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Micro-Scale Control of Oligodendrocyte Morphology and Myelination by the Intellectual Disability-Linked Protein Acyltransferase ZDHHC9

Hey-Kyeong Jeong, Estibaliz Gonzalez-Fernandez, Ilan Crawley, Julia M Coakley, Jinha Hwang, Dale DO Martin, Shernaz X Bamji, Jong-Il Kim, Shin H Kang, Gareth M Thomas

Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States • Department of Biomedical Science, Seoul National University College of Medicine, Seoul, Republic of Korea • Department of Biology, University of Waterloo, Waterloo, Canada • Department of Cellular and Physiological Sciences, Life Sciences Institute and Djavad Mowafaghian Centre for Brain Health, University of British Columbia, Vancouver, Canada • Department of Neural Sciences, Lewis Katz School of Medicine at Temple University, Philadelphia, United States

eLife Assessment:

This study provides an in-depth exploration of the impact of X-linked ZDHHC9 gene mutations on cognitive deficits and epilepsy, with a particular focus on the expression and function of ZDHHC9 in myelin-forming oligodendrocytes (OLs). These **valuable** findings offer insights into ZDHHC9-related X-linked intellectual disability (XLID) and shed light on the regulatory mechanisms of palmitoylation in myelination. The experimental design and analysis of results are **solid**, providing a reference for further research in this field.

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Abstract

Mutations in the X-linked *ZDHHC9* gene cause cognitive deficits in humans, with a subset of patients suffering from epilepsy. X-linked intellectual disability (XLID) is often ascribed to neuronal deficits, but here we report that expression of human and mouse ZDHHC9 orthologs is far higher in myelinating oligodendrocytes (OLs) than in other CNS cell types. *ZDHHC9* codes for a protein acyltransferase (PAT), and we found that ZDHHC9 is the most highly expressed PAT in OLs. Wild type ZDHHC9 localizes to Golgi outposts in OL processes, but other PATs and XLID mutant forms of ZDHHC9 are restricted to OL cell bodies. Using genetic tools for OL progenitor fate tracing and sparse cell labeling, we show that mice lacking *Zdhhc9* have grossly normal OL development but display extensive morphological and structural myelin abnormalities. Consistent with the hypothesis that these deficits are OL-autonomous, they are broadly phenocopied by acute *Zdhhc9* knockdown in cultured

conditions. Finally, we found that ZDHHC9 palmitoylates Myelin Basic Protein (MBP) in heterologous cells, and that palmitoylation of MBP is impaired in the *Zdhhc9* knockout brain. Our findings provide critical insights into the mechanisms of *ZDHHC9*-associated XLID and shed new light on the palmitoylation-dependent control of myelination.

Introduction

Mutations in the *ZDHHC9* gene cause X-linked Intellectual Disability (XLID)(1–3). XLID-associated *ZDHHC9* mutations include nonsense mutations and missense mutations affecting key amino acids, as well as splice site mutations, insertions, and deletions that result in frameshifts. Importantly, all these genetic changes are predicted to be loss-of-function. These findings suggest that ZDHHC9 is essential for higher brain function.

Given that many XLID genes are neuronally enriched (4–10), an *a priori* hypothesis is that ZDHHC9 acts in neurons. However, patients with *ZDHHC9* mutations have grossly normal gray matter (GM) but reduced brain white matter (WM) volume, especially in the corpus callosum (CC) (1–3). *ZDHHC9* mutations are also linked to cerebral palsy, a condition in which intellectually disability is often associated with WM impairment (WMI) (11–14). These findings raise the possibility that ZDHHC9 may be crucial for normal WM development and/or proper function of oligodendrocytes (OLs), the myelin-forming CNS glia abundant in WM. Notably, *Zdhhc9* knockout (KO) mice exhibit WM volume reductions and seizure-like activity (15, 16), as well as behavioral phenotypes seen in other mouse models of ID (15). Thus, *Zdhhc*9 KO mice have the potential to serve as an excellent model for human ZDHHC9 mutations. However, their phenotype is yet to be fully characterized.

ZDHHC9 codes for a protein acyltransferase (PAT), an enzyme that catalyzes the modification of protein cysteine residues with palmitate and related fatty acids. This process, termed palmitoylation (also known as *S*-palmitoylation or *S*-acylation), plays a critical role in targeting proteins to specific subcellular membrane locations (17). Nearly 30 years ago, it was reported that major myelin proteins in the nervous system, including Proteolipid protein (PLP), Myelin basic protein (MBP), Myelin-associated glycoprotein (MAG) and Myelin oligodendrocyte glycoprotein (MOG), are palmitoylated (18, 19). However, at that time, methods were lacking to define roles of palmitoylation of these proteins in myelin formation and function. Later proteomic studies revealed many other palmitoylated myelin proteins (20–23), and an isolated study suggested that palmitoylation targets myelin proteins to the plasma membrane of cultured OLs (24). Despite these findings, little is known regarding how myelin protein palmitoylation is regulated and the functional importance of this process for higher brain function.

We hypothesized that ZDHHC9-dependent palmitoylation plays a critical role in proper myelination and WM formation. Consistent with this notion, we report that ZDHHC9 is the most highly expressed PAT in mouse and human OLs. Moreover, ZDHHC9 localizes to puncta in OL processes that are likely Golgi outposts, whereas other PATs tested and *ZDHHC9* XLID mutant forms are restricted to OL cell bodies. These findings may explain why *ZDHHC9* loss of function cannot be compensated by other PATs. In mice lacking *Zdhhc9*, we did not detect changes in OL lineage cell generation or total MBP expression levels, but we found that at the micro-scale *Zdhhc9* KO OLs are dysmorphic and myelin ultrastructure is highly abnormal, with both hypo- and hypermyelination of axons in WM tracts. We show that with the help of Golga7, a ZDHHC9 partner protein that also localizes to Golgi outposts, ZDHHC9 robustly palmitoylates the major myelin protein MBP, and that MBP palmitoylation is impaired in *Zdhhc9* KO brain. Palmitoyl- and total levels of another myelin protein, Myelin-associated Glycoprotein (MAG) are also affected by *Zdhhc9* KO, suggestive of a broader deficit in myelin protein regulation and organization in these mice. Interestingly, the XLID mutant ZDHHC9-*P150S* still palmitoylates MBP in a Golga7-dependent

manner in transfected non-neuronal cells, raising the possibility that impaired subcellular localization in OLs, rather than impaired catalytic activity, may cause or contribute to WM abnormalities in some cases of *ZDHHC9*-associated XLID. Together, our findings provide new insights into mechanisms of *ZDHHC9*-associated XLID and the palmitoylation-dependent control of myelination, a process first reported decades ago, but about which almost nothing is known.

Results

Cell-Specific Transcriptomics Reveals That ZDHHC9 and Its Partner Protein GOLGA7 Are Enriched in Oligodendrocytes in the Mouse and Human Brain

To assess *Zdhhc9* expression in OLs, we performed FACS using cerebral cortex (CTX) of P30 *Mobp-EGFP* BAC transgenic (Tg) mice, which express EGFP only in mature OLs (**Fig. 1A**; (25, 26)). We isolated RNA from the $EGFP^+$ OLs and performed OL-specific RNA sequencing (RNA-Seq). Total RNA was also extracted from the cortices of the same mice without OL sorting and used for a separate bulk RNA-Seq. Bioinformatics analysis indicated that genes previously known to be highly expressed in myelinating OLs (14) were highly enriched in our OL-specific RNA preparations (**Fig 1B**). In contrast, expression levels of genes specific to other neural cell types and endothelial cells were very low, confirming that our transcriptomic dataset is highly OL-specific (**Fig. 1B**).

We then extracted fragments per kilobase of transcript per million mapped reads (FPKM) values for all ZDHHC family PATs from our OL-specific RNA-Seq dataset. We found that *Zdhhc9* is the most highly expressed PAT in OLs (FPKM ~ 180), with mRNAs for *Zdhhc14*, *Zdhhc17*, and *Zdhhc20* present at lower but detectable levels (FPKM 30 ~ 70) (**Fig.1C**). The remaining PATs were expressed at far lower levels (FPKM <10). These results suggest that ZDHHC9 is the predominant PAT in myelinating OLs.

ZDHHC9 activity requires a conserved partner, Golgi Complex Protein-16 (gene name *Golga7*) (16, 27, 28). We found that *Golga7* was also abundantly expressed in OLs, at far higher levels than other Golga family members, including *Golga7*'s closest paralog, *Golga7b* (**Fig. 1D**). FPKM values for *Zdhhc9* and *Golga7* were far lower in total RNA preps obtained from whole cortices without specific cell type isolation (**Fig. 1E**, left Heatmap), suggesting that high *Zdhhc9* and *Golga7* expression is an OL-specific feature, rather than a general characteristic of CNS cells. Consistent with this notion, examination of another CNS cell type-specific RNA-Seq dataset, obtained with sorted cells from P17 brain cortices via immunopanning (29), confirmed high and specific expression of *Zdhhc9* and *Golga7* in myelin-forming OLs (**Fig. 1E**, right Heatmap). Moreover, examination of a human RNA-Seq study (30) revealed that, like mouse CNS, both *ZDHHC9* and *GOLGA7* are enriched in human OLs, compared to any other PATs or other GOLGA family members, respectively. (**Fig 1F**). Together, these findings raise the possibility that ZDHHC9, in concert with GOLGA7, plays an important role in OLs.

Wild type ZDHHC9 and GOLGA7 Localize to Oligodendrocyte Processes *In Vitro,* Unlike Other PATs or ZDHHC9 Mutants

As a first step toward understanding ZDHHC9 function in OLs, we sought to define the subcellular localization of transfected HA-tagged ZDHHC9 (HA-ZDHHC9) in cultured OLs. We also compared the subcellular localization of HA-ZDHHC9 with that of HA-tagged forms of ZDHHC3, ZDHHC7 and ZDHHC17, three other ZDHHC-PATs that are implicated in nervous system regulation and which are expressed in OLs *in vivo* (**Fig. 1E**, (29, 31–36)).

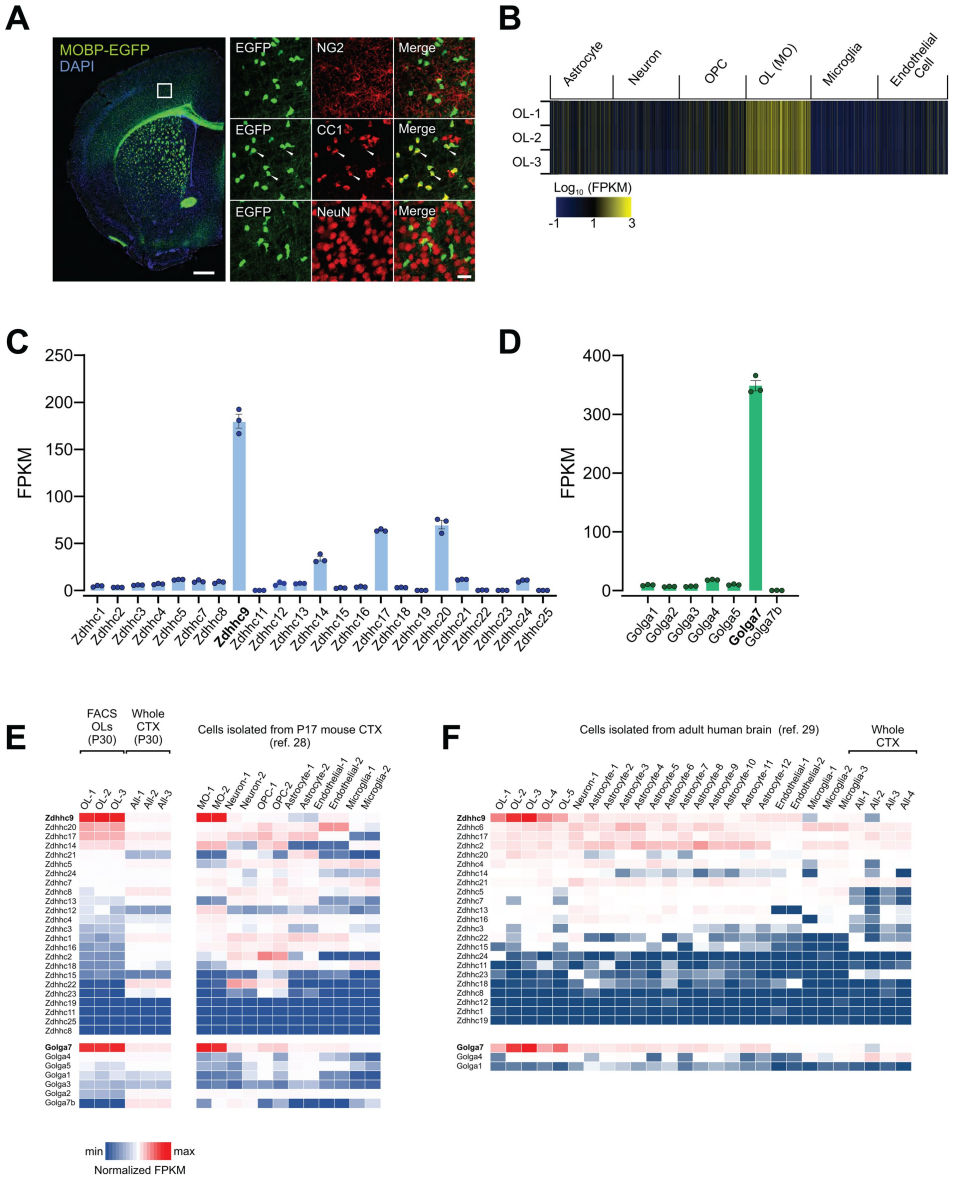

Figure 1

Oligodendrocyte-specific transcriptomics reveals OL-enriched expression of ZDHHC9 and GOLGA7.

A: Fluorescent image of widespread EGFP expression in the brain of *Mobp-EGFP* mice (Left). Confocal images of EGFP and other cell-specific markers (NG2 for OPC, CC1 for OLs, and NeuN for neurons) (Right). White arrowheads indicate colocalization of $EGFP^+$ cells and CC1-immunoreactivities. Boxed area in left panel corresponds to the region used to acquire confocal images for NG2 and CC1 in right panel. Scale bars: 500 (left) and 20 (right) μm. ***B:*** Heatmap for expression levels of previously reported cell type-specific gene clusters from the OL-specific RNA-Seq results. $EGFP^+$ OLs were isolated from cortices of three *Mobp-EGFP* mice with FACS, and their RNAs were used for RNA-Seq. ***C, D:*** FPKM values of ZDHHC-protein acyltransferases (PATs) (C) and Golgin subfamily A members (D) expressed in OLs. ***E:*** Heatmap of relative expression of all PATs and Golgin subfamily A member genes in mouse OLs and other brain cells according to data from this study (left) and Zhang et al., 2014. ***F:*** Heatmap of relative expression of all PATs and Golgin subfamily A member genes in human OLs and other brain cells according to Zhang et al., 2016.

After three days of culture in differentiation medium (**Fig 2A**), cultured OLs were extensively ramified and showed strong MBP immunofluorescence ('OL3D'; Fig S1A). We transfected these cultured OLs and fixed them 9h later to minimize the likelihood of ZDHHC-PAT expression itself altering OL morphology and affecting HA-PAT subcellular localization (**Fig 2A**). Under these conditions, HA-ZDHHC3, HA-ZDHHC7, and HA-ZDHHC17 were only detected in OL cell bodies, consistent with their restriction to the somatic Golgi in other cell types (32, 35, 37–39) (**Fig 2B, D**). In contrast, HA-ZDHHC9 was detected both in OL cell bodies and in discrete puncta in OL processes (**Fig. 2B, D**). Within OL cell bodies, ZDHHC9 partially colocalized with the Golgi marker GM130 (Fig. S1A, B). Consistent with this Golgi localization, ZDHHC9 also colocalized in part with additional Golgi markers (TGN38, Giantin; Fig S1C, D). However, these markers were not detected in OL processes and therefore did not colocalize with ZDHHC9 in this latter location (Fig. S1A-D).

We also compared the subcellular distribution of wild type ZDHHC9 (ZDHHC9WT) with that of XLID-associated ZDHHC9 mutants (R96W; R148W; P150S) (40, 41). In contrast to HA-ZDHHC9WT, these XLID-associated HA-ZDHHC9 mutants did not localize to OL processes (**Fig. 2C, D**). These results raise the possibility that dysregulated subcellular localization of ZDHHC9, rather than, or in addition to, reduced catalytic activity (42) is a causative factor in *ZDHHC9* loss of function mutations in XLID.

We also sought to define the localization in OLs of Golga7, which directly binds and enhances function of ZDHHC9 in other cell types (16, 27, 28). Consistent with this role, myc-tagged Golga7 (myc-Golga7) colocalized extensively with HA-ZDHHC9 in both OL cell bodies and in discrete puncta in OL processes (Fig. S2A). We then sought to further define the identity of these discrete ZDHHC9/Golga7-positive puncta. In neurons, specialized Golgi outposts and Golgi satellites can function as acentrosomal microtubule-organizing centers (MTOCs) in dendrites (43–47). Interestingly, Golgi outposts were also recently reported to be present in OLs (48), and a subset of OL Golgi outposts can be marked by the proteins TPPP and Mannosidase II (ManII) (45, 47, 48). Consistent with their assignation as Golgi outposts, ZDHHC9-positive puncta in OL processes colocalized with GFP-tagged ManII (ManII-GFP) and, to a lesser extent, with Flag-tagged TPPP (TPPP-Flag) (Fig. S2B, C).

The OL processes in our culture condition, most of which are MBP+ even at this comparatively short time point post-differentiation (Fig S1A) go on to develop into large lipid-rich membranous sheets (Fig S1A). In vivo, these OL processes form a spiral membrane expansion on axons (i.e. myelination) *in vivo* (49). ZDHHC9's localization to Golgi outposts/satellites in processes of cultured OLs suggest its potential role in myelin formation (myelination) and/or maintenance. Moreover, this function of ZDHHC9 might not be shared with other PATs tested and may be impaired in XLID-associated mutant forms of ZDHHC9.

No detectable changes in Oligodendrocyte Development or Gross Myelination in *Zdhhc9* KO Mice

To address whether *Zdhhc9* regulates OL development and myelination *in vivo*, we examined the brains of *Zdhhc9* KO mice. Histological observations of brain sections from 6-week-old male mice revealed no apparent difference in MBP-labeled OL processes between WT control and *Zdhhc9* KO mice (**Fig. 3A**). OL numbers in CTX and the corpus callosum (CC), assessed using OL markers aspartoacylase (ASPA) or Quaking 7 (recognized by antibody CC1), also did not detect any significant difference between the two groups (Fig. S3A-C). To quantify OLs more precisely, we crossed *Zdhhc9* KO with *Mobp-EGFP* mice and counted EGFP-labeled OLs. Again, we did not detect significant differences in $EGFP^+$ OL density between the two genotypes in three examined CNS regions (CTX, CC, and spinal cord), at two different ages (P28, P56; (**Fig. 3B, C**)). We also detected no difference in the density of $NG2^+$ OL progenitor cells (OPCs) between WT and *Zdhhc9* KO mice (**Fig. 3D, E**).

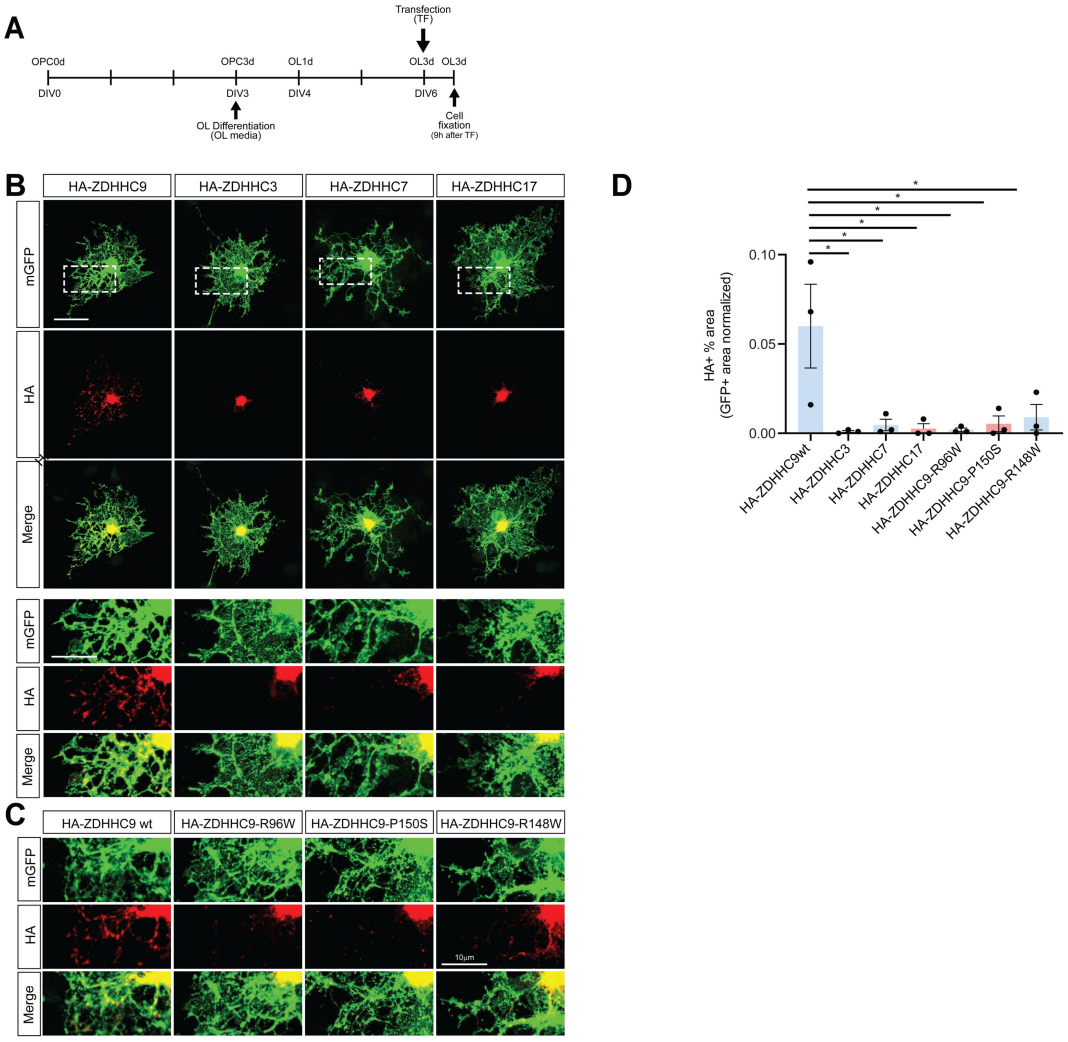

Figure 2

HA-ZDHHC9WT localizes to puncta in OL processes, but other PATs, and XLID mutant forms of ZDHHC9, are restricted to OL cell bodies.

A: Experimental timeline. ***B:*** Images of morphologically mature OLs transfected as in *A* to express the indicated HA-tagged PATs and immunostained with the indicated antibodies. Lower panels show enlarged images of the boxed regions in upper panels. ***C:*** Images as in lower panels of *B* of OLs transfected to express WT HA-ZDHHC9 or the indicated XLID mutant forms of ZDHHC9. ***D:*** Quantified data confirms that HA-ZDHHC9 WT occupies a greater percentage of the total OL area (GFP+ area) compared with other PATs, or with HA-ZDHHC9 XLID mutants. *: p=0.032 (HA-ZDHHC9WT vs HA-ZDHHC3); p=0.0053 (HA-ZDHHC9WT vs HA-ZDHHC7); p=0.004 (HA-ZDHHC9WT vs HA-ZDHHC17); p=0.0036 (HA-ZDHHC9WT vs HA-ZDHHC9-R96W); p=0.0058 (HA-ZDHHC9WT vs HA-ZDHHC9-P150S); p=0.0047 (HA-ZDHHC9WT vs HA-ZDHHC9-R148W), Data are from 3-5 cells per condition, pooled from n=3 cultures.

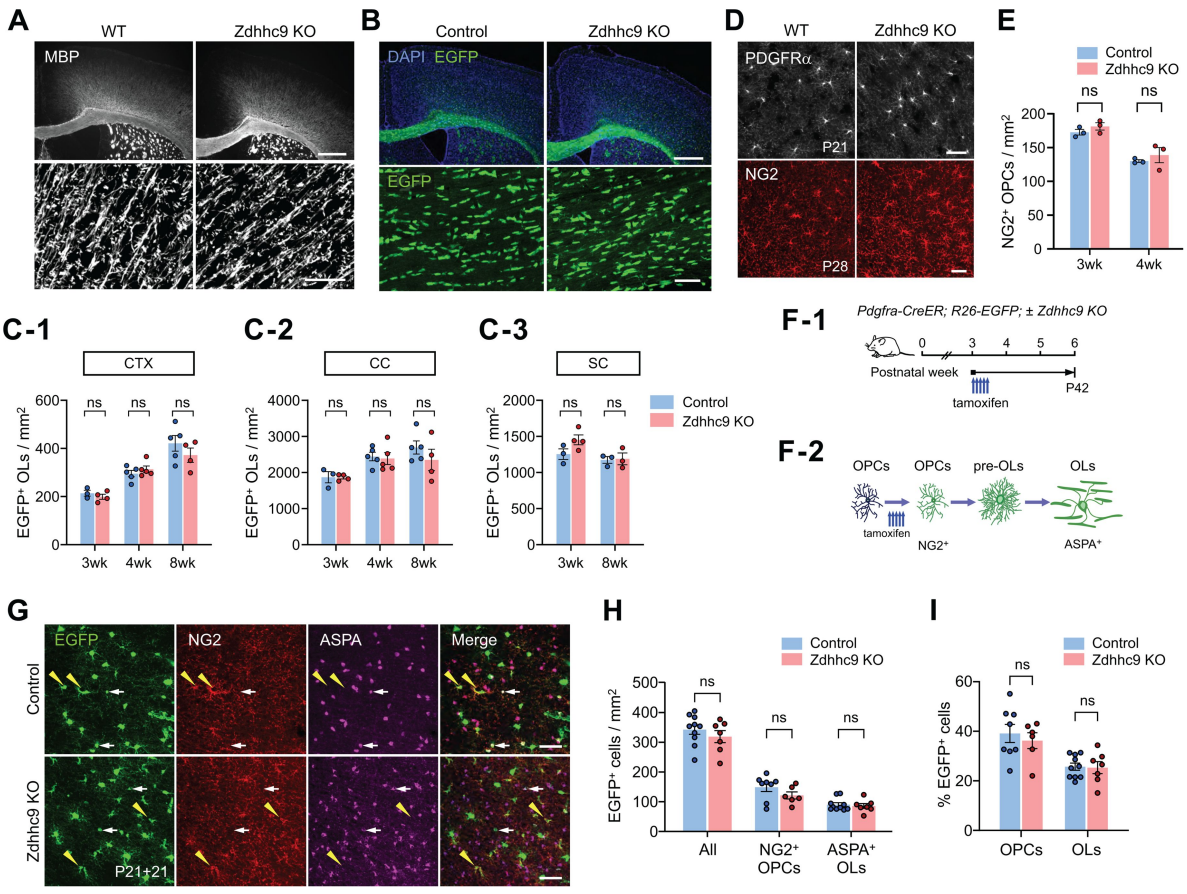

Figure 3

No detectable gross abnormality in oligodendrocyte development in *Zdhhc9* KO mice.

A: Fluorescent (upper) and confocal (bottom) images of MBP immunostaining in the brain of 6-week-old WT and Zdhhc9 KO mice. MBP confocal images were taken from layers IV/V of CTX. Scale bars: 500 (upper panel) and 20 (bottom) μm. ***B:*** Fluorescent (upper) and confocal (bottom) images of EGFP in the brain of 8-week-old control (Mobp-EGFP) and Zdhhc9 KO (Mobp-EGFP; ZDHHC9y/-) male mice. EGFP confocal images were taken from the CC. Scale bars: 500 (upper panel) and 50 (bottom) μm. ***C1-C3:*** Quantification of EGFP+ OLs in CTX, CC, and spinal cord (SC) from 3-week (P21), 4-week (P28) and 8-week (P56)-old control and Zdhhc9 KO Mobp-EGFP mice (n=3 – 5 mice for each group). ***D:*** Confocal images of cortical PDGFRα+ OPCs from 3-week-old male mice (upper panel) and NG2+ OPCs from 8-week-old male mice (lower panel). Scale bar: 50 μm. ***E:*** Quantification of the density of NG2+ OPCs in CTX of 3-week and 4-week-old WT and Zdhhc9 KO mice (n=3 per group). ***F-1:*** Experimental scheme for OPC fate tracing. Pdgfra-CreER; RCE and Pdgfra-CreER; RCE; Zdhhc9 KO mice received tamoxifen injections starting at P21 for a duration of 3 days, and their brains were collected at P42. ***F-2:*** Schematic diagram illustrating the stepwise changes in genetically labeled OPCs during fate tracing. ***G:*** Confocal images of EGFP, NG2, and ASPA in CTX of control and Zdhhc9 KO Pdgfra-CreER; RCE mice (P21+21). Yellow arrowheads: overlapping signals between EGFP and NG2; white arrows: overlapped signals between EGFP and ASPA. Scale bars: 50 μm. ***H:*** Quantification of EGFP+NG2+ OPCs and EGFP+ASPA+ OLs. ***I:*** Percentage of NG2+ OPCs and ASPA+ OLs among EGFP+ cells. Data are mean ± SEM (*E, H, I*). For *H* and *I*, N= 10 (control) or 7 (Zdhhc9 KO) male and female mice. Two-way ANOVA and subsequent pair-wise comparisons were performed with Šidák's multiple comparison tests for (*C, E, H, I*). ns: not significant.

We next asked whether *Zdhhc9* loss affects the rate of oligodendrogenesis (maturation of OPCs to OLs). To address this question, we crossed *Zdhhc9* KO mice with *Pdgfra-CreER; R26-EGFP* (RCE) mice (50) and analyzed the fates of genetically labeled OPCs. Control (*Pdgfra-CreER; RCE*) and KO (*Pdgfra-CreER; RCE*; *Zdhhc9*$^{y/-\text{ or }-/-}$) mice received tamoxifen injections at P21 and were sampled 3 weeks later (P21+21) (**Fig. 3F-1**). This allowed us to label PDGFRα$^+$ OPCs with EGFP at P21 and track their differentiation. Newly differentiated EGFP$^+$ OLs from the previously labeled EGFP-labeled OPCs were analyzed (**Fig. 3F-2**, G). However, we did not detect differences in the number of newly formed EGFP-labeled ASPA$^+$ OLs and % of EGFP$^+$ OLs (**Fig. 3H, I**) and the ratio of OLs to OPCs among EGFP-labeled cells (data not shown) between the two groups. These results suggest that *Zdhhc9* KO does not alter oligodendrogenesis in either the young or mature CNS, at least using the markers and methodologies that we employed.

Zdhhc9 KO Impairs Microstructure of Oligodendrocyte Processes

Although we did not detect differences in overall oligodendrogenesis or myelin production, we reasoned that loss of *Zdhhc9* might still affect the targeting of specific myelin proteins to the membrane, resulting in irregular formation of processes in OLs or abnormalities in myelin. To address this possibility, we used a sparse genetic OL labeling method, crossing *Mobp-iCreER*; *mT/mG* mice to *Zdhhc9* KO mice. By P56, a small degree of tamoxifen-independent (leaky) Cre activity leads to spontaneous expression of membrane-anchored EGFP (mEGFP) in a subset (~5%) of cortical OLs in these mice. The sparsely EGFP-labeled brain sections were then imaged with confocal microscopy to detect mEGFP signal in CTX and to subsequently trace OL processes (**Fig. 4A**). The morphology of individual OLs was then reconstructed as a 3D skeleton (**Fig. 4B**). Sholl analysis of these reconstructions revealed that the overall OL process complexity was slightly increased in *Zdhhc9 KO* mice (**Fig. 4C**). More surprisingly, the primary and secondary branch process were longer in *Zdhhc9* KO than control mice (**Fig. 4D**).

We also analyzed the processes of individual OLs in raw images from these sections. In control mice, the EGFP-labeled processes originating from one OL soma were of similar thickness, with a uniformly smooth structure. However, the thickness of mEGFP$^+$ processes in *Zdhhc9* KO mice was far more heterogenous, with several spheroid-like swellings (**Fig. 5A, B**). The abnormal thickness of EGFP$^+$ OL processes may reflect dysregulated axon recognition by OLs. Notably, the abnormal spheroid-like swellings on OL processes were distinguished from OL somas as they lack signal for DAPI or Olig2 (**Fig. 5C**, Fig. S4). Careful tracing of EGFP$^+$ OL processes connected to DAPI$^+$ cell body and quantification of spheroid-like swellings devoid of DAPI signal (**Fig. 5C-1**, **C-2**) revealed significant structural abnormalities in OL processes in *Zdhhc9* KO mice (**Fig. 5D, E**). These results indicate that, in contrast to its lack of effect at the gross level, *Zdhhc9* loss greatly alters the structure of individual OL processes at the microscopic level, presumably due to abnormal OL membrane expansion.

Impaired Myelination in *Zdhhc9* KO Mice

The non-uniformity of *Zdhhc9* KO OL processes suggests that *Zdhhc9* loss may disrupt the typical bias of myelination based on axon caliber and/or the spreading of the OL membrane along the axon. To address this issue, we examined the extent and pattern of axonal myelination using electron microscopy (EM) (**Fig. 6A**). In electron micrographs from P56 CC, the number of axons did not differ between WT and *Zdhhc9* KO mice (**Fig. 6A, B**). However, while most axons were evenly myelinated in WT mice, myelin patterns of axons in *Zdhhc9* KO mice were highly abnormal; with many large axons unmyelinated (**Fig. 6A, C**) and a subset of small diameter axons (< 0.5 μm) appearing to be hypermyelinated (**Fig. 6A, D**). Consistent with the latter finding, *g*-ratios of these small diameter axons were smaller in *Zdhhc9* KO mice (**Fig. 6E, F**).

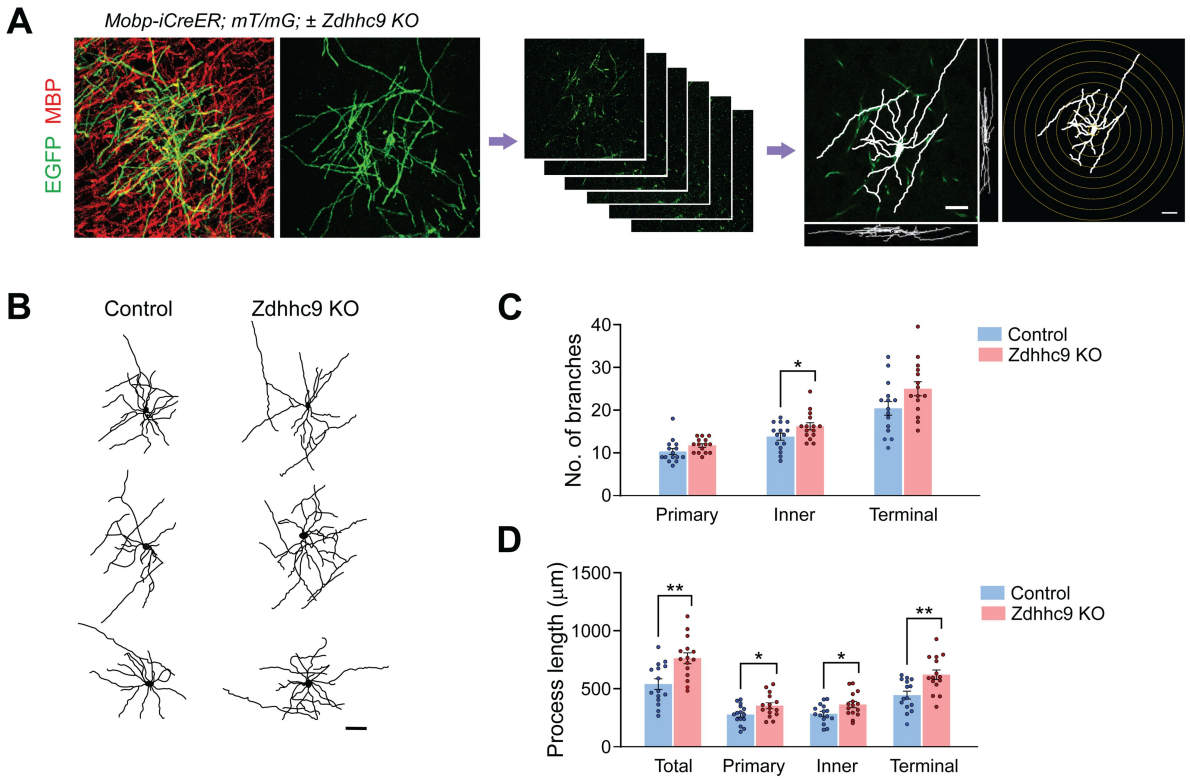

Figure 4

Genetic sparse cell labeling reveals altered complexity of oligodendrocyte processes in *Zdhhc9* KO mice.

A: Experimental flow of the sparse genetic labeling of OLs, from acquisition of confocal images of mEGFP from CTX in P56 *Mobp-iCreER; mT/mG* mice to 3D OL process tracing to Sholl analysis. mEGFP signal allows for detailed process morphology of individual OLs, compared to more complex 'bulk' MBP signal. ***B:*** Representative results from tracing of OL processes. Scale bars: 20 μm. ***C, D:*** Branch numbers (C) and process length (D) were compared between control (*Mobp-iCreER; mT/mG*) and Zdhhc9 KO (*Mobp-iCreER; mT/mG; Zdhhc9 KO*) mice. N=15 OLs (5 OLs per mouse, three mice per group). Two-way ANOVA and pair-wise comparisons were performed with Šidák's multiple comparison tests (C and D). ns: not significant. *, p <0.05; **, p <0.01.

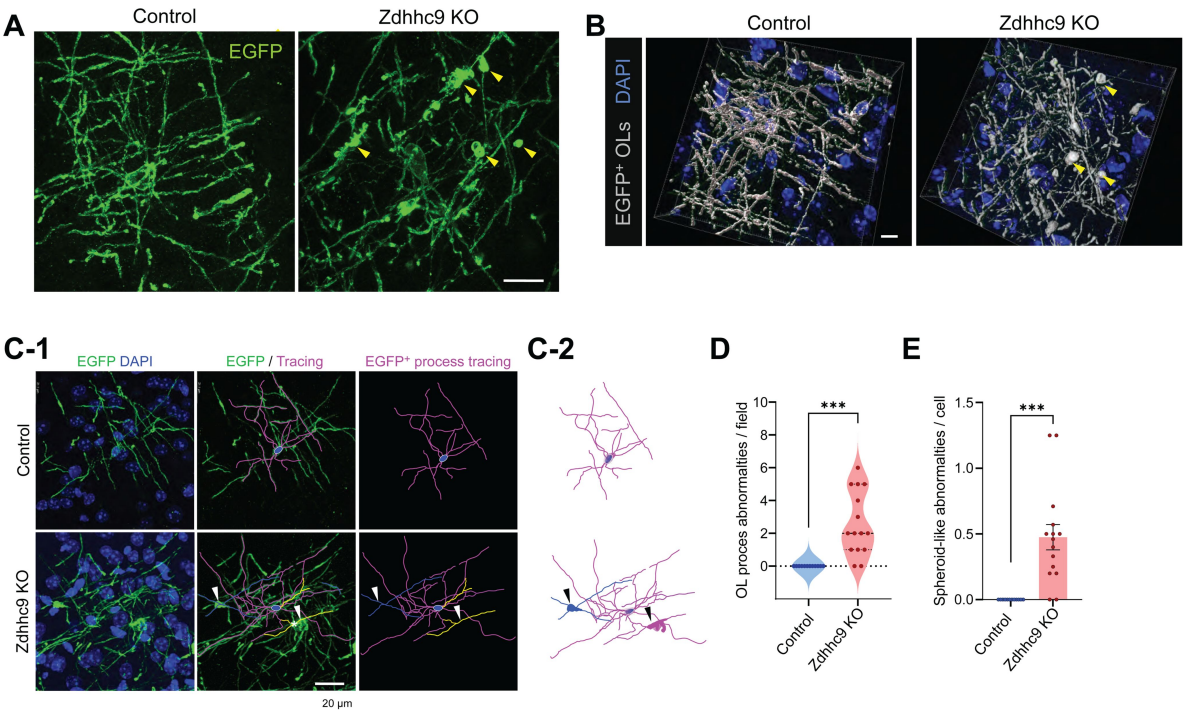

Figure 5

Abnormal oligodendrocyte processes in *Zdhhc9* KO mice.

A: Confocal microscopy of mEGFP in P56 *Mobp-iCreER; mT/mG* mice reveals morphological abnormalities, such as non-homogenous EGFP$^+$ cell processes and spheroid-like membrane folding (yellow arrowheads). Scale bar: 20 μm. ***B:*** 3D-reconstruction of EGFP$^+$ OLs and DAPI$^+$ nuclei from the images shown in (A) with the Imaris software. Scale bar: 7 μm. ***C:*** OL processes and connected OL cell body were traced. Two different cells are shown in magenta and green. Arrowheads indicate spheroid-like swelling. ***D, E***: Quantification of process abnormalities per field (D) and per cell (E). N=15 cells from 3 mice per group. Student's t-test. ***, $p < 0.001$.

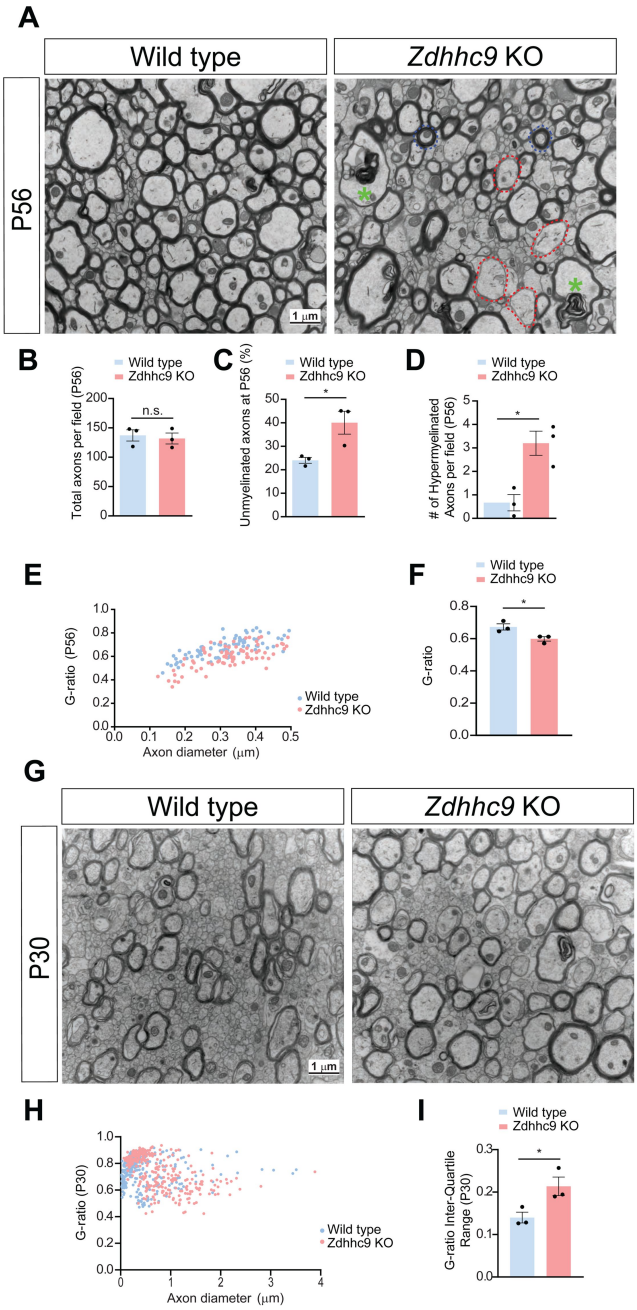

Figure 6

Altered myelination in *Zdhhc9* KO corpus callosum *in vivo*.

A: Electron micrographs of corpus callosum axons from P56 male mice of the indicated genotypes. Wild type axons are uniformly myelinated, but in *Zdhhc9* KO some large axons are hypomyelinated (red dotted outline), while a subset of small axons appear hypermyelinated (blue dotted outline). There is also frequent dysmyelination (green asterisk). ***B:*** Quantification of images from *A* reveal no change in total number of axons in *Zdhhc9* KO. n.s.: not significant (p=0.7117, t test, N=3 mice per genotype). ***C:*** Percentage of unmyelinated axons from mice of the indicated genotypes (N=3 mice per genotype; *;p=0.0036, t test). ***D:*** number of hypermyelinated small axons per field in corpus callosum images from mice of the indicated genotypes (N=3 mice per genotype; *; p=0.0150, t test). ***E:*** G-ratio of small diameter axons (0.1-0.5 µm) from mice of the indicated genotypes. ***F:*** Average G-ratio from *E* confirms hypermyelination of small diameter axons in P50 *Zdhhc9* KO mice. ***G:*** as *A*, but from P30 mice. Deficits in myelination are already evident at this time. ***H:*** As *E*, but for all axons from *G*. ***I:*** Increased heterogeneity of myelination in *Zdhhc9* KO corpus callosum at P30, represented by an increased interquartile range of G-ratios (*;p=0.0425, t test).

Next, we inquired whether the dysmyelination observed in young adult (P56) *Zdhhc9* KO mice resulted from impaired initial myelination or from impaired myelin maintenance or active demyelination. At P30, a time at which myelination is actively ongoing, EM images showed a broadly similar extent of myelination across all axons in WT mice (**Fig. 6G, H**). However, in *Zdhhc9* KO mice, both hypo- and hypermyelination of axons was already noticeable at this developmental stage (**Fig. 6G**), which was evident from an increased interquartile range of *g*-ratios (**Fig. 6H, I**). These findings suggest that *Zdhhc9* loss results in dysmyelination and, thus, that ZDHHC9 is required for proper initial myelination of individual axons.

Evidence supporting a cell-autonomous role of ZDHHC9 in Oligodendrocyte Maturation

Zdhhc9 is constitutively deleted in the KO mice, so OL morphological abnormalities and myelination deficits seen in this line might be caused by loss of action of ZDHHC9 in other cell types. As a first step to testing whether phenotypes in *Zdhhc9* KO mice are cell-autonomous, we delivered lentivirus expressing GFP plus a specific shRNA (16) to knock down *Zdhhc9* in WT OPCs in culture and induced OPC-to-OL differentiation one day later (**Fig. 7A**). *Zdhhc9* knockdown OLs had significantly reduced expression of MBP, compared to OLs infected to express a scrambled shRNA (**Fig. 7B-D**).

We asked whether this failure to express and distribute MBP is due to impaired commitment to the OL lineage in culture. However, *Zdhhc9* knockdown OLs still expressed 2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP), an early myelin-specific protein of developing OLs (51) (Fig. S5). This result suggests that ZDHHC9 is not required for initial differentiation of OPCs to OLs, consistent with the normal number of OLs seen in *Zdhhc9* KO mice *in vivo* (**Fig. 3C**, Fig. S3C). However, consistent with the reduced and restricted MBP expression, *Zdhhc9* knockdown OLs were also morphologically immature (**Fig. 7E**) with a reduced degree of branching confirmed by Sholl analysis (**Fig. 7F**). These data indicate that *Zdhhc9* loss cell-autonomously impairs OL maturation *in vitro*.

A ZDHHC9-Golga7 PAT Complex Directly Palmitoylates MBP

Finally, we sought to identify potential ZDHHC9 substrates in OLs. Myelination of CNS axons by OLs requires the targeting of myelin proteins, including MBP, PLP, MOG and MAG, to the myelin membrane. These myelin proteins are all palmitoylated (20, 24, 52–54), but the PAT(s) that controls myelin protein palmitoylation was not previously identified. We thus used a non-radioactive palmitoylation assay, acyl biotin exchange (ABE) (55, 56), to determine whether ZDHHC9 can directly palmitoylate MBP in co-transfected HEK293T cells. Palmitoylation of 21.5 kDa MBP (one of two MBP isoforms that contains a cysteine residue and is thus capable of undergoing palmitoylation) was very low when transfected alone. MBP palmitoylation remained very low when either HA-ZDHHC9 or myc-Golga7 were co-transfected in isolation (**Fig. 8A, B**). However, MBP palmitoylation was greatly increased by co-transfection of HA-ZDHHC9 and myc-Golga7 (**Fig. 8A, B**).

To examine whether ZDHHC9 palmitoylates MBP *in vivo*, we used ABE to purify palmitoyl-proteins from forebrain WM tissue (CC and striatum) of WT and *Zdhhc9* KO mouse brains. Consistent with *in vivo* immunostaining results (**Fig. 3A**), *Zdhhc9* KO did not affect total MBP levels measured by western blotting (all isoforms assessed together; **Fig 8C, D**). Two MBP bands were detected in ABE (palmitoyl) fractions, which based on their molecular weights, likely represent the 17.0 and 21.5 kDa isoforms of MBP (57). Like 21.5 kDa MBP, the 17.0 kDa form of MBP contains a cysteine residue and may be subject to palmitoylation (57). Importantly, palmitoylation, but not total expression, of both the 17 kDa and 21.5 kDa MBP isoforms was significantly reduced in *Zdhhc9* KO mice (**Fig. 8C, D**). Total and palmitoyl-levels of MAG were also significantly reduced in *Zdhhc9* KO mice, although the palmitoyl:total ratio of MAG was not. In the same samples, neither palmitoyl-, total, nor palmitoyl:total levels of Cadm4 were affected in *Zdhhc9* KO mice. This latter

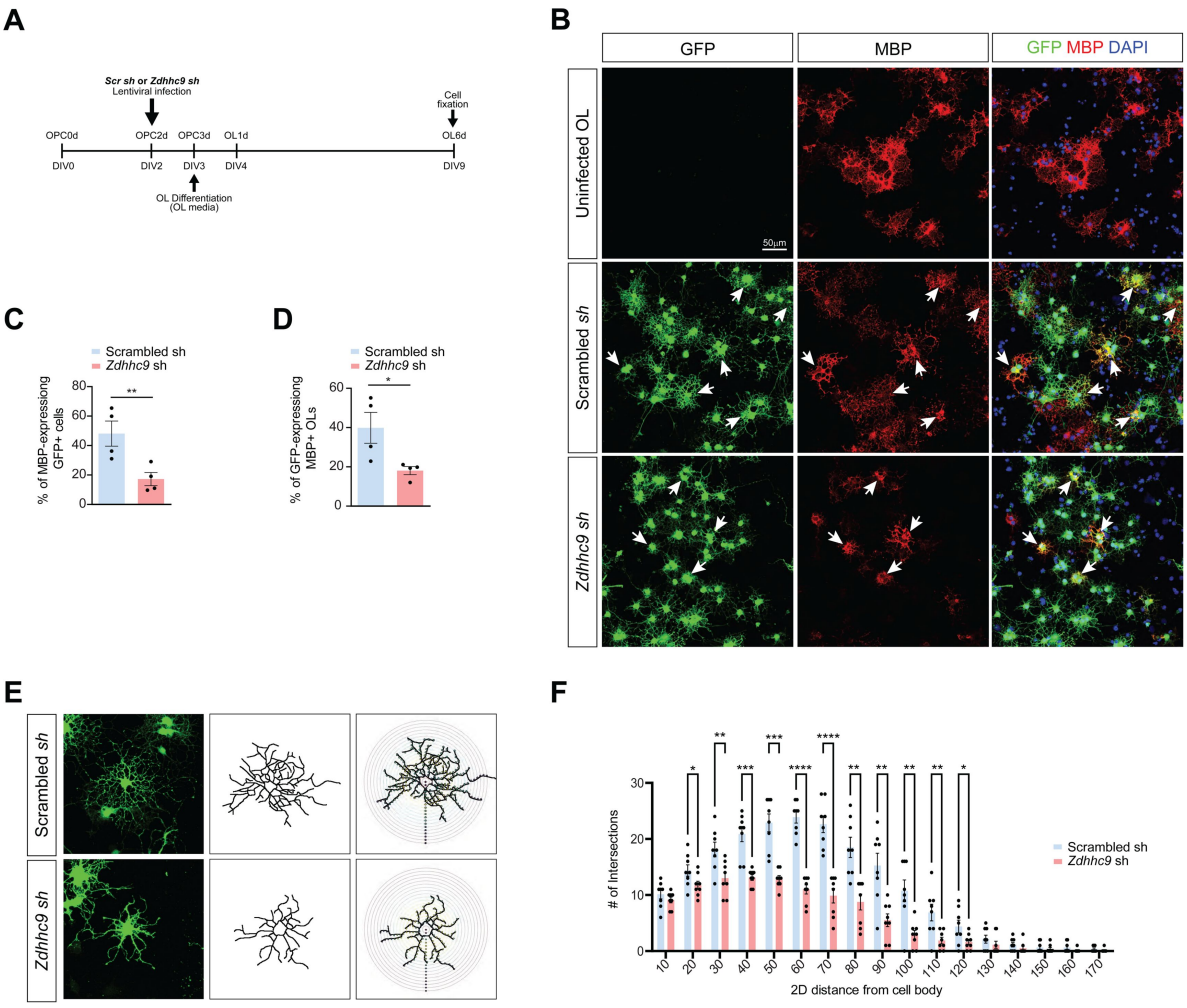

Figure 7

ZDHHC9 loss cell-autonomously impairs maturation of cultured OLs.

A: Timeline of experiment. ***B:*** Immunofluorescent images of cultured OLs, after infection with the indicated lentiviruses and fixation as in *A*. ***C:*** Quantified data from *B* reveal that *Zdhhc9* knockdown reduces the percentage of GFP-expressing (virally infected) MBP^+ cells i.e., mature OLs (**; p=0.0067, t-test, n=4 individual cultures per condition). ***D:*** Likewise, *Zdhhc9* knockdown reduces the percentage of MBP-expressing GFP+ cells. (*; p=0.0359, t-test, n=4 individual cultures per condition). ***E:*** *Left:* Images of individual OLs after infection and fixation as in *B. Middle column:* reconstructed outline of individual OLs. *Right column:* Images from middle column with superimposed concentric circles for Sholl analysis. ***F:*** Sholl analysis from OLs reconstructed as in *E* confirms reduced morphological complexity of *Zdhhc9* knockdown OLs. n=8 cells per condition. *:p<0.05; **:p<0.01; ***:p<0.001; ****:p<0.0001, individual t tests.

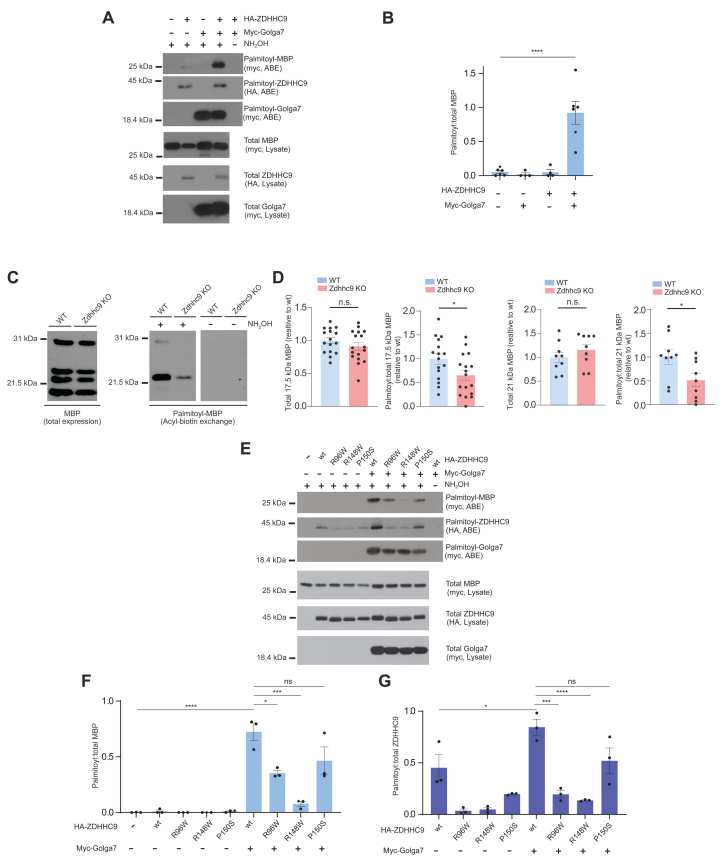

Figure 8

ZDHHC9 palmitoylates MBP in cultured cells and *in vivo,* and certain ZDHHC9 XLID mutants display residual PAT activity towards MBP.

A: Western blots of palmitoyl- and total proteins from HEK293T cells transfected to express the indicated cDNAs. ***B:*** Quantified data from C confirms that co-transfection of HA-ZDHHC9 and myc-Golga7 greatly increases MBP palmitoylation. No signal for palmitoyl-MBP is seen in the absence of the key reagent hydroxylamine (NH_2OH), confirming assay specificity. ****:p<0.0001, ANOVA with Dunnett's multiple comparison test. ***C:*** Western blots to detect MBP in total lysates and ABE fractions from forebrain WM (CC and striatum) from mice of the indicated genotype. ***D:*** Quantified data from *C* confirms that *Zdhhc9* loss reduces palmitoyl, but not total, levels of 17 kDa and 21.5 kDa MBP isoforms. palmitoyl:total 17.5 kDa isoform, n=15-16 per genotype; **:p=0.0078, palmitoyl:total 21 kDa isoform, n=8 per genotype,. 'N' number is lower for 21.5kDa MBP because in a subset of experiments this isoform was not efficiently extracted and was hence undetectable. ***E:*** As *A,* expect that HEK293T cells were transfected to express HA-tagged ZDHHC9wt or XLID mutant forms from **Figure 2**, plus the indicated additional proteins. Western blots of palmitoyl-fractions (isolated by ABE assay) and total proteins are shown. ***F:*** Quantified data from *E* for palmitoylation of MBP confirm that palmitoylation of MBP by ZDHHC9-*P150S* and ZDHHC9wt do not differ significantly and that palmitoylation of MBP by ZDHHC9-*R96W* is less impaired than that by ZDHHC9-*R148W* (****:p<0.0001; ***:p<0.001; *:p<0.05; n.s.; not significant, ANOVA with Dunnett's multiple comparison test). ***G:*** Quantified data from *E* for autopalmitoylation of ZDHHC9 confirm that ZDHHC9wt and ZDHHC9-*P150S* autopalmitoylate to broadly similar extents but that autopalmitoylation of both ZDHHC9-*R96W* and ZDHHC9-*R148W* is significantly lower than that of ZDHHC9wt ((****:p<0.0001; ***:p<0.001; *:p<0.05; n.s.; not significant, ANOVA with Dunnett's multiple comparison test).

finding is consistent with a report ascribing Cadm4 palmitoylation to a different PAT (58). Together, these results suggest that ZDHHC9 directly palmitoylates MBP with the support of Golga7 and that *Zdhhc9* loss impairs MBP palmitoylation. In addition, *Zdhhc9* loss impacts other myelin protein levels and/or regulation.

Finally, we compared the ability of ZDHHC9wt and ZDHHC9 XLID mutants to palmitoylate MBP. We found that MBP palmitoylation by the XLID mutant ZDHHC9-*R148W* is almost undetectable in cotransfected heterologous cells and was minimally increased by additional cotransfection of Golga7. Surprisingly, though, ZDHHC9-*R96W* palmitoylated MBP only approximately 50% as effectively as ZDHHC9wt in the presence of Golga7. Moreover, ZDHHC9-*P150S* did not differ significantly from ZDHHC9wt in its ability to palmitoylate MBP in the presence of Golga7. These findings suggest that certain ZDHHC9 XLID mutants are still capable of palmitoylating substrates, at least in heterologous cells. Moreover, autopalmitoylation (sometimes used as a surrogate marker of ZDHHC-PAT activity) of ZDHHC9wt and ZDHHC9-*P150S* also did not differ significantly in the presence of Golga7, suggesting that ZDHHC9-*P150S* may have sufficient catalytic activity to drive palmitoylation of an array of substrates. These findings further suggest that dysregulated subcellular localization may be a more important factor than altered catalytic activity in certain cases of *ZDHHC9*-associated XLID.

Discussion

Genetic factors are central to intellectual disability (59, 60), as exemplified by the increasing number of known XLID-associated genes (61–64). However, knowledge of the cellular/molecular processes controlled by XLID-associated gene products is often limited. The cognitive deficits and epilepsy that are hallmarks of *ZDHHC9*-associated XLID are often ascribed to malformations of neocortical gray matter (65–69). Indeed, ZDHHC9 is expressed in a subset of forebrain neurons (70), and '*Zdhhc9* knockdown' primary hippocampal neurons display impaired dendritic branching and an altered ratio of excitatory-to-inhibitory synapses (16). However, there is an increasing appreciation that ID can also result from impaired WM formation and/or function (71–75). Consistent with this notion, both ZDHHC9 and its partner protein Golga7 are far more highly expressed in OLs than in other CNS cell types, in both mice and humans (**Fig. 1E, F**, (29, 31)). In support of a key role for ZDHHC9 in normal WM formation and function, we found that *Zdhhc9* KO does not affect axon number in the CC, but greatly impacts microscale OL morphology and myelin ultrastructure (**Figs. 4** - **6**). We recognize that using conventional *Zdhhc9* KO mice, in which *Zdhhc9* is globally deleted, cannot directly address whether impaired myelination in these mice is due to a cell-autonomous role for ZDHHC9 in OLs, a role for ZDHHC9 in neurons, or a combination of these factors. However, the impaired OL morphology and maturation seen in culture conditions after acute *Zdhhc9* loss (**Fig. 7**) supports the first of these possibilities. While a conditional KO mouse could more directly help test the OL-autonomous role of ZDHHC9 in vivo, in this study we focused on conventional KO mice to more accurately mirror the situation in human patients with *ZDHHC9* loss or mutation.

It is perhaps surprising that, despite the clear changes in OL morphogenesis, we did not detect changes in overall numbers of OPCs or OLs in *Zdhhc9* KO mice. However, we cannot exclude the possibility that such changes could be revealed by different methods. In addition, different OL subtypes express different subsets of other marker genes (75) and it remains possible that *Zdhhc9* loss preferentially directs OL maturation towards or away from one or more of these subtypes. Furthermore, we cannot exclude the possibility that assessment of other brain regions might also reveal differences in OPC and/or OL numbers in *Zdhhc9* KO mice. Lastly, we also note that acute *Zdhhc9* loss in cultured OLs causes more striking phenotypes than seen with KO mice *in vivo*. However, it is not uncommon for acute knockdown to cause more dramatic effects than

germline KO, possibly due to longer-term compensatory mechanisms *in vivo* (76, 77). Together, though, our findings suggest that OL and WM abnormalities could significantly contribute to *ZDHHC9*-associated XLID.

We also recognize that although *Zdhhc9* loss affects OL morphology both in vitro and in vivo, the specific nature of these changes differs between the two systems (compare **Fig 4** and **Fig 7**). However, several non-mutually exclusive factors could account for these differences. First, morphology in vivo may well be influenced by the axons and/or other extrinsic components around each OL that are not present in our primary cultures. Second, OL growth in vivo is highly 3-dimensional, whereas growth in culture is largely 2-dimensional – it may be difficult to support formation of spheroids (by definition, a 3-dimensional structure) in the latter situation. Finally, ZDHHC9 is absent in vivo from the beginning of development until the time points examined, whereas in our cultured OL experiments, Zdhhc9 shRNA is virally delivered to OPC cultures at DIV2 and likely acutely affects Zdhhc9 expression predominantly in committed OLs (following the switch to differentiation medium at DIV3). These differences may also affect the ability of other PATs or, potentially, palmitoylation-independent subcellular processes, to compensate for Zdhhc9 loss. Further experiments, perhaps involving longitudinal imaging of the development of fluorescently labeled OLs in the two systems, could provide more insights into how *Zdhhc9* loss affects OL morphological elaboration.

Based on behavioral milestones, patients with *ZDHHC9*-associated XLID have been diagnosed as young as two years of age (41), suggesting that ZDHHC9 is important for higher brain function during early development. Consistent with this notion, we observed dysmyelination (both hyper and hypomyelination) in P30 mice, approximately equivalent to a 3-year-old human (78). These findings suggest that the myelin abnormalities observed in adult *Zdhhc9* KO mice (**Fig. 6**) are due to impaired initial myelination, rather than impaired myelin maintenance, and further support the use of *Zdhhc9* KO mice as a model for human *ZDHHC9*-associated XLID.

Although difficult to prove causation in a global, conventional KO line, the myelin abnormalities in *Zdhhc9* KO mice are also associated with behavioral impairments that have some overlap with human patients with *ZDHHC9*-associated XLID. Most notably, *Zdhhc9* KO mice have seizures, reminiscent of the rolandic epilepsy reported in many human patients with *ZDHHC9* mutation (1, 16). *Zdhhc9* KO mice also have impaired latency to reach a hidden platform in the Morris Water Maze (MWM), a task designed to assess spatial learning (15). However, the KO mice do not differ significantly in reference memory in a subsequent probe trial, suggesting that, despite the increased latency, they do learn the location of the platform as effectively as wt (15). In addition, performance in both the open field and in an elevated plus maze (EPM, in which the KO mice spend significantly more time in the open arms) suggest that *Zdhhc9* KO mice also have decreased levels of anxiety (15). Decreased anxiety is reported in several other mouse models of ID (79–83). Finally, *Zdhhc9* KO mice spend significantly less time on a hanging wire task (15), a phenotype consistent with hypotonia, a phenotype also reported in patients with *ZDHHC9* mutation (2, 3, 40). Like reduced anxiety, hypotonia is seen in several human neurodevelopmental conditions and relevant mouse models (see (15) and references therein for a fuller discussion). While either OL-specific conditional KO of *Zdhhc9* or OL-specific rescue in a KO background would be required to determine causation. However, these findings are consistent with a model in which the impaired myelination in the absence of *Zdhhc9* causes behavioral abnormalities reminiscent not just of *ZDHHC9* mutation in humans, but also in other forms of ID.

Zdhhc9 is also expressed at higher levels than other PATs in OLs (**Fig 1C, E, F**), potentially explaining much of the impact of *Zdhhc9* loss in these cells. However, it is also intriguing that ZDHHC9wt localization in OLs differs markedly from that of other PATs examined, and of three ZDHHC9 XLID mutants (**Fig 2**). These findings suggest that specific subcellular localization of ZDHHC9wt is central to the role of this PAT in OLs and why explain why other PATs, and ZDHHC9 XLID mutant forms cannot compensate for loss of ZDHHC9wt. This notion is further supported by

our findings that the XLID-associated *P150S* and, to a lesser extent, *R96W* mutant forms of ZDHHC9 are still capable of palmitoylating MBP in cotransfected cells. Indeed, despite its reduced catalytic activity in an in vitro assay (42), ZDHHC9-*P150S* does not differ significantly from ZDHHC9wt in its ability to palmitoylate MBP, or to autopalmitoylate, in our cell-based assay. This residual autopalmitoylation, which was also observed in (42), suggests that ZDHHC9-*P150S* might still be capable of palmitoylating MBP and/or other substrates in OLs, if it were to be targeted to the correct subcellular location. Impaired subcellular targeting thus represents an additional possible contributory factor, not mutually exclusive with impaired catalytic activity, to explain impairments in ZDHHC9-assciated XLID. More broadly, these results emphasize the importance of precise subcellular localization in palmitoylation-dependent regulation, a key take home message from prior studies by ourselves and others e.g. (76, 84, 85)

What, then, is the identity of the puncta in OL subcellular processes to which ZDHHC9wt can localize, but which other PATs and ZDHHC9 XLID mutants cannot? Our immunostaining results with subcellular organelle markers suggest that these puncta represent Golgi outposts or satellites, and that ZDHHC9's key partner Golga7 also localizes to this location (**Fig. 2**, Fig. S2)(47, 48). Golgi outposts have long been known in neurons (86), and we previously reported that ZDHHC9 localizes to such structures in primary hippocampal neurons (16). Golgi outposts were also recently described in OLs (46). However, the function of these structures in OLs remains to be fully determined, so the role(s) of ZDHHC9 at this subcellular organelle is challenging to infer. Nonetheless, we speculate that ZDHHC9 could be involved in either or both of the best-described roles of Golgi outposts, protein glycosylation and microtubule nucleation, for the following reasons. First, ZDHHC9-positive Golgi outposts in OL processes are also positive for the enzyme Mannosidase II, suggesting they represent sites at which protein glycosylation is regulated (Fig. S2B). In addition, although ZDHHC9 colocalization with TPPP, a protein implicated in microtubule nucleation, is less apparent (Fig. S2C), knockout/knockdown of either ZDHHC9 (**Figs. 4** and **7**) or TPPP (48) affects branching and complexity of OL processes, a process that involves microtubule nucleation. However, more investigation is needed to determine the functions of Golgi outposts in OLs, in order to define the associated contribution of ZDHHC9.

Another intriguing question is whether and how the loss of ZDHHC9 action at Golgi outposts might contribute to the myelination deficits we observed in *Zdhhc9* KO mice (**Figs. 5** and **6**). Our sparse genetic labeling studies revealed that *Zdhhc9* KO OL processes are distended, with numerous spheroid-like swellings (**Fig. 5**). We reason that these spheroids are enclosed by the OL lipid membrane because if the membrane were ruptured, the EGFP signal would likely diffuse. This in turn suggests that the caliber of the OL process at the position of the spheroid is grossly abnormal i.e. the membrane has hyper-expanded. Given that OL membrane growth during myelination extends in two directions i.e. spiral growth to the axonal surface and longitudinal growth along the axon, it is possible that spheroid-like structures are formed by uneven myelin growth. We recognize that we cannot yet conclude whether and how spheroid formation might relate to the myelination deficit in Zdhhc9 KO mice that we observe by EM (**Figure 6**). However, it is not inconceivable that the hypo- and hypermyelination of *Zdhhc9* KO axons seen in single-plane EM cross-sections (**Fig. 6**) represents this same distension phenotype, observed using a different method. Although beyond our current technical abilities, we speculate that EM-based reconstruction in 3 dimensions would reveal regions of both hypo- and hypermyelination of individual callosal axons in *Zdhhc9* KO mice.

How, though, might *Zdhhc9* loss cause dysmyelination? Several myelin proteins are palmitoylated, and we found that a ZDHHC9/Golga7 complex palmitoylates the major myelin protein MBP and that MBP palmitoylation is reduced in forebrain WM of *Zdhhc9* KO mice (**Fig. 8**). Given that palmitoylation can sort proteins to the myelin membrane (24), the simplest explanation might be that impaired palmitoylation of MBP, and potentially other myelin proteins, in the absence of ZDHHC9 affects myelin structure per se. It would be of considerable interest to investigate whether and how *Zdhhc9* loss alters the distribution of specific MBP splice. However, the anti-MBP

antibody used in this study recognizes all forms of MBP, not just the specific splice variants whose palmitoylation is affected by *Zdhhc9* loss. Specifically assessing nanoscale distribution of these splice variants would require a way (e.g. anti-MBP splice form-specific antibodies that are compatible with immuno-EM) to distinguish these variants from other, non-palmitoylated forms of MBP. Developing such an antibody could greatly facilitate future studies of nanoscale MBP distribution in the presence and absence of ZDHHC9.

We also observed slight but significant reductions in palmitoyl- and total levels of MAG in the absence of ZDHHC9 (Fig S6A, B), which may also contribute to impaired myelination. In contrast, ZDHHC9 loss did not alter palmitoyl- or total levels of Cadm4, a myelin protein whose palmitoylation is ascribed to the Golgi-localized PAT ZDHHC3 (58)(Fig S6C, D). This latter finding suggests that, although ZDHHC9 loss may affect additional myelin proteins, gross, widespread dysregulation of myelin protein levels and/or palmitoylation in the absence of ZDHHC9 is unlikely. Importantly, several mutant forms of ZDHHC9 fail to localize to Golgi outposts in OLs and lead to XLID, even when showing apparently normal catalytic activity. This latter observation is consistent with a model in which ZDHHC9 action at OL Golgi outposts is critical for normal myelination. An additional factor to consider, however, is that the key ZDHHC9 substrate(s) may not be the myelin proteins themselves, but rather other proteins that act at Golgi outposts to direct myelin proteins to the correct region of the myelin membrane and/or to ensure uniform distribution of myelin proteins within that membrane. Another non-mutually exclusive possibility is that ZDHHC9 acts at Golgi outposts but indirectly, for example to drive the expression of myelin protein genes. A comparison of WT and *Zdhhc9* KO palmitoyl-proteomes could help determine which of these possibilities (which are not mutually exclusive) best accounts for impaired myelination in the absence of ZDHHC9.

Interestingly, despite their marked differences in basal catalytic activity (as assessed by autopalmitoylation), wt and all XLID forms of ZDHHC9 appear to show enhanced activity (measured by both auto- and MBP palmitoylation) in the presence of Golga7, suggesting that the association with Golga7 (which also localizes to Golgi outposts) is central to ZDHHC9 activity. This model is also highly consistent with the biased expression of Golga7 in OLs, compared to other CNS cell types (**Fig 1E**, **1F**). Moreover, XLID-associated mutant forms of ZDHHC9 also show reduced protein stability and are impaired in their ability to form complexes with Golga7 (also known as Golgi Complex Protein 16kDa; GCP16)(28). ZDHHC9 XLID mutations may thus affect ZDHHC9 trafficking to Golgi outposts per se but alternatively, or in addition, may decrease formation/stabilization of ZDHHC9/Golga7 complexes at these locations. Either or both of these mechanisms, which are not mutually exclusive, may contribute to impaired MBP palmitoylation and/or myelination deficits caused by *ZDHHC9* mutation.

In summary, we reveal an unexpectedly non-heterogeneous expression of *Zdhhc9* in both mice and humans, with biased expression in mature OLs. Moreover, within OLs, WT ZDHHC9 localizes uniquely compared to other PATs examined, and to XLID-associated forms of ZDHHC9. Although we did not detect changes in overall OL numbers or gross myelin staining, we found that *Zdhhc9* loss greatly impairs OL morphology and myelination at the microscale. These findings provide new insights into mechanisms of *ZDHHC9*-associated XLID and into other conditions marked by WMI.

Materials and Methods

Mice

Zdhhc9 knockout mice (B6;129S5-Zdhhc9tm1Lex/Mmucdm) were originally obtained from Mutant Mouse Resource and Research Center (MMRRC), UC Davis and were previously described (16). Mice were transferred from University of British Columbia to Temple University School of

Medicine for this study. Female heterozygous *Zdhhc9* knockout mice were bred with male wildtype C57BL/6 mice to obtain male *Zdhhc9* hemizygous knockout and male wild-type mice as littermate controls, which were used for experiments in **Figures 6** and **8**. Experiments in **Figs. 3** - **5** used a mixture of male *Zdhhc9* hemizygous KO (*Zdhhc9*$^{y/-}$) and female *Zdhhc9* homozygous KO (*Zdhhc9*$^{-/-}$) mice. *Mobp-EGFP* BAC Tg (26) (generated by GENSAT; MMRRC stock #030483-UCD, *RRID:MMRRC_030483-UCD*), *R26-CAG-LSL-EGFP (RCE)* (80) (MMRRC stock #032037-JAX, *RRID:MMRRC_032037-JAX*) and *Mobp-iCreER* mice (87) were described previously and obtained from Dr. Dwight Bergles (Johns Hopkins University). *Pdgfra-CreER*^{TM} BAC Tg ((88); *RRID:IMSR_JAX:018280*) and mT/mG mice (89) were purchased from the Jackson Laboratory. Mice were housed in a barrier facility with a 12h:12h light: dark cycle, were provided food and water *ad libitum* and were checked daily by ULAR staff. All procedures involving vertebrate animals followed National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of Temple University.

Fluorescence-activated cell sorting (FACS)

After the whole brain was isolated from one-month-old *Mobp-EGFP* mice, one hemisphere of the forebrain was chopped with a blade into small pieces. The brain cells were further dissociated using a Neural Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instruction. After enzymatic digestion, the cells were mechanically dissociated with gentle pipetting and suspended in Hank's Balanced Salt Solution (GIBCO). The cell suspension was passed through a 40-µm cell strainer (Corning). Cells were re-suspended in 0.5% FBS in HBSS and isolated with BD influx (BD Biosciences) at the Flow Core Facility of Temple University School of Medicine. The other hemisphere of each mouse brain was used for total RNA isolation.

RNA Sequencing

Total RNAs were extracted from the FACS-isolated EGFP^{+} OLs with RNeasy Micro kit (Qiagen). For total RNA extraction from the other hemisphere forebrain (without OL sorting), we homogenized the brains with Trizol reagent (Invitrogen). All RNA samples were subjected to Quality Control with Bioanalyzer (Agilent), and the RNA samples whose RIN was higher than 8 were used for subsequent applications. Ten ng of total RNA was used in the Ovation RNA-Seq System v2 (NuGEN) to prepare cDNA library. Following manufacturer's instructions, total RNA and primer were incubated at 65 °C for 5 min, followed by first-strand cDNA synthesis (4 °C for 1 min, 25 °C for 10 min, 42 °C for 10 min, 70 °C for 15 min, and then cooling to 4 °C) and second strand cDNA synthesis (4 °C for 1 min, 25 °C for 10 min, 50 °C for 30 min, 80 °C for 20 min and then cooling to 4 °C). RNAClean XP bead purification was performed. Single Primer Isothermal Amplification (SPIA) was used to amplify cDNAs. QIAquick cleanups (Qiagen) were eluted in 30 µl low EDTA TE buffer and quantified via Nanodrop 1000. 100 ng amplified SPIA cDNA was sonicated using a Covaris E210 (50 µl, duty cycle 10%, intensity 5, 200 burst/sec, 45 sec) to shear samples to 350bp. The libraries were prepared using the TruSeq DNA LT Sample Prep Kit (Illumina) according to manufacturer's instructions. Samples were purified using sample purification beads. End repair reaction (at 30 °C for 30 min) was followed by bead purification and size selection for 350bp insert. dA-tailing (37 °C for 30 min, 70 °C for 5 min, 4 °C for 5 min) and adapter ligation with barcoded adapters were performed (30 °C for 10 min), followed by bead purification and PCR amplification (95 °C for 3 min; 8 cycles of 98 °C for 20 sec, 60 °C for 15 sec and 72 °C for 30 sec; 72 °C for 5 min). A final bead purification was performed, and libraries were quantified using the Agilent Bioanalyzer High Sensitivity DNA assay. For the forebrain total RNA library (all cell RNA-seq), 500 ng of RNA was used for library generation with TruSeq Stranded mRNA Library Prep Kit (Illumina). Sequencing was performed using an Illumina HiSeq 2000.

RNA-Seq Data Analyses

RNA-seq reads were aligned to a mouse reference genome (mm10) by the STAR alignment tool (v2.4.0) with default option and PCR duplicate reads were removed using the Picard Tools (v1.124). To quantify expression levels for each gene, we counted the number of aligned fragments for each gene using HTSeq-0.6.1 with parameters (-s no, -r pos, -f bam, -m intersection-nonempty and -t exon) according to the Ensembl mouse transcript annotation (GRCm38.74 version) and calculated the FPKM (Fragments Per Kilobase per Million mapped reads) values of each gene. For heatmap visualization, the FPKM values for each gene were color-coded with the Microsoft Excel color scales.

Accession Codes

The RNA-seq files have been uploaded to the European Nucleotide Archive (ENA) under accession code PRJEB19341.

Tamoxifen Administration

Cre activity was induced with tamoxifen (Sigma-Aldrich, Cat# T5648) administration to *Pdgfra-CreER* mice. Tamoxifen was dissolved (20 mg/ml) in a mixture of sunflower seed oil-ethanol (10:1), and then ethanol was evaporated in a vacuum concentrator for 30 min. Forty mg/kg (b.w.) of tamoxifen was intraperitoneally (i.p.) injected twice daily with at least a 6 hour interval between injections. A total of 8 doses of tamoxifen was injected into the *Pdgfra-CreER mice; RCE;* ± *Zdhhc9 KO* mice between P21 and P24.

Rat Primary OPC Culture and Differentiation

Primary mixed glial cultures were prepared from P1 rat pups, as previously described (90). Briefly, cortices were isolated and digested with papain and DNase I, followed by mechanical dissociation. Cells were resuspended in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin. Cells were plated in T75 flasks coated with poly-D-lysine and medium was replaced every other day. Under these conditions, a mixed population of glial cells survives and proliferates, but neuronal cells do not survive. After 7~10 days, these mixed glial cultures were shaken overnight (14~16 hours). Detached cells were added to uncoated petri dishes, to which microglia preferentially adhere but OPCs do not. The OPC-enriched supernatant was then plated on poly-D-lysine coated coverslips. OPCs were maintained in defined OPC media containing PDGF (10 ng/ml) and bFGF (5 ng/ml). The following day, fresh OPC medium was added, and the medium was refreshed every other day. To induce OPC differentiation, OPC medium was replaced by defined OL medium containing triiodothyronine (T3, 30 ng/ml and T4, 40 ng/ml), CNTF (10 ng/ml) and NT3 (1 ng/ml).

Molecular Biology

Wildtype mouse *Zdhhc3*, *Zdhhc7*, *Zdhhc9* and *Zdhhc17* cDNAs (all with N-terminal HA tag) were a kind gift from Dr. Masaki Fukata (National Institute of Physiological Sciences, Okazaki, Japan) and were subcloned into lentiviral expression vector (FEW) downstream of the EF1α promoter and an N-terminal HA tag, as described (37). XLID mutant forms of *Zdhhc9* (human point mutations introduced into mouse cDNA) were generated as *BsrGI – AfeI* gBlock fragments (Integrated DNA Technologies) and were used to replace the wildtype *Zdhhc9 BsrGI – AfeI* fragment *via* standard subcloning. Mouse *Golga7* and MBP (21.5kDa isoform) cDNAs were synthesized as *XhoI-NotI* gBlock fragments and subcloned into a modified FEW vector downstream of an N-terminal myc epitope tag. Mouse *Tppp* cDNA was synthesized (Genewiz) and subcloned into modified FEW vector upstream of a C-terminal Flag tag. C-terminally GFP-tagged Mannosidase cDNA was obtained from Addgene (plasmid #160905) and used without additional subcloning. Membrane-

bound GFP (mGFP) cDNA was generated by appending the N-terminal 40 amino acids of MARCKs to eGFP (91) as a *PspXI – HindIII* fragment, which was then subcloned into FEW vector with no additional tag.

Lentiviral Vectors and Lentivirus Preparation

VSV-G pseudotyped lentiviruses were prepared as described (56), except that viruses were collected in DMEM containing 0.1% (v/v) FBS and were added to cultured cells without ultracentrifugation.

Lentiviral Infection and OL Differentiation

For *Zdhhc9* knockdown, OPCs were infected at two days *in vitro* (DIV2) with lentiviral vectors carrying *Zdhhc9* shRNA or a control scrambled shRNA (16). OPC media was refreshed at 4 hours and one day post-infection, and then the medium was replaced with defined OL medium at one day post-infection for differentiation. The OL culture medium was then refreshed every other day. Infected OLs were fixed at 9 DIV ('OL 6 days') for immunostaining.

Transfection of Cultured OPCs

To define the subcellular localization of ZDHHC PATs and other proteins in OLs, differentiated OLs at 6 DIV ('OL 3 days') were transfected with plasmids using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific) according to the manufacturer's recommendations. Briefly, plasmid DNA (diluted in Opti-MEM, GIBCO, Thermo Fisher Scientific, Waltham, MA) was mixed with 1.5ul of PLUS™ Reagent and incubated for 5 min at room temperature (RT). 1ul of Lipofectamine® LTX (diluted in opti-MEM) was added to pre-incubated DNA and incubated for 30 min at RT for generation of DNA-lipid complex. This DNA-lipid complex was applied to the cells for transfection, and OLs were fixed 9 hours later and processed for immunostaining.

Immunocytochemistry (ICC)

Coverslips containing OL cells were rinsed with 1x Recording buffer (25mM HEPES pH7.4, 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 30mM Glucose) and cells were fixed for 20 min in PBS containing 4% paraformaldehyde (PFA)/ 4% sucrose. Coverslips were washed 3X 10 min in phosphate buffered saline (PBS) and blocked for 1 hr at room temperature with 10% (v/v) normal goat serum (NGS; Thermo Fisher Scientific, Waltham, MA) in PBST containing 0.15% Triton X-100. Cells were then incubated in blocking solution with primary antibodies overnight at 4°C, washed 3X 10 min in PBS, incubated in secondary antibodies in blocking solution for 1 hour at RT, washed 3X 10 min in PBS, and mounted on microscope slides using FluorSave reagent (Millipore Sigma). Confocal images were captured with a laser scanning confocal microscope (Leica TCS SP8) and LAS X software.

Quantification of ICC images

To quantify distribution of HA-tagged PATs in OL processes (**Fig. 2**), confocal images of HA (PAT) and GFP (cell fill) signal were thresholded. The same threshold values for each channel were used across all images. Using the Sync Windows tool in ImageJ/Fiji, the cell body area was traced in the GFP channel image and deleted from both images. Both images were then inverted and the ADD function was used to generate an image of the HA+/GFP+ signal. Using the Analyze particles tool (with settings 0-infinity pixels sq and 0.00-1.00 circularity), the combined area occupied by the HA+/GFP+ puncta was calculated and normalized to the total extra-somatic GFP+ area from the GFP+ image of the same cell.

The percentage of GFP^+/MBP^+ cells (**Fig. 7B**) was counted manually without thresholding, using the same criteria for all conditions. Sholl analysis in **Fig 7E, F** was performed by manually reconstructing the morphology of individual OLs in ImageJ/Fiji as a binarized image. OL nuclei

were considered the center, and concentric circles were drawn with an interval of 10 μm and the number of intersections calculated using the ImageJ/Fiji Sholl Analysis plug-in.

The percentage of morphologically immature OLs (Fig. S5) was calculated manually based on identification of GFP^+/CNP^+ cells (committed OLs) that did not show the 'pancake'-like morphology of mature OLs and plotted as a percentage of all GFP^+/CNP^+ cells per field. Morphologically immature OLs also typically lacked MBP staining (as in **Fig 7B**) but this property was not used in the Fig S5 analysis.

Immunofluorescence of Brain Sections

Mice were anesthetized with pentobarbital sodium (70 mg/kg, i.p) and transcardially perfused with PBS followed by 4% PFA. Mouse brains were post-fixed overnight at 4°C and transferred to 30% sucrose in PBS at 4°C. Brain tissues were frozen in Tissue-Tek optimum cutting temperature (O.C.T.) compound (Sakura, Cat# 4586) and sectioned using a Leica Biosystems CM1950 Cryostat. Three different thicknesses of brain or spinal cord sections were used: 20 μm for MOBP-EGFP-based or ASPA-staining dependent OL quantification, 35 μm for OPC fate analysis, and 50 μm for mEGFP-based OL morphological analysis. Brain and spinal cord sections were stained in a free-floating manner. Sections were permeabilized with 0.3% (w/v) Triton X-100 and blocked with blocking solution (5% (v/v) normal donkey serum, 0.3% (w/v) Triton X-100) for 1 hour at RT. Sections were then incubated in blocking solution containing primary antibodies at 4°C overnight. On the next day, sections were rinsed with PBS 3X 5 min and incubated with secondary antibodies and DAPI (1:1,000) in blocking solution at RT for 2 hours. Sections were rinsed in PBS 3X 5 min and then mounted onto slide glasses with a mounting medium with ProLong antifade (Thermo Fisher Scientific, P36970). Image acquisition of the immunostained brain sections was performed with a wide-field fluorescent microscope AxioImager M2 (Zeiss) or a laser scanning confocal microscope TCS SP8 (Leica).

Morphological Analysis of Oligodendrocytes

The settings for OL tracing were kept the same for all the samples across genotypes. Confocal images were captured for sparsely labeled randomly chosen $EGFP^+$ OLs from the CTX (layers IV–VI). Images of labelled cells were imported into Fiji and traced without z-projecting the stacks. Sholl analysis was performed using Fiji plug-in. For Sholl analysis of OLs in vivo, the OL nuclei were considered the center, and concentric circles were drawn with an interval of 5 μm. Imaris 9.9 (Oxford Instrument) software was used for 3D surface rendering of OLs with representative confocal images.

Electron Microscopy and *g*-ratio Analysis

Mice were anesthetized with pentobarbital sodium (100 mg/kg, i.p) and transcardially perfused with PBS followed by 2.5% PFA, 2% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4). Mouse brains were post-fixed overnight at 4°C and transferred to 0.1 M phosphate buffer at 4°C. Brains were dehydrated using a graded ethanol series and embedded into Embed-812 (EMS). Thin sections were prepared, stained with uranyl acetate and lead citrate and visualized with an electron microscope (JEOL 1010 electron microscope fitted with a Hamamatsu digital camera) at the University of Pennsylvania Electron Microscopy Resource Laboratory. 15,000X magnified images and Fiji Plug-in and ImageJ/Fiji (92) were used for the g-ratio analysis of myelin. To analyze potentially hypermyelinated small axons in P56 mice, g-ratio was calculated using the ratio of inner-to-outer diameter (distance values derived from pixel intensity along plot profile generated in ImageJ/Fiji) of each myelinated axon. To analyze myelination of all axons in P30 mice, inner and outer diameters of axons were traced manually in ImageJ/Fiji and the resultant diameters calculated.

Acyl Biotinyl Exchange Assay

HEK cells were transfected using a calcium phosphate-based method as described (56). Cells were lysed 8h post-transfection and ABE assays performed as in (56). For WM tissue collection from *Zdhhc9* knockout and littermate control mice, mice were anesthetized with pentobarbital sodium (100 mg/kg, i.p). Brains were isolated and briefly rinsed with ice-cold HBSS buffer (no calcium, no magnesium). Using a Mouse Brain Slicer, 1mm block coronal brain sections were prepared on ice and WM-enriched tissues including corpus callosum and striatum were dissected from slices under a dissection microscope. Dissected WM tissue was homogenized in a glass-teflon dounce homogenizer (20 strokes, 200 rpm) in 4mM HEPES, 0.32M sucrose, containing freshly diluted Protease Inhibitor Cocktail (PIC; Roche). Homogenized samples were transferred to a fresh tube, rapidly brought to room temperature, and solubilized by addition of 1/10 volume of 10% (w/v) SDS. Samples were centrifuged for 10 minutes at 4°C at 13,000 rpm to pellet insoluble material and protein concentrations in supernatants determined by BCA assay (Pierce). Protein concentrations were normalized by addition of homogenization buffer containing SDS and PIC and were used for ABE assay as described (56).

Quantification and Statistical Analysis

All data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). In all graphs the mean is shown, and error bars indicate standard error of the mean (SEM). For the quantitative comparisons of OL lineage cells, OPC fate analysis, and OL process complexity, two-way ANOVA and Šidák's multiple comparison test were used. For spheroid structure comparison, unpaired student's t-test was used.

Antibodies

The following antibodies, from the indicated sources, were used for ICC

Anti-HA-Tag (Rb IgG), Cell Signaling technology #3724 (C29F4), 1:100

anti-HA.11 Epitope Tag (mouse IgG1), Covance HA.11 #MMS-101P, 1:100

GFP (Rb IgG), Invitrogen #A11122, 1:1000

GFP (mouse IgG2a), Invitrogen #A11120 (3E6), 1:500

Myc (mouse IgG1), Cell signaling #92013(E7F9B), 1:200

MBP (chicken IgG), Aves Lab #MBP, 1:1000

CNP (Rb IgG), Phosphosolutions #325-CNP, 1:1000

Secondary antibodies for ICC were Alexa Fluor 488-, 568- or 647-conjugated goat IgG against mouse IgG1 or IgG2a, rabbit or chicken (Thermofisher Scientific, 1:500).

The following antibodies, from the indicated sources, were used for Immunofluorescence

anti-APC (mouse IgG2b) EMD Millipore #OP80 (CC1), 1:50

anti-ASPA (Rb IgG) GeneTex #GTX113389, 1:500

anti-GFP (goat IgG) Rockland, #600-101-215, 1:500

anti-MBP (Rb IgG) Cell Signaling Technology #78896 (D8X4Q), 1:500

anti-NG2 (guinea pig IgG) gift from Dr. Bergles, Johns Hopkins University, 1:4000

Olig2 (goat IgG) R&D #AF2418, 1:500.

Secondary antibodies for immunofluorescence were Alexa Fluor 488-, Cy3-, Cy5-, or Alexa Fluor 647-conjugated donkey IgG against goat, rabbit, or guinea pig (Jackson ImmunoResearch, 1:500).

The following primary antibodies, from the indicated sources, were used for western blotting:

anti-HA.11 Epitope Tag (mouse IgG1), Covance HA.11 #MMS-101P, 1:5000

anti-MAG (Rb IgG) Cell Signaling Technology #9043, 1:500

anti-MBP (Rb IgG) Cell Signaling Technology #78896 (D8X4Q), 1:500

anti-Myc (rabbit) Cell Signaling Technology #2278 (71D10), 1:500

anti-Cadm4 / SynCAM4 (mouse) Antibodies Inc.#75-247 1:100

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Additional files

Supplementary Material

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Author information

Hey-Kyeong Jeong*
Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States

*Equal contribution

Estibaliz Gonzalez-Fernandez*
Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States

*Equal contribution

Ilan Crawley
Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States

Julia M Coakley
Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States

Jinha Hwang
Department of Biomedical Science, Seoul National University College of Medicine, Seoul, Republic of Korea

Dale DO Martin
Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States, Department of Biology, University of Waterloo, Waterloo, Canada
ORCID iD: 0000-0002-2407-1597

Shernaz X Bamji
Department of Cellular and Physiological Sciences, Life Sciences Institute and Djavad Mowafaghian Centre for Brain Health, University of British Columbia, Vancouver, Canada

Jong-Il Kim
Department of Biomedical Science, Seoul National University College of Medicine, Seoul, Republic of Korea
ORCID iD: 0000-0002-7240-3744

Shin H Kang
Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States, Department of Neural Sciences, Lewis Katz School of

Medicine at Temple University, Philadelphia, United States

For correspondence: shin.kang@temple.edu

Gareth M Thomas
Shriners Hospital Pediatric Research Center, Lewis Katz School of Medicine at Temple University, Philadelphia, United States, Department of Neural Sciences, Lewis Katz School of Medicine at Temple University, Philadelphia, United States
ORCID iD: 0000-0003-3183-8431

For correspondence: gareth.thomas@temple.edu

Editors

Reviewing Editor
Klaus-Armin Nave
Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany

Senior Editor
John Huguenard
Stanford University School of Medicine, Stanford, United States of America

Reviewer #1 (Public review):

Summary:
Having shown that acyltransferase ZDHHC9 expression is far higher in myelinating oligodendrocytes (OLs) than in other CNS cell types, Jeong and colleagues focus on exploring the role of ZDHHC9 in myelinating OLs in particular in the palmitoylation of several myelin proteins. This study is relevant in the context of X-linked intellectual disability as it suggests a more relevant role for myelinating glia than previously thought. It also provides useful insights the mechanisms of ZDHHC9-associated XLID and on the palmitoylation-dependent control of myelination.

Strengths:
Well written paper
In general good data quality
Use of transgenics strategies (in addition to the ZDHHC9 KO) strengthen the data and claims

Weaknesses:
A few claims might have needed better experimental support but new data and revised discussion sections addressed some of these weaknesses

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Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *In this work Jeong and colleagues focus on exploring the role of the acyltransferase ZDHHC9 in myelinating OLs in particular in the palmitoylation of several myelin proteins.*

> *After confirming the specific enrichment of the Zdhhc9 transcript in mouse and human OLs, the authors examine the subcellular localization of the protein in vitro and observed that in comparison with other isoforms, ZDHHC9 localizes at OLs cell bodies and at discrete puncta in the processes. These observations (Figures 1 and 2) led the authors to hypothesize that ZDHHC9 plays an important role in myelination. No gross changes were detected in OL development in Zdhhc9 KO mice and analyses from P28 Zdhhc9 KO mice crossed with Mobp-EGFP reporter mice did not show changes in EGFP+ OL differentiation (Figure 3).*
>
> *However, and given the observed subcellular localization of ZDHHC9 in OL processes (Figure 2) and the observation that the percentage of unmyelinated axons is increased in Zdhhc9 KO (Figure 6), early time points to examine the differentiated pools of OLs and their capacity to extend processes/contact axons need to be considered.*

We appreciate this point, but due to the order in which experiments were performed, the ZDHHC9 KO mouse colony that we maintained after initial submission of this work contains homozygous MOBP-EGFP, but not the mT/mG transgene that would be most optimal for the proposed experiment. We hope the reviewer appreciates that it would take considerable time and effort regarding mouse breeding to cross out the MOBP and add back the mT/mG. We nonetheless appreciate the importance of the point raised and therefore examined an earlier developmental time point (P21, 3 weeks) to quantify OLs and NG2+ OPCs. In our updated Fig 3C1-C3, we use Mobp-EGFP mice to show that Zdhhc9 KO does not significantly affect the number of EGFP+ OLs at this time point in the cortex, corpus callosum and spinal cord. We also show that in corpus callosum, Zdhhc9 KO does not significantly affect the number of NG2+ OPCs at this earlier time point (Fig 3D, E). Furthermore, immunostaining to detect BCAS1, a marker of pre-mature OLs, also revealed no qualitative difference with ZDHHC9 loss at P21. We show representative images from these BCAS1 experiments in an updated Fig S3. While these new experiments do not address the morphology of OLs in Zdhhc9 KO, they do provide further evidence that deficits in myelination in young Zdhhc9 KO mice (Figure 6) are not likely due to gross differences in OPC or OL numbers during development.

> *Maturation of OL in Zdhhc9 KO was examined by crossing Zdhhc9 KO with Pdgfra-CreER;R26- EGFP and following the newly EGFP-labelled OPCs following tamoxifen administration. No changes in the numbers of EGFP+ OL were detected. The authors concluded that the loss of ZDHHC9 does not alter oligodendrogenesis in either the young or mature CNS. The authors observed defects in Zdhhc9 KO OL protrusions that they attributed to abnormal OL membrane expansion (Fig 4 and 5). Can they show evidence for this?*

This is an important point, and we appreciate the opportunity to explain the reasoning behind our initial statement more fully, while noting that other explanations are possible. Fig 5B (an Imaris-assisted reconstruction using the EGFP cell fill/morphology marker) highlights large spheroid-like distensions along OL processes. We reason that these spheroids are enclosed by the OL lipid membrane because if the membrane were ruptured, the EGFP signal would likely diffuse. This in turn suggests that the caliber of the OL process at the position of the spheroid is grossly abnormal i.e. the membrane has hyper-expanded. Given that OL membrane growth during myelination extends in two directions, i.e., spiral growth to the axonal surface and longitudinal growth along the axon, it is possible that spheroid-like structures are formed by uneven myelin growth. We recognize that we cannot yet conclude whether and how spheroid formation might be linked to the myelination deficit that we observe in Zdhhc9 KO mice. However, defining the subcellular mechanism for spheroid formation may provide further insights into this issue. We have therefore largely retained the original statement but have added the reasoning above to our revised Discussion.

> *The authors report that Zdhhc9 KO primary and secondary branches in OL were longer, some contained spheroid-like swellings and the OL protrusion complexity was higher. However, these data is partially contradictory to what they show in OL differentiation experiments in vitro (Fig 7). There is also no evidence for increased membrane expansion in Zdhhc9 knockdown myelin forming cells in culture. How to reconcile this?*

We appreciate the reviewer's interest in this issue. Several non-mutually exclusive factors could account for the differences in OL morphology in vitro versus in vivo caused by Zdhhc9 loss. First, morphology in vivo may well be influenced by the axons and/or other extrinsic components around each OL that are not present in our primary cultures. Second, OL growth in vivo is highly 3-dimensional, whereas growth in culture is largely 2-dimensional – it may be difficult to support formation of spheroids (by definition, a 3-dimensional structure) in the latter situation. Finally, Zdhhc9 is absent in vivo from the beginning of development until the time points examined, whereas in our cultured OL experiments, Zdhhc9 shRNA is virally delivered to OPC cultures at DIV2 and likely acutely affects Zdhhc9 expression predominantly in committed OLs (following the switch to differentiation medium at DIV3). These differences may also affect the ability of other PATs or, potentially, palmitoylation-independent subcellular processes, to compensate for Zdhhc9 loss. We have more fully explained these points in our revised Discussion.

> ***Reviewer #2 (Public Review):***
>
> *This study provides an in-depth exploration of the impact of X-linked ZDHHC9 gene mutations on cognitive deficits and epilepsy, with a particular focus on the expression and function of ZDHHC9 in myelin-forming oligodendrocytes (OLs). These findings offer crucial insights into understanding ZDHHC9-related X-linked intellectual disability (XLID) and shed light on the regulatory mechanisms of palmitoylation in myelination. The experimental design and analysis of results are convincing, providing a valuable reference for further research in this field. However, upon careful review, I believe the article still needs further improvement and supplementation in the following aspects:*
>
> *(1) Regarding the subcellular localization experiment of ZDHHC9 mutants in OL, it is currently limited to in vitro cultured OL, lacking validation in vivo OL or myelin sheath. Additionally, it is necessary to investigate whether the abnormal subcellular localization of ZDHHC9 mutants affects their enzyme activity and palmitoylation modification of substrate proteins.*

This is an important point but is technically challenging to address in vivo as it would likely require delivery of AAV to express ZDHHC9wt and XLID mutants specifically in OLs, preferably in the absence of endogenous ZDHHC9. We hope the reviewers would agree that this experiment is beyond the scope of the current study. However, we did compare the ability of ZDHHC9wt and XLID mutants to palmitoylate MBP, and to autopalmitoylate (sometimes used as a surrogate measure of PAT activity) in transfected heterologous cells. Although we recognize that this over-expression system is less physiological than a native OL, it has the benefit of being able to readily compare transfected wt vs mutant forms of ZDHHC9 with minimal contribution from endogenous ZDHHC9. Intriguingly, using this system, we found that autopalmitoylation activity of the XLID ZDHHC9-P150S mutant does not differ significantly from that of ZDHHC9wt, and that this mutant is still capable of palmitoylating MBP. Moreover, the R96W mutant, while impaired in autopalmitoylation, still palmitoylated MBP approximately 50% as effectively as ZDHHC9wt in our cell-based assay. These findings suggest that ZDHHC9-P150S and, probably, ZDHHC9-R96W mutants might still be able to palmitoylate substrates in OLs if they were properly localized. This possibility in turn suggests that impaired subcellular targeting in addition to, or instead of, impaired catalytic activity, may be a key factor in certain cases of ZDHHC9-associated XLID. We have expanded

our Figure 8 (new panels 8E-G) to show these additional experiments and have summarized the conclusions above in our revised Discussion. We thank the reviewer for suggesting that we further investigate this issue.

> *(2) The experimental period (P21+21 days) using genetic labeling to track the development of myelinating cells may not be long enough. It is recommended to extend the observation time and analyze at more time points to more comprehensively reflect the impact of Zdhhc9 KO.*

We appreciate this point from the reviewer but, regrettably, we did not maintain the PdgfraCreER; R26-EGFP; Zdhhc9 KO mouse line and hope the reviewer appreciates that it would take considerable time and effort to rederive this line and then perform the suggested extended time course experiments. However, we note for the reviewer that our preliminary studies did not reveal any effect of Zdhhc9 KO on the number of MOBP-EGFP+ OLs in 6-month-old mice (not shown), consistent with a model in which Zdhhc9 loss does not affect OPC-OL commitment per se.

> *(3) The author speculates that Zdhhc9 may regulate myelination by affecting the membrane localization of specific myelin proteins, but lacks direct experimental evidence to support this. It is suggested to detect the expression and distribution of relevant proteins in the myelin of Zdhhc9 KO mice.*

We share the reviewer's interest in this point but realized that it is more technically challenging to address than might be initially thought. The main protein we would implicate and seek to test is MBP, but we already found that there is no gross change in MBP distribution in vivo in Zdhhc9 KO mice (Fig 3A). However, an anti-MBP antibody recognizes all forms of MBP, not just the specific splice variants whose palmitoylation is affected by ZDHHC9 loss. Specifically assessing nanoscale distribution of these splice variants would require a way (e.g. anti-MBP splice form-specific antibodies that are compatible with immuno-EM) to distinguish these variants from other, non-palmitoylated forms of MBP. Although such an antibody could be an important tool, we hope the reviewers would agree that developing and characterizing such a reagent is beyond the scope of the current study.

We do, however, note that the lack of gross change in MBP distribution and levels in Zdhhc9 KO mice is consistent with the relatively mild phenotype of these mice, compared with shiverer (shi/shi) mice, in which MBP is completely lost. In shiverer, CNS compact myelin is almost absent (PMID: 671037; PMID: 88695; PMID: 460693) and, as the name suggests, mice display a shivering gait, and exhibit seizures and early death. In contrast, Zdhhc9 mice show only subtle behavioral deficits (PMID: 29944857). These differences are all consistent with a model in which Zdhhc9 KO mice, despite their significantly reduced MBP palmitoylation (Fig 8) have grossly normal distribution and levels of MBP when all splice variants are assessed (Fig 3, Fig 8). It is not inconceivable that Zdhhc9 KO mice have a nanoscale change in the distribution of MBP, particularly of specific palmitoylated splice variants, within myelin that profoundly affects myelin ultrastructure, without grossly altering MBP distribution. However, an alternative and not mutually exclusive possibility is that aberrant palmitoylation of other Zdhhc9 substrates accounts for, or contributes to, the abnormalities in myelin at the ultrastructural level. Addressing this issue would require a multi-pronged approach, not just to assess palmitoylation and distribution of such proteins in Zdhhc9 KO, but also to test whether they are direct Zdhhc9 substrates, in order to rule out indirect effects. We hope reviewers would agree that this is best left to a separate study. However, in our revised Discussion we now summarize what can be inferred regarding Zdhhc9-dependent effects on total and splicevariant specific distribution and levels of MBP.

> *(4) Although the article mentions the association of Zdhhc9 with intellectual disabilities, it does not involve behavioral analysis of Zdhhc9 KO mice. It is recommended to supplement some behavioral experimental data to support the important role of Zdhhc9 in maintaining normal cognitive function, enhancing the clinical relevance of the article.*

We appreciate this point from the reviewer. The behavior of the same ZDHHC9 KO mouse line that we used was reported in PMID: 31747610 and in PMID: 29944857. In the former study, Zdhhc9 KO mice were reported to display seizures reminiscent of phenotypes in human patients with ZDHHC9 mutation. The latter study assessed performance of Zddhc9 KO mice in several tasks that test cognitive function. Specifically the KO mice were reported to display "altered behaviour in the open-field test, elevated plus maze and acoustic startle test that is consistent with a reduced anxiety level; a reduced hang time in the hanging wire test that suggests underlying hypotonia but which may also be linked to reduced anxiety [and] deficits in the Morris water maze test of hippocampal-dependent spatial learning and memory.". We have incorporate these findings in our revised Discussion, where we summarize how these phenotypes are common, not just to human patients with ZDHHC9 mutation, but also to other human neurodevelopmental conditions and mouse models in which ID is a common feature.

> *(5) For the abnormal myelination observed in Zdhhc9 KO mice, including unmyelinated large-diameter axons and excessively myelinated small-diameter axons, the article lacks indepth research and explanation on the exact mechanism and mode of action of ZDHHC9 in regulating myelination.*

We share the reviewer's interest in this point but again note that gaining definitive insights into this issue is far from trivial. Convincing evidence of a causative mechanism would require an exhaustive identification of ZDHHC9 in vivo substrates, followed by point mutation of substrate palmitoylation site(s) to determine the extent to which palmitoylation of such protein(s) phenocopies ZDHHC9 loss. Nonetheless, it is possible to break this question down and to summarize what we do and do not know. For example, our experiments in cultured OLs show that ZDHHC9 loss causes call-autonomous deficits in morphological maturation of these cells. We also know that ZDHHC9 loss results in impaired palmitoylation of MBP, a direct substrate for ZDHHC9. Moreover, loss of ZDHHC9 at Golgi outposts in OLs (a phenotype observed with several XLID-associated mutant forms of ZDHHC9, even those with no significant loss of catalytic activity) correlates with intellectual disability. Together, these findings are consistent with a model in which ZDHHC9 action at OL Golgi outposts is critical for normal myelination. However, it is yet to be determined whether the key substrates of ZDHHC9 include MBP, other palmitoyl-proteins that are key constituents of CNS myelin, or proteins whose palmitoylation is important for myelin protein trafficking and targeting. Another non-mutually exclusive possibility is that ZDHHC9 acts at Golgi outposts but indirectly, for example to drive the expression of myelin protein genes. Future experiments, including but not limited to palmitoyl-proteomics in ZDHHC9 (OL-specific) KO mice, will be needed to provide more definitive insights into this issue. We have expanded our Discussion of links between ZDHHC9 mutation and impaired myelination to summarize the above points.

> *(6) The function of ZDHHC9 in OL may be related to the Golgi apparatus, but its exact role in these structures is still unclear. It is suggested to discuss in more detail the role of ZDHHC9 in the Golgi apparatus in the discussion section.*

We appreciate this point, which we considered as related to point (5) above. In our revised Discussion we highlight how ZDHHC9 action at Golgi outposts may involve direct palmitoylation of myelin proteins, palmitoylation of proteins that direct myelin proteins to

the myelin membrane and/or activation of gene expression programs that serve to drive myelination. We further note that these possibilities are not mutually exclusive.

> *(7) More experimental support and in-depth research are needed on the detailed mechanism of how ZDHHC9 and Golga7 cooperatively regulate MBP palmitoylation, and how this decrease in palmitoylation level leads to myelination defects.*

This is another important point – our new experiments suggest that, although some XLID mutations markedly affect ZDHHC9's ability to palmitoylate MBP, others do not, yet all of the mutant forms fail to localize to Golgi outposts. These findings are consistent with a model in which the subcellular location at which ZDHHC9 palmitoylates MBP, and potentially other substrates, is critical for normal myelination. Interestingly, despite their marked differences in basal catalytic activity (as assessed by autopalmitoylation), wt and all XLID forms of ZDHHC9 appear to show enhanced activity (measured by both auto- and MBP palmitoylation) in the presence of ZDHHC9, suggesting that the association with Golga7 (which also localizes to Golgi outposts) is central to ZDHHC9 activity. This model is also highly consistent with the biased expression of Golga7 in OLs, compared to other CNS cell types (Fig 1E, 1F). Moreover, XLID-associated mutant forms of ZDHHC9 also show reduced protein stability and are impaired in their ability to form complexes with Golga7 (also known as Golgi Complex Protein 16kDa; GCP16; PMID: 37035671). Failure of ZDHHC9 XLID mutants to localize to Golgi outposts may thus be due to aberrant trafficking of mutant ZDHHC9 per se, but may also involve impaired association/stabilization of ZDHHC9/Golga7 complexes at these locations. Again, it is possible that either or both of these mechanisms, which are not mutually exclusive, contribute to impaired MBP palmitoylation and/or myelination deficits. We summarize these points in our revised Discussion.

> *In summary, it is recommended that the authors address the above issues through additional experiments and improved discussions to further strengthen the credibility and clinical relevance of the article.*
>
> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *No gross changes were detected in OL development in Zdhhc9 KO mice and analyses from P28 Zdhhc9 KO mice crossed with Mobp-EGFP reporter mice did not show changes in EGFP+ OL differentiation (Figure 3). However, and given the observed subcellular localization of ZDHHC9 in OL processes (Figure 2) and the observation that the percentage of unmyelinated axons is increased in Zdhhc9 KO (Figure 6), ***early time points to examine the differentiated pools of OLs and their capacity to extend processes/contact axons need to be considered***.*

We appreciate this point, but due to the order in which experiments were performed, the ZDHHC9 KO mouse colony that we maintained after initial submission of this work contains homozygous MOBP-EGFP, but not the mT/mG transgene that would be most optimal for the proposed experiment. We hope the reviewer appreciates that it would take considerable time and effort regarding mouse breeding to cross out the MOBP and add back the mT/mG. We nonetheless appreciate the importance of the point raised and therefore examined an earlier developmental time point (P21, 3 weeks) to quantify OLs and NG2+ OPCs. In our updated Fig 3C1-C3, we use Mobp-EGFP mice to show that Zdhhc9 KO does not significantly affect the number of EGFP+ OLs at this time point in the cortex, corpus callosum and spinal cord. We also show that in corpus callosum, Zdhhc9 KO does not significantly affect the number of NG2+ OPCs at this earlier time point (Fig 3D, E). Furthermore, immunostaining to detect BCAS1, a marker of pre-mature OLs, also revealed no qualitative difference with ZDHHC9 loss at P21. We show representative images from these BCAS1 experiments in an updated Fig S3.

While these new experiments do not address the morphology of OLs in Zdhhc9 KO, they do provide further evidence that deficits in myelination in young Zdhhc9 KO mice (Figure 6) are not likely due to gross differences in OPC or OL numbers during development.

> *The authors observed defects in Zdhhc9 KO OL protrusions that they attributed to abnormal OL membrane expansion (Fig 4 and 5). Can they show evidence for this?*

This is an important point, and we appreciate the opportunity to explain the reasoning behind our initial statement more fully, while noting that other explanations are possible. Fig 5B (an Imaris-assisted reconstruction using the EGFP cell fill/morphology marker) highlights large spheroid-like distensions along OL processes. We reason that these spheroids are enclosed by the OL lipid membrane because if the membrane were ruptured, the EGFP signal would likely diffuse. This in turn suggests that the caliber of the OL process at the position of the spheroid is grossly abnormal i.e. the membrane has hyper-expanded. Given that OL membrane growth during myelination extends in two directions, i.e., spiral growth to the axonal surface and longitudinal growth along the axon, it is possible that spheroid-like structures are formed by uneven myelin growth. We recognize that we cannot yet conclude whether and how spheroid formation might be linked to the myelination deficit that we observe in Zdhhc9 KO mice.

However, defining the subcellular mechanism for spheroid formation may provide further insights into this issue. We have therefore largely retained the original statement but have added the reasoning above to our revised Discussion.

> *The authors report that Zdhhc9 KO primary and secondary branches in OL were longer, some contained spheroid-like swellings and the OL protrusion complexity was higher. However, these data is partially contradictory to what they show in OL differentiation experiments in vitro (Fig 7). There is also no evidence for increased membrane expansion in Zdhhc9 knockdown myelin forming cells in culture. How do they reconcile these different findings?*

We appreciate the reviewer's interest in this issue. Several non-mutually exclusive factors could account for the differences in OL morphology in vitro versus in vivo caused by Zdhhc9 loss. First, morphology in vivo may well be influenced by the axons and/or other extrinsic components around each OL that are not present in our primary cultures. Second, OL growth in vivo is highly 3-dimensional, whereas growth in culture is largely 2-dimensional – it may be difficult to support formation of spheroids (by definition, a 3-dimensional structure) in the latter situation. Finally, Zdhhc9 is absent in vivo from the beginning of development until the time points examined, whereas in our cultured OL experiments, Zdhhc9 shRNA is virally delivered to OPC cultures at DIV2 and likely acutely affects Zdhhc9 expression predominantly in committed OLs (following the switch to differentiation medium at DIV3). These differences may also affect the ability of other PATs or, potentially, palmitoylation-independent subcellular processes, to compensate for Zdhhc9 loss. We have more fully explained these points in our revised Discussion.

> *Page 7: "The OL processes in this culture condition correspond to large lipid-rich membranous sheets that form spiral membrane expansion on axons in vivo (49)." At which stage are authors referring to? OL processes are extended in culture before membrane formation and this is not clear here. In a 3-days differentiation culture, most OLs have not yet formed a myelin sheath (eg., Figure 2 in Zuchero et al., 2015, Dev Cell).*

We appreciate the reviewer highlighting this point. We first note that our oligodendrocyte (OL) culture conditions differ from the immunopanning method used by Zuchero et al., 2015 (original reference (Emery and Dugas, 2013)), which may affect the time course and

progression of OL process elaboration and/or myelin sheath formation. We further note that in our cultures most EGFP+ processes are also MBP+ at the time point examined (strictly 3 days plus 9 hours post-differentiation). It thus seems likely that these MBP+ structures largely correspond to the MBP+ wrapping sheaths that occur in vivo, so we have therefore retained our original statement but have added this further explanation.

> *Minor: Figure 6 (Legend): Time points should be indicated throughout the panels.*

We have added this information as requested

> ***Reviewer 2 Recommendations for the Authors:***
>
> *(1) Regarding the subcellular localization experiment of ZDHHC9 mutants in OL, it is currently limited to in vitro cultured OL, lacking validation in vivo OL or myelin sheath. Additionally, it is necessary to investigate whether the abnormal subcellular localization of ZDHHC9 mutants affects their enzyme activity and palmitoylation modification of substrate proteins.*

We thank the reviewer for raising this point. New data in our revised Figure 8 compares autopalmitoylation (sometimes used as a surrogate measure of PAT activity) of ZDHHC9wt and XLID mutants, and their ability to palmitoylate MBP in transfected cells. Intriguingly, we found that autopalmitoylation activity of the ZDHHC9-P150S mutant does not differ significantly from that of ZDHHC9wt, and that this mutant is still capable of palmitoylating MBP. Moreover, the R96W mutant, while impaired in autopalmitoylation, still palmitoylated MBP approximately 50% as effectively as ZDHHC9wt in our cell-based assay. These findings suggest that ZDHHC9-P150S and, probably, ZDHHC9-R96W mutants might still be able to palmitoylate substrates in OLs if they were properly localized. This possibility in turn suggests that impaired subcellular targeting in addition to, or instead of, impaired catalytic activity, may be a key factor in certain cases of ZDHHC9-associated XLID. We have expanded our Figure 8 to show these new experiments and have summarized the conclusions above in our revised Discussion. We thank the reviewer for suggesting that we further investigate this issue.

> *(2) The experimental period (P21+21 days) using genetic labeling to track the development of myelinating cells may not be long enough. It is recommended to extend the observation time and analyze at more time points to more comprehensively reflect the impact of Zdhhc9 KO.*

We appreciate this point from the reviewer but, regrettably, we did not maintain the PdgfraCreER; R26-EGFP; Zdhhc9 KO mouse line and hope the reviewer appreciates that it would take considerable time and effort to rederive this line and then perform the suggested extended time course experiments. However, we note for the reviewer that our preliminary studies did not reveal any effect of Zdhhc9 KO on the number of MOBP-EGFP+ OLs in 6-month-old mice (not shown), consistent with a model in which Zdhhc9 loss does not affect OPC-OL commitment per se.

> *(3) The author speculates that Zdhhc9 may regulate myelination by affecting the membrane localization of specific myelin proteins, but lacks direct experimental evidence to support this. It is suggested to detect the expression and distribution of relevant proteins in the myelin of Zdhhc9 KO mice.*

We share the reviewer's interest in this point but realized that it is more technically challenging to address than might be initially thought. The main protein we would implicate and seek to test is MBP, but we already found that there is no gross change in MBP

distribution in vivo in Zdhhc9 KO mice (Fig 3A). However, an anti-MBP antibody recognizes all forms of MBP, not just the specific splice variants whose palmitoylation is affected by ZDHHC9 loss. Specifically assessing nanoscale distribution of these splice variants would require a way (e.g. am anti-MBP splice form-specific antibody that is compatible with immuno-EM) to distinguish these variants from other, non-palmitoylated forms of MBP. Although such an antibody could be an important tool we hope the reviewers would agree that developing and characterizing such a reagent is beyond the scope of the current study.

We do, however, note that the lack of gross change in MBP distribution and levels in Zdhhc9 KO mice is consistent with the relatively mild phenotype of these mice, compared with shiverer (shi/shi) mice, in which MBP is completely lost. In shiverer, CNS compact myelin is almost absent (PMID: 671037; PMID: 88695; PMID: 460693) and, as the name suggests, mice display a shivering gait, and exhibit seizures and early death. In contrast, Zdhhc9 mice show only subtle behavioral deficits (PMID: 29944857). These differences are all consistent with a model in which Zdhhc9 KO mice, despite their significantly reduced MBP palmitoylation (Fig 8) have grossly normal distribution and levels of MBP when all splice variants are assessed (Fig 3, Fig 8). It is not inconceivable that Zdhhc9 KO mice have a nanoscale change in the distribution of MBP, particularly of specific palmitoylated splice variants, within myelin that profoundly affects myelin ultrastructure, without grossly altering MBP distribution. However, an alternative and not mutually exclusive possibility is that aberrant palmitoylation of other

Zdhhc9 substrates accounts for, or contributes to, the abnormalities in myelin at the ultrastructural level. Addressing this issue would require a multi-pronged approach, not just to assess palmitoylation and distribution of such proteins in Zdhhc9 KO, but also to test whether they are direct Zdhhc9 substrates, in order to rule out indirect effects. We hope reviewers would agree that this is best left to a separate study. However, in our revised Discussion we now summarize what can be inferred regarding Zdhhc9-dependent effects on total and splicevariant specific distribution and levels of MBP.

> *(4) Although the article mentions the association of Zdhhc9 with intellectual disabilities, it does not involve behavioral analysis of Zdhhc9 KO mice. It is recommended to supplement some behavioral experimental data to support the important role of Zdhhc9 in maintaining normal cognitive function, enhancing the clinical relevance of the article.*

We appreciate this point from the reviewer. The behavior of the same ZDHHC9 KO mouse line that we used was reported in PMID: 31747610 and in PMID: 29944857. In the former study, Zdhhc9 KO mice were reported to display seizures reminiscent of phenotypes in human patients with ZDHHC9 mutation. The latter study assessed performance of Zddhc9 KO mice in several tasks that test cognitive function. Specifically the KO mice were reported to display “altered behaviour in the open-field test, elevated plus maze and acoustic startle test that is consistent with a reduced anxiety level; a reduced hang time in the hanging wire test that suggests underlying hypotonia but which may also be linked to reduced anxiety [and] deficits in the Morris water maze test of hippocampal-dependent spatial learning and memory.”. We have incorporate these findings in our revised Discussion, where we summarize how these phenotypes are common, not just to human patients with ZDHHC9 mutation, but also to other human neurodevelopmental conditions and mouse models in which ID is a common feature.

> *(5) For the abnormal myelination observed in Zdhhc9 KO mice, including unmyelinated large-diameter axons and excessively myelinated small-diameter axons, the article lacks indepth research and explanation on the exact mechanism and mode of action of ZDHHC9 in regulating myelination.*

We share the reviewer's interest in this point but again note that gaining definitive insights into this issue is far from trivial. Convincing evidence of a causative mechanism would require an exhaustive identification of ZDHHC9 in vivo substrates, followed by point mutation of substrate palmitoylation site(s) to determine the extent to which palmitoylation of such protein(s) phenocopies ZDHHC9 loss. Nonetheless, it is possible to break this question down and to summarize what we do and do not know. For example, our experiments in cultured OLs show that ZDHHC9 loss causes call-autonomous deficits in morphological maturation of these cells. We also know that ZDHHC9 loss results in impaired palmitoylation of MBP, a direct substrate for ZDHHC9. Moreover, loss of ZDHHC9 at Golgi outposts in OLs (a phenotype observed with several XLID-associated mutant forms of ZDHHC9, even those with no significant loss of catalytic activity) correlates with intellectual disability. Together, these findings are consistent with a model in which ZDHHC9 action at OL Golgi outposts is critical for normal myelination. However, it is yet to be determined whether the key substrates of ZDHHC9 include MBP, other palmitoyl-proteins that are key constituents of CNS myelin, or proteins whose palmitoylation is important for myelin protein trafficking and targeting. Another non-mutually exclusive possibility is that ZDHHC9 acts at Golgi outposts but indirectly, for example to drive the expression of myelin protein genes. Future experiments, including but not limited to palmitoyl-proteomics in ZDHHC9 (OL-specific) KO mice, will be needed to provide more definitive insights into this issue. We have expanded our Discussion of links between ZDHHC9 mutation and impaired myelination to summarize the above points.

> *(6) The function of ZDHHC9 in OL may be related to the Golgi apparatus, but its exact role in these structures is still unclear. It is suggested to discuss in more detail the role of ZDHHC9 in the Golgi apparatus in the discussion section.*

We appreciate this point, which we considered as related to point (5) above. In our revised Discussion we highlight how ZDHHC9 action at Golgi outposts may involve direct palmitoylation of myelin proteins, palmitoylation of proteins that direct myelin proteins to the myelin membrane and/or activation of gene expression programs that serve to drive myelination. We further note that these possibilities are not mutually exclusive.

> *(7) More experimental support and in-depth research are needed on the detailed mechanism of how ZDHHC9 and Golga7 cooperatively regulate MBP palmitoylation, and how this decrease in palmitoylation level leads to myelination defects.*

This is another important point – our new experiments suggest that, although some XLID mutations markedly affect ZDHHC9's ability to palmitoylate MBP, others do not, yet all of the mutant forms fail to localize to Golgi outposts. These findings are consistent with a model in which the subcellular location at which ZDHHC9 palmitoylates MBP, and potentially other substrates, is critical for normal myelination. Interestingly, despite their marked differences in basal catalytic activity (as assessed by autopalmitoylation), wt and all XLID forms of ZDHHC9 appear to show enhanced activity (measured by both auto- and MBP palmitoylation) in the presence of ZDHHC9, suggesting that the association with Golga7 (which also localizes to Golgi outposts) is central to ZDHHC9 activity. This model is also highly consistent with the biased expression of Golga7 in OLs, compared to other CNS cell types (Fig 1E, 1F). Moreover, XLID-associated mutant forms of ZDHHC9 also show reduced protein stability and are impaired in their ability to form complexes with Golga7 (also known as Golgi Complex Protein 16kDa; GCP16; PMID: 37035671). Failure of ZDHHC9 XLID mutants to localize to Golgi outposts may thus be due to aberrant trafficking of mutant ZDHHC9 per se, but may also involve impaired association/stabilization of ZDHHC9/Golga7 complexes at these locations. Again, it is possible that either or both of these mechanisms, which are not mutually

exclusive, contribute to impaired MBP palmitoylation and/or myelination deficits. We summarize these points in our revised Discussion.

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