genes associated with the GO term in our list by the number of genes associated with the GO term in the database.

Statistical Analysis. There was no pre-experimental prediction of the difference between control and experimental groups when the study was designed. Therefore, we did not use computational methods to determine sample size a priori. Instead we use the minimum of mice per genotype and experimental treatment for a total of at least 3 independent experiments to achieve the statistical power discussed by Gauch et al (Gauch, 2006). We used littermate Lrp1 control or Lrp1 cKO or iKO mice for comparison throughout the study. All independent replicas were biological replicas, rather than technical replicas. For each experiment the sample size (n) is specified in the figure legend. Throughout the study independent replicas (n) indicate biological replica. Technical replicas were used to control for the quality of each measurement and were averaged before quantification and the average value was used as (n= 1) biological replica. Unless indicated otherwise, results are represented as mean value ±SEM. For single pairwise comparison, Student's t-test was used and a p-value <0.05 was considered statistically significant. For multiple comparisons, 2-way ANOVA followed by post hoc t-test were used. Numbers and R software (see source code file for details) were used for determining statistical significance and graph plotting. For detailed raw data and statistical report, see source data files for each figure. For image processing and quantification, ImageJ 1.47v software was used for threshold setting, annotation, and quantification.
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Author contribution

J.-P.L., Y.A.M., P.S., and R.J.G. designed research. J.-P.L., Y.A.M., and R.J.G. performed research. J.-P.L. and P.S. analyzed data. J.-P.L and R.J.G. wrote the paper.

Additional information

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**Figure 1:** Inducible global ablation of Lrp1 leads to CNS hypomyelination and reduced remyelination of a chemically induced white matter lesion

**Figure 2:** Lrp1 ablation in the oligodendrocyte (OL)-lineage leads to hypomyelination, nodal defects, and reduced myelin repair

**Figure 3:** Loss of Lrp1 in the OL-lineage attenuates OL differentiation

**Figure 4:** Lrp1 deficient OPCs show reduced levels of free cholesterol

**Figure 5:** In the OL-lineage loss of Lrp1 leads to a reduction in peroxisomes

**Figure 6:** Peroxisome proliferator and cholesterol combined treatment rescues OL differentiation in Lrp1 deficient OLs

**Figure 7:** Working model of LRP1 regulated pathways in the OL-lineage

**Figure 1-figure supplement 1:** Tamoxifen induced global Lrp1 ablation and genotyping

**Figure 1-figure supplement 2:** Global inducible ablation of Lrp1 in neonatal mice leads to CNS hypomyelination and reduced axon caliber

**Figure 1-figure supplement 3:** Lrp1 is important for white matter repair

**Figure 2-figure supplement 1:** Lrp1 ablation in the OL lineage leads to CNS hypomyelination

**Figure 2-figure supplement 2:** Loss of Lrp1 in the OL lineage leads to faulty nerve conduction

**Figure 3-figure supplement 1:** Loss of Lrp1 does not alter OPC proliferation.

**Figure 4-figure supplement 1:** Lrp1 deficient OLs are sensitive to statin treatment but not to exogenously supplied mevalonate.

**Figure 5-figure supplement 1:** Gene ontology (GO) analysis of Lrp1 deficient OPCs revealed enrichment of peroxisomal genes.