CNS myelin protein 36K regulates oligodendrocyte differentiation through Notch

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Numbers: INST 1172/37-1 FUGG, KU2474/13-1; Ministry of Culture and Science of North Rhine-Westphalia, Grant/Award Number: Repatriation Grant to BO

Abstract
In contrast to humans and other mammals, zebrafish can successfully regenerate and remyelinate central nervous system (CNS) axons following injury. In addition to common myelin proteins found in mammalian myelin, 36K protein is a major component of teleost fish CNS myelin. Although 36K is one of the most abundant proteins in zebrafish brain, its function remains unknown. Here we investigate the function of 36K using translation-blocking Morpholinos. Morphant larvae showed fewer dorsally migrated oligodendrocyte precursor cells as well as upregulation of Notch ligand. A gamma secretase inhibitor, which prevents activation of Notch, could rescue oligodendrocyte precursor cell numbers in 36K morphants, suggesting that 36K regulates initial myelination through inhibition of Notch signaling. Since 36K like other short chain dehydrogenases might act on lipids, we performed thin layer chromatography and mass spectrometry of lipids and found changes in lipid composition in 36K morphant larvae. Altogether, we suggest that during early development 36K regulates membrane lipid composition, thereby altering the amount of transmembrane Notch ligands and the efficiency of intramembrane gamma secretase processing of Notch and thereby influencing oligodendrocyte precursor cell differentiation and further myelination. Further studies on the role of 36K short chain dehydrogenase in oligodendrocyte precursor cell differentiation during remyelination might open up new strategies for remyelination therapies in human patients.

KEYWORDS
36K, gamma secretase, Morpholino (MO), Notch signaling, oligodendrocytes, oligodendrocyte precursor cells (OPCs), zebrafish

Abbreviations: MO1, 36K MO; MO1 + RNA, 36K MO co-injected with 36K mRNA; CNRQ, calibrated normalized relative quantities; CNS, central nervous system; control MO, control MO; dpf, days post fertilization; dia, Deltaa; DRG, dorsal root ganglion; FGF, fibroblast growth factor; Gfap, glial fibrillary acidic protein; Her4, hairy related 4; HDAC, histone deacetylase; mRNA, messenger ribonucleic acid; MBP, myelin basic protein; DAPT, N-(3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine t-buty1 ester; PNS, peripheral nervous system; OPCs, oligodendrocyte precursor cells; Plp1, proteolipid protein; q-qRT-PCR, qPCR, quantitative real time polymerase chain reaction; RA, retinoic acid; rt-PCR, reverse transcription polymerase chain reaction; MO2, second translation blocking MO to 36K; Shh, Sonic hedgehog; TSA, TrichostatinA; zfl, zebrafish larvae.

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Myelin sheath is the extension of plasma membrane of glial cells wrapping around neuronal axons (Sherman & Brophy, 2005). These glial cells are called oligodendrocytes in the central nervous system (CNS). Disruption of myelin sheaths in CNS can occur in various demyelinating diseases like multiple sclerosis, myelopathies, leukodystrophies (Franklin & Ffrench-Constant, 2008) and has implications for other neurodegenerative diseases, including secondary axon injury (Edgar & Nave, 2009). As myelination with nodes of Ranvier is a vertebrate specific adaptation (Zalc, 2016), zebrafish is an evolutionary early and more simple model organism for studying myelination in vivo. Still zebrafish have emerged to be a powerful and useful model over the past decade to study myelination and remyelination (Brösamle & Halpern, 2002; Czopka & Lyons, 2011; Kirby et al., 2006; Preston & Macklin, 2015) due to the translucent nature of their embryos and their fast ex-utero embryonic development.

In addition to the common conserved myelin proteins like myelin basic protein (Mbp) (Nawaz, Schweitzer, Jahn, & Werner, 2013) or proteolipid protein (Plp1 or DM20) (Schweitzer, Becker, Schachner, Nave, & basic protein (Mbp) (Nawaz, Schweitzer, Jahn, & Werner, 2013) or proteolipid protein (Plp1 or DM20) (Schweitzer, Becker, Schachner, Nave, & Werner, 2006), 36K (ff13639, Zebrafish International Network [ZFIN] ZDB-GENE-030131-8,104) is a major component of teleost CNS myelin protein (Jeserich, 1983). The closest human homologue to zebrafish 36K is SDR12 (National Center for Biotechnology Information [NCBI] Accession Number: NP_001026889.1), with 61% amino acid identity (Uniprot alignment). The mRNA of SDR12, also referred to as FLJ13639, has been shown to be present in human white matter in the cerebral cortex, while its existence at protein level still remains hypothetical (Morris et al., 2004). In zebrafish, 36K is a membrane associated protein present in both myelin cytoplasm as well as in membrane (Morris et al., 2004). Other proteomic studies have found 36K to be one of the most abundant proteins in zebrafish brain (Gebriel et al., 2014). Despite its abundance, the function of 36K remains unknown. Further, it remains unknown if other enzymatic or structural myelin proteins might replace its function in rodents or humans. 36K has been suggested to belong to the short chain dehydrogenase family, based on homology studies and the conserved cofactor binding domain (Morris et al., 2004). 36K is a highly basic protein similar to Mbp and is also present in compact myelin (Morris et al., 2004), which might suggest a role in myelin compaction similar to that of Mbp (Jeserich & Rauen, 1990). In this study, we used MO® knockdown to further investigate the function of 36K in zebrafish CNS myelin.
denaturation at 95°C, 40 amplification cycles were carried out: 10 s at 95°C and 1 min at 60°C with a ramp rate of 1.6°C/s.

q-rt-PCR analysis was performed using CFX Manager™ and qBase software. All data were normalized to the reference genes and scaled to average hence generating calibrated normalized relative quantities (Helleman, Mortier, Pape, Speleman, & Vandesompele, 2007).

Primer sequences for RT PCR/q-rt-PCR: 36K Forward primer (FP): CACAGCTCAATGAAGGAG; 36K Reverse primer (RP): CACCTTCCGATCCTGATA; 36K Probe: CACCCAGCAAGGACGACATACA; ef1a FP: CAGGAATCACCCTTCTTG; ef1a RP: CTGGCTCAGAATCTTC; ef1a Probe: TCTCTTGCGATTCCA CCGCA; beta-actin FP: GCCAACAGAGAGAAGATG; beta-actin RP: GATGACCACGAAATGAAC; mbpa FP: CTCTGGTGATGGTG; mbpa RP: GCGTAACCCTCATAGATG.

2.6.2 | Larval wholemount

Zfl were anesthetized by immersing in MS222 (1:1,000), followed by immersion in 4% paraformaldehyde (PFA) in PBS overnight. For wholemount staining, larvae were washed with PBS 3x10 min, dehydrated through a series of methanol followed by rehydration. Embryos were then washed in PBST 5 min 3x, equilibrated in Tris buffer (150 mM Tris-HCl pH 9.0) at 70°C for 15 min, permeabilized in 5 μg/ml ProteinaseK in PBS for 50 min, fixed again in 4% PFA for 20 min. They were blocked in 10% normal goat serum (NGS)/2% Bovine serum albumin (BSA)/PBST at 4°C for 4 hr, incubated in primary antibody solution 2% NGS/2% BSA/PBST with anti-Sv2 antibody (deposited to DSHB by Buckley, K.M.) (1:250) at 4°C for 72 hr. They were then washed 6x with PBST at rt before incubation with secondary antibody goat anti-mouse IgG (H + L) Alexa Fluor 594 (1:1,000) for 48 hr and washed again 6x 15 min in PBST. Stained larvae were then mounted in agarose for imaging.

2.7 | Morpholino injections

Antisense oligonucleotide Morpholinos (MOs) from Genetools were dissolved in Ampuwa® Water to 2 mM stock concentrations. A working concentration of 0.15 mM was used for 36K MO1 (ATGCTGAGTTCCGGTACAGAGACAT) and MO2 (GTGGCCCCTCAGCTCTGATG). Control MO was used at a working concentration of 0.25 mM to avoid any differences due to injection or developmental differences. A glass micropipette was filled with the MO working solution and a volume of 1.8 nl was injected into yolk of the embryos when they were between one to four cell stages. A plasmid with Donio rerio 36K cDNA (Source BioScience IRAU969E0658D) was mutated within the MO1 binding site “atggcgttactggagacagcat.” 72 pg of mutated zebrasfish 36K mRNA was co-injected with MO1. Plasmid human SDR12 cDNA (Source BioScience IRAUp991A0379D) was used for generating human SDR12 mRNA.

2.8 | Imaging

For length measurement, images were acquired using a Nikon AZ100 multi-zoom microscope 0.5x objective. The analyst was blinded to the experimental groups during analysis. The body-length of four to eight larvae per image was measured using Fiji.
For all other in-vivo fluorescent images, a custom-built two-photon light sheet microscope (manuscript in preparation) was used unless otherwise mentioned. Embryos were anesthetized in MS222 (Tricain) and each embryo was mounted inside a fluorinated ethylene propylene (FEP) tube with an inner diameter of 1 mm and an outer diameter of 1.6 mm in 1.25% low melting point agarose in 0.3x Danieau. Brightfield images were used to orientate the images and crop two spinal segments (in most cases third and fourth spinal segments, as the images were always taken in the same region above the yolk extension). The observer was blinded before cell counting. Fiji Cell counter plugin (Author: Kurt De Vos, University of Sheffield) was used for counting cells. For dorsally migrated OPCs or oligodendrocytes, all GFP+ cells above and not touching the ventral spinal cord pMN region were counted (represented by dashed lines in the figures). For the fluorescence intensity measurements, mean intensity was taken from 3D size matched average intensity stag projection images from comparable spinal cord regions.

Images showing Sv2 immunostaining were taken with a LaVision Trimscope two-photon point scanning microscope. Embryos were mounted in a drop of 1.25% low melting point agarose in 1x PBS.

For high-throughput imaging using an EnSight© multimode plate reader, embryos were anesthetized in MS222. Larvae were placed in 96-well glass bottom plates with one larva in each well. Larvae were centered in the wells using a small pipette tip so that they laid in the correct orientation to be detected by an automated cell counting algorithm. After placing the 96-well plate in the EnSight© multimode plate reader, with a newly coded algorithm embedded in the Perkin Elmer’s Kaleido© Software (manuscript in preparation), all dorsally migrating cells were automatically detected using the imaging mode.

2.9 | Electron microscopy

Larvae zebrafish were anesthetized in MS222 immersion fixed in glutaraldehyde fixative and then processed as described previously (Maier, Wang-Eckhardt, Hartmann, Gieselmann, & Eckhardt, 2015).

2.10 | Trichostatin A (TSA) treatment

For histone deacetylase inhibition and hence prevention of oligodendrocyte differentiation, embryos were kept in 100 ng/ml TSA in 0.3x Danieau from 36 hpf until stage of analysis as described in (Takada & Appel, 2010). Controls were kept in 0.00001% methanol in 0.3x Danieau as TSA was dissolved in methanol.

2.11 | N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) treatment

For inhibition of Notch, embryos were raised from 24 hpf until stage of analysis in 5 μg/ml DAPT in 0.3x Danieau as described in (Salta et al., 2014) and controls in 0.1% DMSO in 0.3x Danieau.

2.12 | Microarray analysis

For microarray analysis, total RNA was extracted from 30 larvae using TRIzol® RNA Isolation (Invitrogen 15596018). Samples were then column purified using Nucleospin RNA clean up kit. 1 μg of RNA were shipped in RNastable tubes to Oaklabs for ArrayXS zebrafish analysis, where the expression levels of 60,023 genes were measured. All microarrays were in 8x60K format, 8 subarrays/slide. Samples from control MO and MO1 groups at 3 dpf and 5 dpf from six independent experiments were analyzed. Detailed protocol is available from Oaklabs (http://www.Oak-labs.com/). Data were normalized by using ranked median quantiles (Bolstad, Irizarry, Astrand, & Speed, 2002). By default, a Welch’s t-test was used and p-values were adjusted according to the adaptive Benjamini–Hochberg procedure to decrease the number of “false-negatives” (Benjamini & Hochberg, 1995, 2000). Only probes which had a |log2fold| > 0.5 and a p-value of <.05 were considered. Heatmaps from microarray analysis were generated using Heatmapper (Babicki et al., 2016).

2.13 | Behavior experiments

For behavior experiments, larvae from different experimental groups were arranged in 12-well plates with one larva per well. Each 12-well plate had three larvae from each of the four experimental groups. Following acclimatization for 2 hr in the well plates, recordings were done with the same lighting and temperature conditions in the afternoons between 14:00 and 16:00.

For unprovoked behavior test, swim trajectories of the larvae were recorded for 10 min. Recordings were analyzed from the second to ninth min. For stimulated behavior test, a marble weighing ~20 g was dropped from a height of 9.5 cm along a slanted board 15° angle always fixed at the same position. The vibration caused by the marble drop stimulated the larvae to swim (escape) and the first swim episode following the marble drop was analyzed. The test was repeated twice for each larva and the average values were taken so as to exclude the problem of one trial being over representative in the value.

For unprovoked tests, image sequences were reduced so that 96 frames with 5 s separation were analyzed. For stimulated response tests, 30 frames at 30 fps (=1 s) were analyzed from the time point of the marble drop. By manually tracking the position of the larva in each well in all frames using MTrackJ plugin (Meijering, Dzyubachyk, & Smal, 2012) in Fiji, distance of swimming was measured.

2.14 | Thin layer chromatography/analysis of sphingolipids

2.14.1 | Lipid extraction

Whole fish larvae (~100) were homogenized in 1 ml of ice-cold 20 mM Tris-HCl (pH 8.5) using an UltraTurrax T10 basic homogenizer (IKA, Staufen, Germany), followed by centrifugation at 100,000g for 1 hr. The pellet was transferred into Pyrex glass tubes and extracted with 6 ml of chloroform/methanol (2:1; v:v) at 60°C for 4 hr (under
constant stirring). After centrifugation (1,000g for 10 min) supernatant was transferred into a Pyrex glass tube and the pellet was reextracted with 6 ml of chloroform/methanol (1:1; v:v) overnight at 60°C. Samples were again centrifuged and the supernatant from second extraction was combined with the first supernatant to obtain the total lipid extract. The lipid extract was filtered through glass wool and dried under a stream of nitrogen (45°C) and dissolved in 0.5 ml of chloroform/methanol (1:1). Aliquots of these total lipid extracts were examined by TLC.

2.1.4.2 | Alkaline hydrolysis and desalting

The total lipid extract was dried under a stream of nitrogen, dissolved in 2.5 ml of methanol, and sonicated in an ultrasonic bath for 10 min. After adding 625 μl of 4 M sodium hydroxide solution the lipid extract was incubated for 2 hr at 37°C (under constant stirring) to hydrolyze glycerolipids. Alkaline hydrolysis was stopped by addition of 10 μl acetic acid. Extract was dried under a stream of nitrogen, dissolved in 1 ml of methanol, and sonicated in an ultrasonic bath for 5 min. Lipid extract was desalted by reversed phase chromatography. LiChrosorb® RP-18 beads (Merck, Darmstadt, Germany) were filled into Pasteur pipettes (about 1 ml of bead volume) and equilibrated with chloroform/methanol/0.1 M KCl (6:96:94; v:v:v). Lipid extract was mixed with one volume of 300 mM ammonium acetate and passed through the RP-18 column. The column was washed 6-times with water (1 ml), and lipids were eluted with 1 ml of methanol followed by 8-times 1 ml of chloroform/methanol (1:1; v:v). Combined elution fractions were dried under a stream of nitrogen and dissolved in 0.5 ml of chloroform/methanol (1:1; v:v).

2.1.4.3 | Thin layer chromatography

Lipids were separated on HPTLC silica gel 60 plates (Merck, Darmstadt, Germany). Lipids isolated from identical numbers of fish larvae and lipid standards were applied by hand or using an automatic TLC sampler (ATS4; CAMAG, Berlin, Germany). All lipid standards (cholesterol, galactosylceramide, lactosylceramide, sphingomyelin, sulfatide, phosphatidylcholine, phosphatidylethanolamine) were from Sigma-Aldrich. TLCs with total lipid extracts were developed in chloroform/methanol/water (60:27:4; v:v:v; or 70:30:4; v:v:v) to obtain a better separation of free fatty acids and cerebrosides. TLC plates were dried, sprayed with 625 mM cupric sulfate 8% phosphoric acid and heated to 180°C for 5 min (Yao & Rastetter, 1985). TLC plates were scanned using a commercial scanner (Epson Perfection V700 Photo) within 30 min after staining.

2.15 | Mass spectrometry

2.15.1 | Lipid extraction

As described in (Rabionet et al., 2008), fish larvae (~100) were homogenized in 1 ml of ice-cold 20 mM Tris-HCl (pH 8.5) using an Ultraturrax T10 basic homogenizer (IKA, Staufen, Germany), followed by centrifugation at 100,000g for 1 hr. The pellet was resuspended in 100 μl of water and transferred into Pyrex glass tubes. After adding 1 ml of chloroform and 1 ml of methanol (to obtain a ratio of chloroform:methanol:water 10:10:1; v:v:v), samples were sonicated in an ultrasonic bath for 10 min. Samples were then centrifuged for 5 min at 4,000 rpm and the supernatants were transferred into fresh Pyrex glass tubes. Two milliliters of Chloroform/methanol/water (10:10:1; v:v:v) was added to the pellet and the extraction was repeated. Pellet was reextracted with 2 ml of chloroform/methanol/water (30:60:8; v:v:v). Supernatants from the three extractions were combined in Pyrex glass tubes and dried under a stream of nitrogen (at 45°C). The dried lipids were dissolved in 1 ml of chloroform/methanol (1:1; v:v), transferred into safe look Eppendorf tubes and again dried under a stream of nitrogen and stored until analyzed by mass spectrometry.

Solvents and additives for UPLC used were of LC–MS grade. Synthetic and endogenous sphingolipids and phospholipids, that is, NS-, AS-, ND5-, and NP-ceramides, NS-Glucosylceramides, Kerasin, Phrenosin, sphingomyelins, Lactosylceramides, sulfatides, and phosphatidylincholines were obtained from Avanti Polar Lipids, Matreya, Sigma, or Abcam. NS-Cer(d18:1/14:0-, 19:0-, 25:0-, or 31:0), NS-HexCer(d18:1/14:0-, 19:0-, 25:0-, or 31:0), NS-LacCer(d18:1/14:0-, 19:0-, or 27:0), NS-SM4s(d18:1/14:0-, 19:0-, or 27:0), and NS-SM (d18:1/31:0) were self-synthesized (Jennemann et al., 2007; Sandhoff et al., 2002; Seyranetepe et al., 2018).

A methanolic solution containing a mix of internal lipid standards [Cer(d18:1/14:0), Cer(d18:1/19:0), Cer(d18:1/25:0), Cer(d18:1/31:0), GlcCer(d18:1/14:0), GlcCer(d18:1/19:0), GlcCer(d18:1/25:0), GalCer (d18:1/31:0), LacCer(d18:1/14:0), LacCer(d18:1/19:0), LacCer (d18:1/27:0), SM(d18:1/12:0), SM4s(d18:1/14:0), SM4s(d18:1/19:0), SM4s(d18:1/27:0), SM(d18:1/12:0), SM(d18:1/17:0), SM(d18:1/31:0), PC(24:0), PC(28:0), PC(44:0), and PC(48:0)], 750 μl of methanol and 50 μl of water were added to a lyophilized aliquot of the lipid extract corresponding to 40 larva fish. Samples were mixed and sonicated at 37°C for 5 min and subjected directly to LC–MS/MS analytics.

2.15.2 | LC–MS/MS analysis of sphingolipids

Extracted lipids were separated on Waters I class UPLC equipped with Waters CSH C18 column (length 100 mm, diameter 2.1 mm, particle size 1.7 μm) using a gradient starting with 57% solvent A (50% methanol, 50% water) and 43% solvent B (99% 2-propanol, 1% methanol), both containing 10 mM ammonium formiate, 0.1% formic acid and 5 μM sodium citrate (Table S2). UPLC was coupled to an ESI-(QqQ)-tandem mass spectrometer (Waters Xevo TQ-S) for compound detection in +ESI MRM mode except for sulfatides in −ESI MRM mode (Table S3). Samples were injected and processed using MassLynx software, mass spectrometric peaks were quantified according to their peak area ratio with respect to the internal standard using TargetLynx software (both v 4.1 SCN 843) both from Waters Corporation (Manchester, UK). Subsequently, quantification of ceramides, hexosylceramides, lactosylceramides, sulfatides (SM4s), and...
sphingomyelins was adjusted to the length of the acyl-chain and dihydro(G)SL as well as phyto-(G)SL quantification were further adjusted by a factor calculated between the intensities of external ceramides and dihydroceramide or phytoceramide standards of same concentration.

2.16 | Statistical analysis

All graphs generated and the statistical analyses were performed using GraphPad Prism version 6.0. All experiments were repeated at least three times unless otherwise mentioned. In general, \( N \) represents the total number of experiments and \( n \) represents the number fish larvae used in total.

Column statistics were performed to test if the values were Gaussian distributed with D'Agostino and Pearson omnibus normality test. For two experimental groups, two-tailed \( t \)-test was used if the values were normally distributed. If not, Mann–Whitney \( U \) test was used as it is a nonparametric test and would not assume Gaussian distribution. In case of three or more groups, one-way analysis of variance ANOVA was used followed by Bonferroni’s multiple comparison posthoc test if the values were normally distributed. If the values were not normally distributed, Kruskal–Wallis ANOVA with Dunn’s multiple comparison posthoc test was used. For grouped analyses, two-way ANOVA followed by Tukey’s multiple comparison posthoc test was used. Mean with standard error of the mean (SEM) is indicated as variability in all graphs unless otherwise mentioned. Asterisks in the graphs mean: ns when \( p > .05 \), (*) when \( p \leq .05 \), (**) when \( p \leq .01 \), (***) when \( p \leq .001 \) and (****) when \( p \leq .0001 \).

3 | RESULTS

3.1 | Characterization of 36K mRNA and protein expression

36K protein has been shown to be present both within the cell body of oligodendrocytes and in the compact myelin formed by these cells in the adult zebrafish CNS (Morris et al., 2004). To investigate and quantify mRNA expression of 36K in zebrafish larvae (zfl) during development from 1 to 11 days post fertilization (dpf) as well as in adult organs (Figure 1a,b), we performed exon-exon junction spanning.
reverse transcription polymerase chain reaction (rt-PCR). Expression of 36K was found to be strong in adult zebrafish in brain, eyes and spinal cord CNS tissues in comparison to other non-CNS organs. By quantitative real time polymerase chain reaction analysis (q-rt-PCR) using dual labeled probes we could show that in developing zfl 36K expression increases from 3 dpf onward (Figure 1c) until 7 dpf where it reaches a plateau. To characterize 36K protein expression, we had raised custom-made polyclonal antibodies against the C terminus of zebrafish 36K in rabbit (Perbio Thermo Fisher Scientific, Bonn, Germany). For initial validation of these antibodies, we transfected Chinese hamster ovary (CHO) cells with an expression plasmid containing 36K cDNA sequence. 36K expression was specific to the transfected cells where it was found in the entire cytoplasm and the cell membrane (Figure S1a–c). In Western blot, a single band was observed at 36 k-Dalton (kD) only in 36K transfected cells (Figure S1d). A single band at 36 kD was observed in zfl as well as in brain tissue and spinal cord from adult zebrafish (Figure 1d). At 3 dpf, 36K protein is hardly detectable in western blot in whole zebrafish.

![Figure 2](image-url)

**FIGURE 2** Translation blocking MOs specifically knock down 36K protein expression. (a) Anti-36K Western blot on whole larvae tissue showing 36K MO1 knockdown and rescue at 4 dpf. (b) Lateral view of control MO and MO1 injected zfl at 4 dpf. Scale bars: 500 μm. (c) Reduction in body length of the MO larvae at 3, 4, and 5 dpf. Sample sizes: N = 3; n shown in each bar. Two way ANOVA p ≤ .0001 (****) followed by Tukey’s multiple comparisons test for control MO versus MO1 and control MO versus MO2. MO1 versus MO1 + RNA was always significantly different (not shown). Control MO versus MO1 + RNA was not significant at any of the three time-points (p > .05). (d) Zebrafish overview sketch highlighting different CNS regions: shown as red box (e, hindbrain) and as green box (f, spinal cord). (e,f) Comparable sagittal paraffin sections counter stained with hematoxylin depicting anti-36K staining in the hindbrain (e) and spinal cord (f) at 4 dpf in control MO (top; intense staining) and MO1 (bottom; reduced/weak staining) zfl. Scale bars: 50 μm.
larvae (zfl) lysate. In adult zebrafish, we performed immunohistochemistry in paraffin sections where the antibody specifically recognized cells in brain and spinal cord (Figure 1e,f). 36K expression could be observed to co-localize with some olig2+ oligodendrocytes and oligodendrocyte precursor cells (OPCs) in both brain and spinal cord CNS regions at 3 dpf (Figure 1g). At 5 dpf co-localization of 36K with Mbp + myelinating oligodendrocytes could be clearly observed in these regions (Figure S1e).

### 3.2 | 36K MO specifically and efficiently knocks down 36K protein expression

In order to investigate the function of 36K, we designed two independent antisense translation blocking MOs (MO1 and MO2) targeting different sites of the 5' initial regions of the 36K mRNA. Knockdown of 36K protein with 36K MO1 was confirmed by western blot (Figure 2a). MO1 injected fish showed ~80% less 36K protein at 4 dpf in comparison to non-injected (referred to as uninjected) and control Morpholino (control MO) injected fish (Figure S2a). 36K expression level could be rescued when MO1 was co-injected with a 36K mRNA (MO1 + RNA), in which the MO1 binding site was mutated so that MO1 cannot bind to the injected mRNA. In zfl injected with MO1, a reduction in body length was observed from 3 dpf till 5 dpf, which was rescuable by MO1 + RNA injections (Figure 2b,c). This reduction in body length was also observed with the second translation blocking MO to 36K (MO2) (Figure 2c). The human orthologue to zebrafish 36K is SDR12 (Morris et al., 2004). The length phenotype was not rescuable when MO1 was co-injected with the mRNA of SDR12, which MO1 should not be able to bind (Figure S2b). An enlarged pericardial sac and slower yolk consumption were observed in some MO injected zfl (Figure 2b). Somite numbers were unaltered although the body length was reduced. In MO1 injected zfl, the expression of 36K in the

![Figure 3](image-url)

**FIGURE 3** 36K knockdown zfl are less active and less responsive than controls. (a) Representative manually tracked swim trajectories of 5 dpf larvae from different groups during 8 min of swimming. (b) Total distance of swimming of different zfl groups at 5 dpf during 8 min with frames taken every 5 s. N = 3; n shown in each column. Kruskal–Wallis ANOVA p \( \leq \) .0001 (****) followed by Dunn’s multiple comparisons test for control MO versus MO1. (c) Cumulative distribution showing relative frequency (number of occurrence) versus swimming distance during this 8-min trials with a sampling rate of 5 s, which depicts shorter bouts of swimming in MO1 zfl. (d) Percentage of fish that respond to marble drop stimulus. N = 4; twoway ANOVA p \( \leq \) .0001 (****) followed by Tukey’s multiple comparisons test for control MO versus MO1. (e) Swimming distance in responding zfl only is not significantly different. N = 4; n shown in each bar. Kruskal–Wallis ANOVA p-value .8481.
hindbrain (Figure 2d,e) and spinal cord (Figure 2d,f) was virtually absent in comparison to the control MO group.

3.3 | 36K knockout fish are less responsive than controls

We then examined if behavior is functionally altered in the MO1 injected zfl. For this purpose, we performed unprovoked swimming tests at 5 dpf where the zfl swim for 8 min and the total distance of swimming was measured. We found that the fish in MO1 group swam 60% shorter total distance in comparison to the controls (Figure 3a–c). This effect could be rescued in the MO1 + RNA group. Further, in order to check more specifically for neural conduction, we performed stimulated behavior tests in which the zfl escape response as reaction to a marble drop was tested at 4 dpf. The marble fell along a slanted board onto the table next to the dish. Thirty percent fewer of the MO1 injected zfl responded to the stimulus, however when they did respond the “escape” swimming distance was indistinguishable from the controls (Figure 3d,e).

3.4 | 36K knockout leads to reduced myelin, fewer oligodendrocytes and disrupted myelin sheath

In order to examine if there are direct effects on myelin in 36K knockout zfl, we investigated the effects of MO1 with transgenic Tg(mbp:EGFP-CAAX) (Almeida, Czopka, & Lyons, 2011), Tg(mbp:EGFP) (Almeida, Czopka, & Lyons, 2011) and Tg(claudinK:EGFP) (Münzel et al., 2012) fishlines. In the spinal cord of Tg(mbp:EGFP-CAAX) MO1 injected fish, we found disturbed and less CNS-internodal-myelin represented by EGFP fluorescence (Almeida, Czopka, & Lyons, 2011) in comparison to the control MO group at 4 dpf (Figure 4a). The quantification of the mbp:EGFP-CAAX myelin fluorescence at 4 dpf is presented in Figure S3a. In the spinal cord of Tg(mbp:EGFP), fewer Mbpl oligodendrocytes were observed both in the ventral and dorsal spinal cord (Figure 4b–d). ClaudinK is specifically expressed in myelinating oligodendrocytes in the CNS and in Schwann cells in the PNS (Münzel et al., 2012). Similar to Mbpl oligodendrocytes, using Tg(claudinK:EGFP) zfl we observed fewer ClaudinK+ cells again in the dorsal and ventral spinal cord at 5 dpf in the MO1 group (Figure S3b–d). To further quantify expression of myelin genes, we performed q-rt-PCR analysis for mbpa and plp1b. We found that in the MO1 group both myelin genes were down-regulated to about 30–50% of their normal wild type expression at 4 dpf (Figure 4e,f).

In collaboration with Oaklabs GmbH, Hennigsdorf, Germany, we performed mRNA microarray chip analysis of MO1 versus control MO zfl larvae. The obtained gene expression levels also suggest less myelin gene expression in 36K knockdown zfl at 3 dpf (Figure 4g). For the myelin genes mbpa, plp1b, claudinK, and myelin protein zero (mpz) we show this in more detail in Figure S3e–h. Finally, to investigate for effects on myelin sheath compaction, we compared electron micrographs between MO1 and control MO zfl at 5 dpf. We observed unusual cytoplasmic vacuolated structures in between the myelin lamellae of MO1 (Figure 4h).

3.5 | 36K regulates OPC numbers

To assess the number of OPCs following 36K down-regulation, we used Tg(olig2:GFP) zfl (Shin, Park, Topczewska, Mawdsley, & Appel, 2003). OPCs that differentiate into oligodendrocytes migrate from the pMN domain of the spinal cord toward the dorsal region (Ravanelli & Appel, 2015). Myelination starts from late 3 dpf and is highly dynamic at 5 dpf (Brösmale & Halpern, 2002; Kirby et al., 2006). We observed significantly fewer dorsally migrated olig2+ cells in the spinal cord at 3 dpf in the MO1 and MO2 groups in comparison to the control MO and non-injected groups but no differences in cell numbers within the ventral spinal cord (Figure 5a–c, Figure S4a). This effect could be rescued in MO1 + RNA group zfl suggesting that the effect on OPC numbers is specific to 36K down-regulation (Figure 5b). The reduction of dorsal olig2+ cells in the MO1 injected fish might be due to a developmental delay. However, at 5 dpf we still observed fewer dorsally migrated olig2+ OPCs in the MO1 group (Figure 5d,e). Fewer dorsally migrated olig2+ cells in MO1 and MO2 larvae at 3 dpf could also be confirmed with a high-throughput automated protocol using the EnSight© multimode plate reader in which more than 75 larvae were analyzed for each group (Figure S4a). We hypothesized that this reduction in migrating cells in the MO group is due to less differentiation toward a cell state in which these finally become oligodendrocytes. Alternatively, this could be due to reduced proliferation or enhanced apoptosis of OPCs. In zfl spinal cord paraffin sections, we checked for proliferation by immunostaining against the proliferating cell nuclear antigen (Pena; Herce, Rajan, Laettig-Tünemann, Fillies, & Cardoso, 2014; Maga & Hübscher, 2003; Figure 5f). Apoptotic cells were analyzed by immunostaining against Cleaved caspase 3 antibodies (Janicke, Sprengart, Wati, & Porter, 1998; Figure 5i). In addition, we verified the expression data of these genes in the microarray analysis. Interestingly, we observed enhanced cell proliferation within the whole larva CNS in the MO1 treated group but no difference in apoptosis as also shown by our microarray data (Figure 5j,k), hence suggesting less differentiation toward non dividing oligodendrocytes in the 36K MO1 knockdown zfl. For further detail about the cell fate of the unexpected increased cell proliferation, see the next section.

We then asked if there is 36K expression when there is no differentiation toward oligodendrocytes. We treated zfl from 36 hpf until 4 dpf, subsequent to neurogenesis (Ravanelli & Appel, 2015), with TrichostatinA (TSA) a histone deacetylase (HDAC) inhibitor, which has been formerly shown to inhibit migration (Figure 5g) and differentiation of OPCs to oligodendrocytes completely (Takada & Appel, 2010). We saw no 36K expression any more at 4 dpf in the TSA treated larvae at all (Figure 5f), suggesting 36K expression to be specific for differentiating and later also mature oligodendrocytes. We hypothesize that at an early time-point, a meagre amount of 36K is sufficient for enzymatic activity through which oligodendrocyte differentiation is influenced, and only at a later time-point of the larvae development, a large amount of 36K is present in differentiated oligodendrocytes and compact myelin.
3.6 | 36K acts on OPCs through Notch signaling

Several pathways have been described to be involved in neurogenesis and gliogenesis during development of the spinal cord (Briscoe & Novitch, 2008). We commissioned a microarray chip analysis to investigate mRNA expression levels of around 60,000 transcripts in the MO1 versus control MO zfl groups at 3 dpf. From this, we assessed for differences in expression levels of genes, involved in signaling pathways known to influence neurogenesis and gliogenesis. Sonic hedgehog (Shh) pathway has been shown to have a featured role in dorsoventral patterning (Fuccillo, Joyner, & Fishell, 2006). Fibroblast growth factor (FGF) and retinoic acid (RA) have been shown to be involved in posterior fates of neural ectoderm (Kudoh, Wilson, & Dawid, 2002). Notch signaling has been described to promote gliogenesis while suppressing oligodendrocyte differentiation and to be involved in proliferation and apoptosis (Artavanis-Tsakonas, 1999; Fortini, Rebay, Caron, & Artavanis-Tsakonas, 1993; Grandbarbe, 2003; Kim et al., 2008). While Shh, FGF, and RA pathways seem to be
unaffected by the 36K knockdown in our microarray analysis (Figure S5a, Table S1), we could find a pronounced and significant up-regulation of notch (1.4-fold; \( p = .003 \)) (ZFIN ZDB-GENE-990415-173) and the well described Notch target her4 (1.7-fold; \( p = .043 \)) (Takke, Dornseifer, Weizsäcker, & Campos-Ortega, 1999; ZFIN ZDB-GENE-980526-521; Figure 6b,d). We found an up-

**FIGURE 5** 36K knockdown zfl have fewer dorsally migrated OPCs. (a) Representative image depicting lateral view of two spinal segments above the yolk extension at 3 dpf in Tg(olig2:GFP) zfl injected with control MO and MO1 showing dorsal and ventral spinal cord regions with a separation by dotted lines. (b,c) Number of olig2+ cells in two spinal segments (b) in the dorsal spinal cord and (c) in the ventral spinal cord. \( N = 3 \); \( n \) shown in each bar. For (b), Kruskal–Wallis ANOVA \( p \leq .01 \) (**) followed by Dunn’s multiple comparisons test for control MO versus MO1. For (c), Kruskal–Wallis ANOVA \( p > .05 \) (not significant). (d) Representative image depicting lateral view of two spinal segments above the yolk extension at 5 dpf in Tg(olig2:GFP) zfl injected with control MO and MO1. (e) Number of olig2 positive cells in two spinal segments in the dorsal spinal cord. \( N = 3 \); \( n \) shown in each bar. Kruskal–Wallis ANOVA \( p \leq .0001 \) (***) followed by Dunn’s multiple comparisons test for control MO versus MO1. (f) Western blot against 36K using untreated, methanol (control) and TSA treated whole larvae at 4 dpf with \( \beta \)-actin as loading control. There was no 36K band detectable in the TSA treated zfl. (g) Representative lateral view of two spinal segments above the yolk extension at 4 dpf in Tg(olig2:GFP) zfl treated with methanol (control) or TSA. There were no dorsally migrated OPCs detectable in the TSA treated zfl. (h) Representative images of sagittal paraffin sections of 4 dpf larvae injected with control MO and MO1 showing proliferation within the spinal cord labeled with anti-Pcna. Sections were counter stained with hematoxylin. Proliferating cells are marked by pink stars. There were more proliferating cells in the spinal cord of MO1 zfl compared to control MO. Scale bars: 50 \( \mu \)m. (j,k) Expression of (j) pcna and (k) casp3a in control MO and MO1 at 3 dpf from microarray analysis. \( N = 6 \). Unpaired two tailed \( t \) test with Welch’s correction. \( p \)-value .0450 (*) for pcna (j), \( p \)-value .9045 (not significant) for casp3a (k).
regulation in ligands binding to Notch like for example, deltaa (2.0-fold; \( p = .002 \); ZFIN ZDB-GENE-980526-29; Figure 6c), a major ligand that binds to Notch in addition to jagged1a (1.5-fold; \( p = 0.012 \); Louvi & Artavanis-Tsakonas, 2006; ZFIN ZDB-GENE-011128-2). In order to further verify these microarray analysis data, we performed RT-PCR experiments with different amplification cycles and could confirm up-regulations of her4 and deltaa but not notch1a mRNA in the 36K knockdown zfl (Figure S5b–e).

Since Notch signaling is known to influence the general fate of glial cells (Artavanis-Tsakonas, 1999; Fortini et al., 1993; Kim et al., 2008; Louvi & Artavanis-Tsakonas, 2006), TgBAC(gfap:gfap-GFP) zfl (Chen et al., 2009) were used to visualize astrocytes and radial glia. We found increased Glial fibrillary acidic protein (Gfap) expression linked to EGFP in the MO1 injected zfl in comparison to control MO injected zfl (Figure 6a,e,f). This suggests increased differentiation toward astrocytes instead of oligodendrocytes due to up-regulated Notch. The fate of these cells, which are generated by the enhanced proliferation mentioned earlier (Figure 5h,j) can therefore be explained by increased proliferation and differentiation toward astrocytes. This is also supported by an increase (2.0-fold; \( p = .001 \); ZFIN ZDB-GENE-001103-2) in astrocyte specific sox9 (Pompolo & Harley, 2001) expression as shown by microarray analysis (Table S1). To confirm

**FIGURE 6** 36K knockdown zfl have up-regulated Notch targets. (a) Representative images and enlarged selections (red squares) of sagittal paraffin sections depicting spinal cords of 4 dpf zfl injected with control MO and MO1; showing radial glial cells labeled with anti-Gfap antibodies. Sections were counter stained with hematoxylin. Scale bars: 50 \( \mu \)m. (b–d) Microarray analysis at 3 dpf showing up-regulation in MO1 of (b) her4.1 (c) deltaa and (d) notch1a. N = 6. Unpaired two tailed t test with Welch’s correction. p-value .043 (*) for her4.1 (b), p-value .002 (**) for deltaa (c), p-value .003 (***) for notch1a (d). (e) Representative lateral view of two spinal segments above the yolk extension in TgBAC(gfap:gfap-GFP) zfl at 3 dpf injected with control MO and MO1. Scale bars: 40 \( \mu \)m. (f) Mean fluorescence intensity of gfap:gfap-GFP in the spinal cord from average projections acquired from a z stack of 125 \( \mu \)m. Sample sizes: \( N = 4 \); n shown in each bar. Unpaired two tailed t test with Welch’s correction p-value .0032 (**). (g,h) Representative lateral view of four spinal segments above the yolk extension in Tg(-8.4ngn1:GFP) zfl at (g) 3 dpf and (h) 5 dpf injected with control MO and MO1. Ngn + DRG cells are marked by pink stars. Scale bars: 40 \( \mu \)m. (i,j) Fewer ngn1+ DRG neurons in 4 spinal segments in MO1 zfl at (i) 3 dpf and (j) 5 dpf. Sample sizes: \( N = 3 \); n shown in each bar. For (i), Mann–Whitney two tailed test p-value .0008 (**). For (j), Mann–Whitney two tailed test p-value .02760 (*)
FIGURE 7  Legend on next page.
that the increased Gfap expression in MO1 zfl is not because of reactive gliosis, we analyzed macrophages and microglial genes from the microarray analysis and found no change here (Table S1). Dorsal root ganglion (DRG) cells are negatively regulated by Delta-Notch signaling (Cornell & Eisen, 2002). Using Tg(-8.4gn1:GFP) zfl (Blader et al., 2003), we observed significantly fewer (~50%) DRG cells in the MO1 group at 3 dpf as well as at 5 dpf in four analyzed spinal segments (Figure 6g–j). However, motor neurons and myelination in PNS remained unaffected in MO1 zfl (Figure S5a and Figure S6a, Table S1). In order to examine if axonal presynaptic innervation is affected, zfl were stained with Sv2 antibody, a marker for presynaptic terminals (Jonz & Nurse, 2003; Wan et al., 2010) and no obvious difference was found in the MO1 group (Figure S6b). As F3/Contactin is a binding partner for oligodendrocyte Notch (Hu et al., 2003), we also analyzed the expression of contactin from the microarray but found it not to be altered (Table S1). To investigate if Notch up-regulation could be due to miR-132 regulation through the previously described miR-132/Ctbp2 circuit (Salta et al., 2014), we scanned for miR-132, Ctbp2, Sirt1 from our microarray data and also analyzed miR-132 by q-rt-PCR (Figure S6c,d), but found no significant changes here.

3.7 36K knockdown can be rescued by gamma secretase inhibition

Notch receptors can interact with membrane bound ligands from neighboring cells only after Furin site S1 cleavage (Blaumueller, Qi, Zagouras, & Artavanis-Tsakonas, 1997; Kopan, Schroeter, Weintraub, & Nyet, 1996). After a ligand binds to Notch, a conformational change is induced and an ADAM (a disintegrin and metalloprotease) dependent cleavage occurs at site 2 (S2) in the Notch extracellular domain (Brou et al., 2000; Mumm et al., 2000; Geert van Tetering et al., 2009). Following this S2 cleavage, the remaining Notch extracellular truncation targets (NEXT) can be cleaved by presenilin dependent gamma secretase within the outer cell-membrane and the residual Notch intracellular domain (NICD) translocates into the nucleus and regulates the expression of transcription factors (Brou et al., 2000; Louvi & Artavanis-Tsakonas, 2006; Mumm et al., 2000; van Tetering & Vooijs, 2011).

N-(3,5-difluorophenacetyl)-l-alanyl-S-phenylglycine t-butyler ester (DAPT), a well-established gamma secretase inhibitor, has been shown to inhibit the cleavage of Notch to generate NICD and hence indirectly inhibits the activation of the Notch pathway (Geling, 2002). MO injected zfl were bath immersed with DAPT in embryo medium from 24 hpf onward (Figure 7a) so that the period of gliogenesis is covered although initial motor neuron genesis is unaffected as explained in (Salta et al., 2014). The reduced body length phenotype could be rescued partially with 5 μg/ml DAPT (Figure 7b). The number of dorsally migrated olig2 positive cell was fully rescued in the DAPT treated MO1 group in comparison to DMSO treated MO1 group zfl (Figure 7c,d).

Since the 36K knockdown phenotypes can be rescued with a gamma secretase inhibitor, we aimed to further validate increased access/activity of intra membrane gamma secretase cleavage in our MO1 zfl. Due to the lack of suitable antibodies to directly study increased NEXT cleavage by gamma secretase to release NICD in these zfl, we assessed the cleavage of Amyloid precursor protein (App) instead (Krishnaswamy, Verdile, Groth, Kanyenda, & Martins, 2009). Following alpha or beta secretase cleavage of App, gamma secretase cleaves the remaining C terminal fragments (CTFs) further to release the App intracellular domain (AICD). We found decreased CTF and increased AICD in MO1 zfl (Figure 7e,f). This further supports enhanced gamma secretase cleavage in 36K knockdown fish, which is in addition to the up-regulated Notch ligands (deltaa) upstream of gamma secretase cleavage as described above (Figure 6c, Figure S5, Table S1).

Some short chain dehydrogenases (SDRs) have been shown to be involved in lipid metabolism (Kavanagh, Jörnvall, Persson, & Weintraub, 2009). Following this S2 cleavage, the remaining Notch extracellular truncation targets (NEXT) can be cleaved by presenilin dependent gamma secretase within the outer cell-membrane and the residual Notch intracellular domain (NICD) translocates into the nucleus and regulates the expression of transcription factors (Brou et al., 2000; Louvi & Artavanis-Tsakonas, 2006; Mumm et al., 2000; van Tetering & Vooijs, 2011).

**36K knockdown phenotypes can be rescued with gamma secretase inhibitor DAPT.** (a) Timeline showing MO injections at 0–1 hpf, DAPT bath immersion treatment from 24 hpf onward, body length measurements at different time points and olig+ gfp-imaging at 3 dpf. (b) Body length of larvae from different groups at 3, 4, and 5 dpf. Sample sizes: N = 3; n shown in each bar. Two-way ANOVA p ≤ .0001 (****) followed by Tukey’s multiple comparisons test for DMSO MO1 versus DAPT MO1 at different time-points. (c) Representative lateral view of two spinal segments above the yolk extension at 3 dpf in Tg(olf2:GFP) zfl injected with MO1 and treated with DMSO or DAPT. Scale bar: 20 μm. (d) Number of olig2+ cells within two spinal segments in the dorsal spinal cord. Sample sizes: N = 3; n shown in each bar. One-way analysis of variance ANOVA p ≤ .0001 (****) followed by Tukey’s multiple comparisons test for DMSO MO1 versus DAPT MO1. (e) Western blot of different whole zfl at 4 dpf showing App intracellular domain (AICD), cleaved App (CTF), and full-length App (FL) and β-actin for loading control. (f) Quantification of App AICD band strength comparing control MO versus MO1 zfl in different anti-App Western blots normalized to β-actin. Sample sizes: N = 5. Unpaired two tailed t test with Welch’s correction. p-value .0366 (*). (g) Representative TLC showing sphingolipids in control MO and MO1 of 5 dpf zfl together with commercial standards. Weaker band in MO1 comigrating with cerebrosides (GalC) standard is marked by red box. (H) LC–MS/MS analysis of Cer, HexCer, Hex2Cer, and SM4s in Control MO and MO1 larvae at 5 dpf. N = 4. (I) β-actin from our microarray data and also analyzed miR-132 by q-rt-PCR (Figure S6c,d), but found no significant changes here.
Oppermann, 2008; Persson et al., 2009). Since 36K belongs to the SDR family (Morris et al., 2004), we further hypothesized that the distribution of transmembrane Notch ligands as well as the activity of intra membrane gamma secretase cleavage of diverse transmembrane proteins like Notch or APP is altered due to a change in membrane lipids. We therefore performed thin layer chromatography (TLC) to assess changes in lipid composition between control MO and MO1 zfl groups. In total lipids, we did not observe obvious differences (data not shown), however, when sphingolipids were analyzed at 5 dpf, in 36K knockdown zfl we reproducibly observed a weaker band for a lipid comigrating with cerebrosides standard (GalC; Figure 7g, Figure S7a). Analysis of ceramides, hexosylceramides, dihexosylceramides (likely LacCer), and sulfatides (SM4a) by liquid chromatography—tandem mass spectrometry (LC—MS/MS) did not reveal any significant overall changes (Figure 7h). However, the ratio of HexCer containing a saturated sphingoid base (sphinganine) over those containing a monounsaturated sphingoid base (sphingosine) dropped down to two thirds in the 36K MO1 injected zfl (Figure 7i).

4 | DISCUSSION

In this study, we investigate the function of 36K during developmental myelination in the CNS of zebrafish. 36K is one of the most abundant proteins in zebrafish brain white matter (Gebril et al., 2014). In contrast to humans and other mammals, zebrafish can successfully and functionally regenerate and remyelinate CNS axons following injury (Becker & Becker, 2008; MüNZel et al., 2012; MüNZel, Becker, Becker, & Williams, 2014). Although the presence of 36K protein in humans is only hypothetical, its mRNA has been found in CNS white matter (Morris et al., 2004). Thus, it is important to understand the specific role of 36K in zebrafish myelin. We suggest two functions for 36K, one at an early time-point in oligodendrocyte differentiation as described in this study, and possibly another subsequent yet uncharacterized structural function in compaction of CNS myelin. In addition to the expression of 36K in mature oligodendrocytes and compact myelin (Morris et al., 2004), we could detect early expression of 36K in some olig2+ OPCs, in the developing brain-stem region already at 3 dpf and in the spinal cord at 5 dpf. We speculate that for the early developmental function of 36K, only a little amount of protein/enzyme is necessary, whereas for its structural function a large amount of protein might be needed in compact myelin. Since 36K belongs to the short chain dehydrogenase family (Morris et al., 2004), and some SDR have been shown to regulate lipid metabolism (Kavanagh et al., 2008; Persson et al., 2009), we propose that 36K is involved in lipid metabolism as 36K knockdown larvae show alterations in membrane lipid composition. We assume that this lipid alteration, together with the increase in Notch ligands, is responsible for the enhanced activity of prerequisite intramembrane cleavage of gamma secretase substrates and the final cleavage by gamma secretase itself. This causes an up-regulation of Notch signaling and hence influencing oligodendrocyte differentiation as shown in this study (Figure 7j).

In 36K knockdown larvae, the ratio of hexosylceramides containing sphinganine to sphingosine is decreased. This could be either due to an increase in hexosylceramides containing sphingosines or a reduction of hexosylceramides containing sphinganines. The actual molecular mechanism underlying this ratio change is yet to be characterized. Since we do find lipid alterations in 36K knockdown larvae with TLC, we think it is reasonable to suggest that 36K regulates membrane lipid metabolism although this could not yet be characterized at molecular levels. Further work is certainly necessary to estimate to what extent this assumption holds true. Here the main question would be, if 36K either is directly involved in lipid regulation, or whether the changes in lipid composition we describe in this study are secondary to a change or relocation in cell fate during early CNS development.

This means that oligodendrocyte to astrocyte cell fate changes which have been described in this study might be caused by 36K acting on Notch signaling in another way yet to be identified. Furthermore, one cannot completely rule out that Notch up-regulation might solely be influenced by an increase in Notch ligands, which, to a certain extent, has also been observed in our study. It is also possible, but not further investigated, that changes in membrane lipid composition have a direct effect on the distribution and efficacy of Notch ligands within the membrane. The enhanced release of intracellular domain by gamma secretase cleavage could only be shown directly for App but not for Notch. Though increase in Notch ligands should not influence the cleavage in the case of App, on the other hand we cannot completely rule out that the 36K knockdown might influence the prerequisite App processing and, in the end, resulting in increased AICD. Owing to the lack of a suitable Notch antibody in zebrafish we cannot establish if there really exists a sufficient fraction of un-cleaved NEXT in the endogenous wild type situation that could then act as additional substrate for enhanced gamma secretase activity in the knock down situation. On the other hand, increased Notch ligands in the knockdown zfl will possibly increase the availability of NEXT as a substrate needed for gamma secretase. As several reports have shown a connection between lipid alterations and their effect on the activity of gamma secretase within the membrane (Holmes, Paturi, Ye, Wolfe, & Svennerholm & Gottfries, 1994), we so far strongly believe that this might be the case in 36K knockdown as well. The precise molecular mechanisms of how and what lipid alterations does affect gamma secretase in our case is currently unknown and would need further investigation then.

Disruptions in genes involved in ganglioside synthesis have been shown to cause myelination defects (Sheikh et al., 1999). The myelination defects in 36K knockdown zfl suggest a possible deficiency in axonal action potential conduction due to which, the zfl are less responsive than controls in stimulated behavior tests. Reduced startle responses have also been observed in hypo-myelinated mice models (Poggi et al., 2016; Tanaka et al., 2009), which would further support our hypothesis. It might be due to various reasons that only in about 30% of the larvae this startle response discrepancy is observed, for example, myelination is strongly impaired but there is still always
some remaining myelination present in our knockdown model larvae. Furthermore 36K knockdown zf1 swim shorter distance in unprovoked swim tests. However, if 36K knockdown larvae do respond in our stimulated swim tests, they swim equal initial distances as their controls. Our observations therefore neither point toward changes in motor neurons as also shown from microarray analysis nor presynaptic axonal innervation as depicted by anti-Sv2 staining. If motor neurons would have been affected, the larvae would not swim equal distances like controls responding to a stimulus (Sonnack et al., 2015). Altogether this argues in favor of neural conduction and myelination defects rather than muscular defects being causative in 36K knockdown.

Our observations suggest that 36K plays a major role in oligodendrocyte differentiation, as we observe significantly fewer dorsally migrated olig2+ OPCs in 36K knockdown larvae. There is disrupted and less Tg(mbp:EGFP-caax) positive myelin and expression of myelin genes like mbpa and plp1b is reduced in 36K knockdown zfl. This can be explained by OPCs failing to migrate and differentiate into oligodendrocytes in 36K knockdown larvae. Possibly still, it cannot be ruled out that there might solely be an initial migratory defect in the 36K knockdown larvae OPCs and hence for this reason only further differentiation and maturation into myelinating oligodendrocytes is disrupted. On the other hand, we found increased cell proliferation in the CNS of 36K knockdown larvae, and no change in apoptotic cell numbers. This increase can be reasoned by up-regulated Notch signaling that has been found to promote gliogenesis of astrocytes or radial glia while suppressing oligodendrocyte differentiation (Artavanis-Tsakonas, 1999; Fortini et al., 1993; Grandbarbe, 2003; Kim et al., 2008). In accordance with this, we observe increased glial Gfap and sox9 expression in 36K knockdown larvae. As we do not find an up-regulation in genes related to immune response, this increase in Gfap is most likely due to a cell fate change leading to increased astrocytes or radial glia rather than reactive astrogliosis.

Likewise the development of oligodendrocytes from olig2+ OPCs is known to be favored by down-regulation of Delta Notch signaling (Louvi & Artavanis-Tsakonas, 2006; Park & Appel, 2003). DAPT has been shown to indirectly down-regulate Notch by inhibiting gamma secretase (Geling, 2002; Salta et al., 2014). When larvae were treated with DAPT at a concentration known to inhibit Notch up-regulation (Salta et al., 2014), dorsally migrated olig2+ OPC numbers and shorter body length phenotype in 36K knockdown zfl could be rescued while control MO injected larvae remained unaffected. This further suggests an involvement of endogenous 36K early in oligodendrocyte development by inhibiting Notch signaling at the time of initial oligodendrocyte differentiation. A direct or indirect involvement of miRNA-132 in the regulation of oligodendrocyte or glial fate as described in (Salta et al., 2014) linked to the function of 36K could not be found in our study.

Mutations in pescadillo, a gene involved in ribosome biogenesis and proliferation and further described to be required for oligodendrocyte differentiation, have been associated with a shorter body length phenotype (Simmons & Appel, 2012). The length phenotype could be partially rescued when 36K knockdown larvae were treated with DAPT. Hence it might also be attributed to Notch upregulation, as Notch is known to be involved in many other developmental processes.

Custom-made antibodies raised specifically against zebrafish 36K could confirm the localization of 36K mainly in CNS tissue, which is in accordance with previous findings (Morris et al., 2004). The specificity of our custom-made antibodies could be demonstrated in transfected cell culture experiments and also by colocalizations with olig2:GFP expression. We designed two independent translation blocking MOs to knock down the expression of 36K. MO knockdown of 36K could be confirmed by Western blot and immunostaining. 36K knockdown phenotypes including a shorter body length, fewer fish responding in the stimulated behavior test and fewer dorsally migrated OPCs, could be rescued when co-injected with 36K mRNA to which the MO could not bind anymore, signifying the specificity of the MOs. Morpholino injections which induce p53 expression have been described to indicate off-target effects (Robu et al., 2007; Rossi et al., 2015). We did not observe an induction of p53 expression in the MO1 injected larvae, minimizing the possibility of off-target effects. To our knowledge, there is currently no knock out fish line for 36K available. We tried generating CRISPR-Cas9 mutants for 36K, but already failed to prove initial genome editing in the injected larvae. Generating a knockout mutant for 36K would have possibly allowed us to follow the function of 36K at later time points in compaction of myelin as well as in remyelination. We speculate that a complete knockout of 36K might not be viable and would suggest an inducible cell type specific knock-out strategy for the future. This however would involve the challenge of identifying a CNS specific OPC and oligodendrocyte promoter, or rather the 36K promoter itself. Although injecting the Morpholino in a null mutant background to completely rule out any off-target effects would have further confirmed the specificity of the MOs, the usage of multiple MOs against the same target and rescue experiments strongly confirm the specificity of our observations.

Alternatively, to get hands on a zf1 model without differentiated oligodendrocytes, we treated the larvae with TSA, a histone deacetylase inhibitor which inhibits migration and differentiation of oligodendrocytes (Takada & Appel, 2010). In this model we could confirm absence of migrating and differentiating OPCs, as well as absence of 36K in the treated larvae as shown by Western blot. Altogether, presence of 36K in compact myelin (Morris et al., 2004), its significantly high abundance in adult zebrafish brain (Gabriel et al., 2014), and complete absence of 36K when there are no differentiated oligodendrocytes after TSA treatment, suggest a later structural function for 36K possibly in compaction of myelin even after the stage of initial development. This structural function in compaction of myelin could be similar to the role of Mbp in the major dense line as previously suggested by (Jeserich & Rauen, 1990) since both of them are highly basic proteins (Morris et al., 2004).

In conclusion, our observations demonstrate a role for 36K in oligodendrocyte differentiation through Notch signaling pathway, in addition to a yet unknown possible structural function later in compact myelin. Notch signaling is affected by 36K knockdown in two possibly interconnected ways. First, this involvement is most likely
due to an enzymatic role of 36K in lipid metabolism. Since membrane lipid composition is altered in 36K knockdown zfl, gamma secretase activity is enhanced and Notch signaling is up-regulated. Second, this is due to an up-regulation of Notch ligands in 36K knockdown zfl, although the direct cause for the transcriptional up-regulation of these ligands remains unknown. Both of these will lead to an up-regulation of Notch signaling. In turn, fewer oligodendrocytes differentiate, leading to less myelin in 36K knockdown larvae. Understanding the function of 36K in zebrafish myelin improves the background knowledge for usage of zebrafish as a model for CNS myelination. This could eventually provide further opportunities to better understand demyelinating diseases and remyelination in human CNS.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ACKNOWLEDGMENTS

We are grateful for NRW Repatriation grant to B.O. and a German Research Foundation (DFG) equipment grant (INST 1172/37-1 FUGG) for a multi-photon microscope setup. This work was further supported by a DFG grant to U.K. (KU2474/13-1). We thank Prof. Dr. D. Lyons and Prof. Dr. C. G. Becker, Edinburgh and the European Zebrafish Resource Centre (EZRC), Karlsruhe for transgenic fish lines. We are thankful to the Zebrafish Core Facility (Bonn Medical Faculty) and BIGS Neuroscience Graduate School. We acknowledge Dr. N. Garbow, PerkinElmer for the support with the EnSight© multimode plate reader. We thank Prof. Dr. K. Schilling, Prof. Dr. E. Kostenis, Prof. Dr. S. Baader, Dr. A. Toledo, all members of the Odermatt lab and the group of Prof. Dr. V. Stein for support and helpful discussions.

AUTHOR CONTRIBUTIONS

Project conception, writing: B.N. and B.O.; Imaging: B.N., A.H.; Immunostaining: B.N., A.J., Ö.Y; EnSight© measurements: B.N., F.H., E.M.; Lipids: B.N., M.E., R.S.; TEM data: B.N., A.Z., D.H.; Establishing q-rt-PCR: B.N., H.K.; mir-132 qPCR: B.N., A.C., B.E.; Review and editing: B.N., B.O., A.H., M.E., R.S., D.H., B.E.; Resources: B.O., U.K., M.E., R.S., D.H., B.E.; Supervision: B.O., U.K.

DATA AVAILABILITY STATEMENT

Microarray data and all other relevant data are available within the article or from the authors upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: NAGARAJAN ET AL. (2020). CNS myelin protein 36K regulates oligodendrocyte differentiation through Notch. Glia. 2020:68:509–527. https://doi.org/10.1002/glia.23732