Comparison of zebrafish and mice knockouts for Megalencephalic Leukoencephalopathy proteins indicates that GlialCAM/MLC1 forms a functional unit

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Abstract

Background: Megalencephalic Leukoencephalopathy with subcortical Cysts (MLC) is a rare type of leukodystrophy characterized by astrocyte and myelin vacuolization, epilepsy and early-onset macrocephaly. MLC is caused by mutations in MLC1 or GLIALCAM, coding for two membrane proteins with an unknown function that form a complex specifically expressed in astrocytes at cell-cell junctions. Recent studies in Mlc1−/− or Glialcam−/− mice and mlc1−/− zebrafish have shown that MLC1 regulates glial surface levels of GlialCAM in vivo and that GlialCAM is also required for MLC1 expression and localization at cell-cell junctions.

Methods: We have generated and analysed glialcama−/− zebrafish. We also generated zebrafish glialcama−/− mlc1−/− and mice double KO for both genes and performed magnetic resonance imaging, histological studies and biochemical analyses.

Results: glialcama−/− shows megalencephaly and increased fluid accumulation. In both zebrafish and mice, this phenotype is not aggravated by additional elimination of mlc1. Unlike mice, mlc1 protein expression and localization are unaltered in glialcama−/− zebrafish, possibly because there is an up-regulation of mlc1 mRNA. In line with these results, MLC1 overexpressed in Glialcam−/− mouse primary astrocytes is located at cell-cell junctions.

Conclusions: This work indicates that the two proteins involved in the pathogenesis of MLC, GlialCAM and MLC1, form a functional unit, and thus, that loss-of-function mutations in these genes cause leukodystrophy through a common pathway.

Keywords: MLC1, GLIALCAM, Megalencephalic leukoencephalopathy, Myelin, Astrocyte, Zebrafish

Background

One of the most important functions that astrocytes perform is buffering the increase in potassium that occurs during neuronal firing to help restore baseline conditions [1]. Astrocytes buffer excess potassium through different pathways in a still undefined manner: mainly via the Na+, K+, ATPase pump, but also using the Na+, K+, Cl− co-transporter, the potassium channel Kir4.1 and through gap-junction dependent processes [2]. It has also been suggested that the CIC-2 chloride channel may play a role in glial potassium accumulation [3, 4]. Animal models deficient in proteins involved in this process (Kir4.1, CIC-2, Cx32/Cx47, Cx30/Cx43) show several defects in potassium clearance, increased neuronal excitability and presence of vacuoles in myelin [5–8]. Since water movement is parallel to ion flow, it is possible that vacuoles are a consequence of an impaired ion uptake. Additionally, potassium and water entry into astrocytes also causes cellular swelling. A swelling-
dependent chloride channel named VRAC (for Volume-Regulated Anion Channel) strongly expressed in astrocytes is then activated, releasing chloride and osmolytes from the cell, thus changing the driving force for water movement and restoring the astrocyte's original size [9].

A similar phenotype to what is present in knockout animals of genes involved in potassium clearance [5–8] has been observed in patients affected with Megalencephalic Leukoencephalopathy with subcortical Cysts (MLC), a rare type of leukodystrophy [10]. MLC is characterized by astrocyte and myelin vacuolization, epilepsy and early-onset macrocephaly [11]. The epilepsy and the presence of vacuoles in MLC patients suggested a possible defect in potassium handling [10]. MLC is caused by mutations in either MLC1 [12] or GLIALCAM [13]. MLC1 encodes for a membrane protein with eight predicted transmembrane domains (MLC1), which is specifically expressed in astrocytes at cell-cell junctions, including the Bergmann glia of the cerebellum and highly enriched in their perivascular endfeet contacting the blood brain barrier (BBB) [14, 15]. GlialCAM is an adhesion molecule of the immunoglobulin superfamily expressed predominantly in astrocytes and oligodendrocytes [15, 16].

The pathophysiological mechanisms leading to MLC are unclear [17]. Apart from the phenotype of MLC patients, some experimental evidence suggest that GlialCAM/MLC1 have a role in potassium clearance: i) GlialCAM is an auxiliary subunit of the CIC-2 chloride channel [18]. GlialCAM makes CIC-2 an ohmic channel due to a change in its gating mechanism [19], which allow mediating chloride influx at depolarized potentials [15], as expected for a chloride channel involved in potassium uptake; ii) in astrocyte cultures, localization of GlialCAM, MLC1 and CIC-2 at cell-cell junctions depend on extracellular potassium [20]; iii) mice models deficient for Mlc1 or Glialcam display altered brain potassium dynamics [21] and iv) astrocytes deficient in MLC1 or GlialCAM show reduced VRAC activity [22–24]. Even though this experimental evidence suggested the involvement of MLC1 and GlialCAM proteins in potassium uptake, the molecular basis of these defects is unclear, as the precise functions of MLC1 of GlialCAM are still unknown.

The biochemical relationships between MLC1 and GlialCAM are also not well defined. In cultured cell lines such as HeLa cells, MLC1 cannot reach cell junctions without GlialCAM, whereas GlialCAM expressed alone is located at cell-cell junctions [25]. In agreement with this in vitro data, mice deficient in Glialcam show a mislocalization of Mlc1 [15, 16]. On the other hand, MLC1 expressed alone in cell lines can reach the plasma membrane [26–28], while in Glialcam knockout mice, Mlc1 is not present at the plasma membrane and Mlc1 protein levels are reduced [15, 16]. Considering that in primary astrocytes, GlialCAM improves the plasma membrane localization of MLC1-related mutants of MLC1 that present folding defects, it has been suggested that GlialCAM has two putative roles: bringing MLC1 at cell-cell junctions and stabilizing MLC1 [22].

Unexpectedly, both mice [14, 15] and zebrafish [29] deficient in MLC1 also show a mislocalization of GlialCAM in astrocytes and oligodendrocytes. However, this mislocalization is observed in Bergmann glia [29] but not in astrocytes surrounding blood vessels [25] in humans. Furthermore, in astrocyte cultures from Mlc1−/− mice, GlialCAM is not mislocalized, but it loses its localization at cell-cell junctions after incubating astrocytes with a depolarizing solution [29]. According to this, it has been suggested that the mislocalization of GlialCAM when MLC1 is not present depends on the extracellular potassium concentration by an undefined mechanism involving signal transduction processes [20, 23, 30, 31].

In summary, although MLC1 and GlialCAM proteins form a complex located at cell-cell junctions, the biochemical role of each protein in this complex is not well defined. In the present work, with the aim of understanding this relationship, we have generated and analyzed zebrafish deficient in glialcama as well as zebrafish and mice deficient in both proteins. Two orthologous genes for GlialCAM have been described in zebrafish (glialcama and glialcamb), although previous results suggested that glialcama is the orthologous gene of GLIALCAM [29]. The characterization of these models has provided new insights into the molecular basis of GlialCAM and MLC1 interactions.

Methods
Zebrafish maintenance
Zebrafish were kept at the animal facility in Bellvitge Campus, University of Barcelona, under standard conditions at 28 °C, 14 h/10 h light/dark period. AB or AB/TL strains were used in all the experiments. All experimental procedures conformed to the European Community Guidelines on Animal Care and Experimentation and were approved by animal care and use committees.

Generation of glialcama knockout zebrafish
We designed a pair of TALE nucleases to target two sequences at the beginning of glialcama exon1: TGGTCT CTAAGTGAGGGCA (where the start codon is underlined) and TGAAAGAATGGCTGTCTC, leaving a 20 bp spacer: GAGCAGGAGGATCATGCAA (BsrBI restriction site underlined). Plasmids containing the TALE nucleases were synthesized by GeneArt (then Life Technologies), and then cloned by Gateway into pCS2-destination vector. Plasmids were linearized with KpnI
and mRNAs were synthesized with mMessage mMACHINE (Ambion). One hundred pg of each TALE Nuclease mRNA were injected into one cell zebrafish embryos, DNA was isolated from pooled embryos at 3dpf and the target sequence amplified with the following primers: GCCCTGAGTGACAATTAT and AAACGTACACACGTCGACAC to check if the BsrBI restriction site was lost due to the action of the TALE nucleases and the subsequent mistakes made by the cellular repair mechanisms. The remaining embryos were raised to adulthood and crossed with wild-type animals. The heterozygosity of their offspring was confirmed by PCR and High Resolution melting Analysis (HRMA) on a StepOne PCR machine (Invitrogen). These F1 embryos were raised to adulthood, tail clipped and genotyped. PCR products were cloned by TA cloning into the pGEM-T vector (Promega). Individual colonies were sequenced using T7 and SP6 primers to characterize the mutations generated.

Molecular biology
Plasmids used were constructed using standard molecular biology techniques employing recombinant PCR and the multisite gateway system (Life Technologies). The integrity of all cloned constructs was confirmed by DNA sequencing.

RT-PCR
Adult zebrafish were euthanized using an overdose of tricaine (MS222, Sigma). Adult tissues were quickly dissected and flash-frozen in liquid nitrogen. Total RNA was isolated with TRIzol and retrotranscribed using random hexamers with the SuperScript IV system (Life Technologies). The oigonucleotides pairs used for qPCR are the following: Rpl13a (internal control), sense: TCTGGAGGACTGTAAGAGGTATGC, anti-sense: TC TGGAGGACTGTAAGAGGTATGC; mlc1, sense: GCA CGTTCAGTGGACAACTG, anti-sense: CACAAATCAT TGGGCTTCAG; glialcama, sense: CCCACCCACC AAGACTAAGC, anti-sense: CATCCTCACTGGAACGC CATCTG; glialcamb, sense: AGACCGGATCTTGG TGTTTGA, anti-sense: TAGGCTCATCCACAGTAGTA GATTGA.

qPCR was performed with SYBR Select reagent (Life Technologies) in a StepOne apparatus (Life Technologies). Three experiments were analyzed, with three replicates in each experiment. The expression levels were normalized using the comparative Ct method normalized to the internal control genes. The final results were expressed as the relative messenger RNA (mRNA) levels as indicated in the corresponding figures, taking into account the efficiency of each primer with the Pfaffl method.

Histological staining methods in zebrafish
Fish were deeply anesthetized in 0.1% tricaine methanol sulfonate (Sigma, MS-222) in fresh water and fixed by vascular perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Fish heads were post-fixed in the same fixative for at least 24h at room temperature. Next, brains and eyes were extracted, cryopreserved in 30% sucrose in PB, frozen with liquid-nitrogen-cooled methylbutane and cut in a cryostat. Transverse sections (12–14 μm thick) were collected onto gelatinized slides.

For immunohistochemistry, sections were rinsed in saline phosphate buffer (PBS) and sequentially incubated at room temperature with: (1) normal goat serum (NGS, Sigma, 1:10 in PBS) for 1 h; (2) primary antibody or cocktail of primary antibodies, overnight (for antibodies and dilutions, see below); (3) PBS for 15 min; (4) secondary fluorescent antibody or cocktail of fluorescent antibodies for 1 h (for antibodies and dilutions, see below); (6) PBS for 15 min. Incubations with primary and secondary antibodies were made at room temperature in a humid chamber. Finally, sections were mounted using 50% glycerin in PB.

Primary antibodies and dilutions used in the study were: rabbit anti-zebrafish mlc1 (1:100) and rabbit anti-zebrafish glialcama (1:100). The secondary antibody used was goat anti rabbit- Alexa Fluor 488 (Invitrogen, 1:500). All dilutions were done in 10% NGS in PBS. Negative controls omitting incubation with primary antibody were performed, showing no unspecific immunoreactivity.

Sections were first observed in a Nikon Eclipse Fluorescent microscope and then selected sections of were imaged in a Nikon A1R confocal microscope. Confocal and fluorescent data was processed and analysed using ImageJ software.

MRI imaging in zebrafish
Magnetic resonance microimaging (μMRI) of Zebrafish was performed on a vertical wide-bore 7 T Bruker Avance 300WB spectrometer, with a 1000 mT-m⁻¹ actively shielded imaging gradient insert (Bruker Biospin GmbH, Germany). The system was interfaced to a Linux PC running Topspin 2.0 and ParaVision 3.2 software (Bruker Biospin GmbH, Germany). For RF excitation and detection, a birdcage radio-frequency (RF) coil with an inner diameter 10 mm was used. For μMRI, adult zebrafish were euthanized and fixed in 4% buffered paraformaldehyde (Zinc Formal-Fixx, ThermoShandon, UK) for 7 days and subsequently embedded in Fomblin (Solvay Solexis, Inc.) to avoid any artefacts that may arise due to magnetic susceptibility differences at air–tissue boundaries. The magnetic field homogeneity was optimized by shimming before each μMRI measurement. For position determination and selection of the desired region, each session of measurements began with a
multislice orthogonal gradient-echo sequence. Subsequently, high resolution $T_2$ weighted images were acquired by using a rapid acquisition with relaxation enhancement (RARE) sequences with repetition time (TR) = 3000 ms; effective echo time (TE) = 18 ms; RARE factor = 4; slice thickness 0.2 mm; field of view $1.2 \times 1.2$ mm; image matrix of $256 \times 256$ pixels, resulting in a spatial resolution of 47 μm.

For transverse relaxation time ($T_2$) measurement, a standard multi-slice multi-echo (MSME) sequence was used. This sequence is based on the Carr-Purcell Meiboom-Gill (CPMG) sequence, where transverse magnetization of a 90° pulse is refocused by a train of 300° pulses generating a series of echoes. The following imaging parameters were used: nominal flip angles = 90° and 180°, and a train of 12 echoes with TEs ranging from 8.17 ms to 98 ms with 8.17 ms echo-spacing; TR = 2 s, slice thickness 0.5 mm; number of slices 8 and a matrix size $256 \times 256$ pixels.

For calculation of $T_2$ relaxation time, regions of interest (ROIs) were drawn at various locations within the zebrafish brain using an image sequence analysis (ISA) tool package (Paravision 5, Bruker). Another ROI in the muscle was used as an internal control. Monoexponential fitting was then used to calculate $T_2$ using a mono-exponential fit function [$y = A + C \exp(−t/T_2)$], where $A$ = Absolute bias, $C$ = signal intensity, $T_2$ = transverse relaxation time. Means and standard deviation for $T_2$ relaxation times for each ROI were calculated.

For measurement of brain areas, the desired telencephalon and whole brain regions were drawn on the image and areas were computed using an image sequence analysis (ISA) tool package (Paravision 5, Bruker). The data were exported to OriginPro v. 8 (OriginLab, Northampton, MA, USA) for further analysis and percentage of Telencephalon with respect to whole brain area was calculated. One-way ANOVA (Bonferroni’s post-test) for comparison of mean between each group was performed. Levene’s test was performed for homogeneity of variance analysis.

**Mouse studies**

The generation of Glialcam$^{-/-}$ and Mlc1$^{-/-}$ mice has been previously described [15]. For histological analyses of brains, mice were perfused with 4% PFA/PBS and organs were postfixed overnight. Haematoxylin–eosin staining was performed on 6 μm paraffin sections of brains.

Mouse primary astrocyte cultures were prepared from cortex and hippocampus, which were removed from newborn mice. Astrocyte cultures were prepared from 0 to 1 day old OF1 mice. Cerebral cortices were dissected and the meninges were carefully removed in cold sterile 0.3% BSA, 0.6% glucose in PBS. The tissue was trypsinized for 10 min at 37 °C and mechanically dissociated through a small bore fire-polished Pasteur pipette in complete DMEM medium (Dulbecco’s Modified Eagle’s Medium with 10% heat-inactivated fetal bovine serum (Biological Industries), 1% penicillin/streptomycin (Invitrogen) and 1% glutamine (Invitrogen) plus 40 U/ml DNase I (Sigma)). The cell suspension was pelleted and re-suspended in fresh complete DMEM, filtered through a 100-μm nylon membrane (BD Falcon) and plated into 75 cm$^2$ cell culture flasks (TPP). When the mixed glial cells reached confluence, contaminating microglia, oligodendrocytes and precursor cells were dislodged by mechanical agitation and removed. Astrocytes were plated in 6-well plates, at density of 4·10$^5$ cells per well, or in poly-D-lysine-coated cover slips at 7.5·10$^4$cells in 24-well plates. Medium was changed every 3 days. In order to obtain astrocyte cultures arrested in the cell cycle, medium was replaced and cytosine β-D-arabinofuranoside (AraC, Sigma) (2 μM) was added. Cultured astrocytes were identified by their positive GFAP (Glial Fibrillary acid protein) staining (Dako), being > 95% of cells GFAP positive.

For Western blot studies, astrocyte lysates were prepared by homogenization of cells in PBS containing 1% Triton X-100 and protease inhibitors: 1 μM Pepstatin and Leupeptin, 1 mM Aprotinin and PMSF, incubated for 1 h at 4 °C and centrifugated. Supernatants were quantified using BCA kit (Pierce) and mixed with SDS loading sample buffer. After SDS PAGE, membranes were incubated with primary antibodies: anti-MLC1 (1:100), anti-GlialCAM (1:100) and anti-β-Actin (1:10000, Sigma) and secondary antibodies: HRP-conjugated anti-rabbit and anti-mouse (1:10000; Jackson). Quantification of Western blots was performed by ImageJ at different exposition times to ensure linearity.

**Results**

**Generation and characterization of zebrafish glialcama knockout**

We previously described that the teleost-specific genome duplication yielded two glialcama paralogues: glialcama and glialcamb [29]. Experimental evidence suggests that glialcama and not glialcamb exerts similar functions to its orthologue GlialCAM: i) when expressed transiently in cell lines, glialcama is detected in cell junctions, while glialcamb is intracellular [29]; ii) glialcama is able to target MLC1 and CIC-2 to cell junctions in cell lines, but not glialcamb [29]; iii) glialcama modifies the functional properties of human and zebrafish CIC-2 proteins expressed in Xenopus oocytes, whereas glialcamb reduces CIC-2 function [32]; iv) it has been shown that in mlc1$^{-/-}$ glialcama is mislocalized [29], as happens with GlialCAM in Mlc1$^{-/-}$ mice [15] and v) mlc1 and
glialcama could be co-immunoprecipitated (Additional file 1: Figure S1).

Using TALEN nucleases (see Methods), we generated a zebrafish glialcama knockout line that carries a deletion of 7 nucleotides (Δ7) in the first exon of the glialcama gene. The deletion changes the open reading frame after the seventh amino acid and causes a premature stop codon at amino acid 28 (Fig. 1a). To verify that this mutation abolished the glialcama protein, we assayed its expression in brain extracts from wild-type, heterozygous and homozygous glialcamaΔ7 adult fish siblings (Fig. 1b). No glialcama protein expression could be detected in homozygotes, validating glialcamaΔ7 as a glialcama knockout line (glialcama<sup>ben</sup>), which we will refer to as glialcama<sup>Δ7−/−</sup> zebrafish from now on. As with the Glialcam<sup>−/−</sup> mouse or the mlc1<sup>−/−</sup> zebrafish [15, 29], the homozygous glialcama<sup>Δ7−/−</sup> zebrafish turned out to be viable and fertile, with the expected mendelian ratio among adult descendants. Previous immunofluorescence experiments detected similar localization of glialcama and mlc1 in radial glial cell bodies and their processes in the brain (Fig. 1c and [29]) and in the retina, where they are highly expressed at Müller glia end-feet at the inner limiting membrane (Fig. 1e and [29]). We verified that the previously observed glialcama localization was specific, as immunofluorescence studies confirmed no expression in the glialcama<sup>Δ7−/−</sup> fish neither in the brain nor in the retina (Fig. 1d and f, respectively).

Comparison of mouse and zebrafish MLC knockout phenotypes

Histopathology of brain sections from Mlc1<sup>−/−</sup> and Glialcam<sup>−/−</sup> mice revealed the presence of vacuolization mainly in fibre tracts of the cerebellum [15, 16, 21, 33]. In addition, measurements of brain volume revealed that the whole brain is bigger in MLC knockout models than in its wild-type littermates [14, 16, 29]. No major differences were found in the vacuolization phenotype between both mice models [15]. Regarding the zebrafish models, in mlc1<sup>−/−</sup> animals, MRI showed that the telencephalon is larger in comparison to the wild-type, and there are several lesions due to increased fluid in the telencephalon and mesencephalon [29].

Therefore, we analysed the brain phenotype of glialcama<sup>Δ7−/−</sup> zebrafish by MRI. Furthermore, glialcama<sup>Δ7−/−</sup> and mlc1<sup>−/−</sup> zebrafish were pair-wise mated to obtain animals knockout for both genes or knockout for a single gene and heterozygous for the other. Sagittal (Fig. 2a) and coronal (Fig. 2b) MR images of wild-type and various zebrafish mutants were obtained and analysed. We observed similar lesions in glialcama<sup>Δ7−/−</sup> zebrafish to what has been previously observed for the mlc1<sup>−/−</sup> zebrafish [29]. Analysis of T<sub>2</sub> relaxation time in the healthy and damaged brain regions showed similar values for lesions and the ventricles, indicating that lesions were due to increased fluid (Additional file 2: Figure S2). Furthermore, as in mlc1<sup>−/−</sup> animals [29], the size of the telencephalon relative to the whole brain was also larger in the glialcama<sup>Δ7−/−</sup> (Fig. 2c and Additional file 3: Table S1). These results indicate that the lack of glialcama causes two typical MLC features: megalencephaly and increased fluid accumulation. Furthermore, they also suggest that glialcamb does not compensate for the lack of glialcama. For this reason, we did not analyze whether glialcamb could be co-immunoprecipitated with mlc1 and we did not generate glialcamb<sup>−/−</sup> fish.

We further compared the phenotype of the single knockout zebrafish for one gene with the single knockout/heterozygous or the double knockout. No statistical differences were observed in the percent area of telencephalon after normalizing versus whole brain size neither in the amount of damaged brain regions (Fig. 2c and Additional file 3: Table S1).

To study if this was also the case in mice, we analysed the extent of myelin vacuolization in fibre tracts of the cerebellum in single or double knockout mice for Mlc1 and Glialcam (Fig. 3). Additional loss of Glialcam in Mlc1<sup>−/−</sup>/Glialcam<sup>−/−</sup> mice did not increase the degree of vacuolization over that observed for Mlc1<sup>−/−</sup> or Glialcam<sup>−/−</sup> mice. As previous studies on double knockout mice for both Clcn2 and Glialcam revealed that incremental effects on vacuolation are readily observed [15], we conclude that no such incremental effects occurred in Glialcam / Mlc1 double knockout mice.

Thus, in both animal models (mice and zebrafish) deletion of both genes simultaneously did not exacerbate the brain phenotype of the single knockouts.

Expression and localization of mlc1 is unaltered in zebrafish glialcama<sup>Δ7−/−</sup>

We then analysed the expression of glialcama, glialcamb and mlc1 in the brain of glialcama<sup>Δ7−/−</sup> fish by quantitative real-time PCR (Fig. 4a). We observed that mRNA levels of glialcama and glialcamb were not changed. In contrast, the levels of mlc1 messenger RNA in the brain were increased in glialcama<sup>Δ7−/−</sup> zebrafish. It is interesting to point out that no changes in Mlc1 messenger RNA levels were observed in Glialcam<sup>−/−</sup> mice [15].

In mice, Mlc1 protein levels are strongly decreased or absent in Glialcam<sup>−/−</sup> [15, 16]. We wondered whether the expression of mlc1 might be also changed in glialcama<sup>Δ7−/−</sup> zebrafish. Unexpectedly, mlc1 protein levels were unchanged in glialcama<sup>Δ7−/−</sup> zebrafish (Fig. 4b).

In mice, ablation of GlialCAM strongly reduced the labelling for Mlc1 and changed its localization in Bergmann glia and astrocytic end-feet along blood vessels [15, 16]. We then studied mlc1 localization in the brain and retina of wild type and glialcama<sup>Δ7−/−</sup> fish by
immunofluorescence. We observed no detectable differences in mlc1 localization between wild type and glialcama−/− fish either in the brain (Fig. 4c-d) or in the retina (Fig. 4e-f).

Mlc1 is mislocalized in primary astrocytes from Glialcam−/− mice

We wondered whether the differences observed in MLC1 expression and localization between glialcama−/−...
zebrafish and Glialcam<sup>−/−</sup> mice could be investigated in primary astrocyte cultures. In mouse primary astrocytes, lack of GlialCAM (Fig. 5a and c) caused a reduction of Mlc1 protein, as detected by Western blot (Fig. 5c) and a mislocalization of Mlc1, as it could not be detected in astrocyte junctions (Fig. 5b). Defects in Mlc1 protein expression and localization were rescued by expression of human GlialCAM using adenoviruses that expressed the protein (Fig. 5b and c). Thus, mouse primary Glialcam<sup>−/−</sup> cultures...
recapitulated the Mlc1 expression defect and localization observed in vivo.

Zebrafish mlc1 or human MLC1 overexpressed in primary astrocytes from Glialcam\(^-/-\) mice are located in cell junctions

We next investigated in Glialcam\(^-/-\) mouse primary astrocytes what reasons could explain the differences observed between mice and zebrafish regarding MLC1 protein levels and localization. As zebrafish are kept at 28 °C, which is a lower temperature than the temperature mice are kept (37 °C), we reasoned that stabilization of Mlc1 by GlialCAM might not be necessary at lower temperatures. To test this hypothesis, we incubated mouse primary astrocytes at 28 °C overnight and assayed Mlc1 localization (Fig. 6a) and protein levels (Fig. 6b). However, no changes were observed at lower temperatures, suggesting that the stabilization of Mlc1 by GlialCAM is not temperature-dependent.

We then reasoned that the zebrafish mlc1 protein might not need glialcama for its stabilization at the plasma membrane, unlike their orthologs in mice and human. To test this hypothesis, we constructed an adenovirus expressing zebrafish mlc1 and infected Glialcam\(^-/-\) mouse primary astrocytes. Interestingly, zebrafish mlc1 was located at astrocyte junctions, suggesting that it may not need Glialcama for targeting to astrocyte junctions (Fig. 6c). To test that this was not the case for their human orthologous, we repeated the same experiment now with an adenovirus expressing human MLC1.

Fig. 3  Myelin vacuolization in Glialcam\(^-/-\), Mlc1\(^-/-\) and Glialcam\(^-/-\)/Mlc1\(^-/-\) mouse models. Haematoxylin-eosin staining of sagittal sections of the cerebellum of 19- and 61-week-old mice showed similar levels of myelin vacuolization in Glialcam\(^-/-\), Mlc1\(^-/-\) and Glialcam\(^-/-\)/Mlc1\(^-/-\) animals. As a control we show the same area of a wild-type mouse at 19 weeks. The inset shows the percentage of vacuolization in double KO animal versus the vacuolization observed in Glialcam KO animals (n = 3) and Mlc1 KO animals (n = 3) considering each age independently, without subtracting the minor vacuolization observed in wild-type animals. Data were analyzed by GraphPad Prism software. In order to compare the different groups (dKO vs Glialcam\(^-/-\) and dKO vs Mlc1\(^-/-\)), one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni’s multiple comparison test was used. ns: not significative. Scale bar, 400 μm
Unexpectedly, human MLC1 overexpressed in Glialcam−/− astrocytes was also located at astrocyte junctions (Fig. 6d).

Discussion

In this work, we have obtained and characterized a glialcama knockout in zebrafish. The knockout displays megalencephaly and fluid accumulation, indicating that glialcama and not glialcamb, is the functional ortholog gene of GlialCAM in zebrafish. We do not know which could be the role of glialcamb in zebrafish. However, in vitro studies suggest the possibility that it may act as a negative regulator of MLC1 and CIC-2 [29, 32]. Taking into account that overexpression of MLC1 has been reported to be toxic in mice [33], there could be regulatory mechanisms inhibiting MLC1 function, such...
as interaction with glialcam in zebrafish, although experimental evidence to support this hypothesis is lacking.

We also show that additional disruption of mlc1 in glialcama knockout zebrafish or in Glialcam knockout mice does not potentiate the vacuolating phenotype characteristic of MLC disease, indicating that loss-of-function mutations in these genes cause leukodystrophy through a common pathway. Previous [13] and recent [11] reports indicate that the phenotype of patients with mutations in MLC1 is the same to those with recessive mutations in GLIALCAM. Thus, this genetic evidence in humans, together with biochemical studies in mice and zebrafish models of the disease and in vitro studies that indicated GlialCAM and MLC1 interaction, indicate that these proteins need to form a complex to carry out their physiological role. The situation is completely different for the ClC-2 protein. First, genetic evidence indicates that defects in MLC1 or CLCN2 lead to different diseases [34]. Second, the vacuolating phenotype of Clcn2−/− mice increased after additional disruption of Glialcam [15]. Thus, we proposed that defects in CIC-2 might contribute partially to the MLC phenotype, but it is not the only reason to explain the phenotype of MLC patients.

Fig. 5 Mlc1 is mislocalized in primary Glialcam−/− astrocytes. Localization of GlialCAM (a) and Mlc1 (b) in primary astrocytes from wild-type (WT, left), Glialcam−/− (middle) and Glialcam−/− complemented with adenoviruses expressing human GlialCAM (right). In WT and complemented astrocytes, GlialCAM and Mlc1 are located at cell-cell junctions (arrowheads). Scale bar: 10 μm. (c) GlialCAM and Mlc1 protein levels primary astrocytes from wild-type (WT, left), Glialcam−/− (middle) and Glialcam−/− complemented with an adenovirus expressing human GlialCAM. Actin served as a loading control. Two other independent experiments gave similar results. Densitometric analysis (*n = 3) indicates that Mlc1 levels were reduced in astrocytes from Glialcam−/− mice and expression was recovered after expression of GlialCAM using adenoviruses. *p < 0.05 vs wild-type astrocytes.
The fact that the MLC1/GlialCAM complex is a functional unit is evident in the zebrafish knockout for glialcama, in which mlc1 protein is neither reduced nor mislocalized but yet it displays an MLC-like phenotype. In clear contrast, lack of Mlc1 in mice or mlc1 in zebrafish causes GlialCAM and glialcama mislocalization, respectively. Surprisingly, this localization defect could only be observed in primary cultured astrocytes from mouse after incubation with a depolarizing solution [29, 30]. Possibly, the mislocalization of GlialCAM when MLC1 is absent is a consequence of an unknown depolarization-dependent regulatory mechanism.

We speculate that mlc1 protein levels and localization in zebrafish are unaltered in the glialcama−/−, because in the zebrafish knockout there is an up-regulation of mlc1 mRNA, which does not occur in the Glialcam knockout mice. In agreement with this hypothesis, in primary Glialcam−/− astrocytes, where endogenous MLC1 is mislocalized, zebrafish or human MLC1 overexpressed are located at cell-cell junctions, suggesting that perhaps MLC1 overexpression compensates for lack of GlialCAM stabilizing effect.

Unlike in astrocytes, however, MLC1 overexpressed in cell lines without GlialCAM is never located at cell-cell junctions [25]. Possibly, in astrocytes, MLC1 may reach cell junctions not only by its interaction with GlialCAM, but also with the help of other proteins that may not be present in non-astrocyte cell lines.

Conclusions
This work has provided new insights into the molecular interplay that exists between GlialCAM and MLC1, confirming that both proteins form a functional unit that is physiologically relevant. These results also indicate that in order to understand the molecular roles performed by the MLC1/GlialCAM complex, it is important to work at physiological protein levels, due to the fact that their overexpression may cause non-physiological effects [33].

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13023-019-1248-5.

Additional file 1: Figure S1. Co-immunoprecipitation of glialcama and mlc1 from brain zebrafish. Immunoprecipitation of glialcama from
solubilized brain extracts (S9) using an anti-glialcam monoclonal antibody coupled to Sepharose-A beads (IP ±). Uncoupled beads were used as negative control (IP -). The supernatant (SN) of both purifications is included. mlc1 was detected by Western blot. Another experiment gave similar results.

Additional file 2: Figure S2. T2 relaxation time measurement in the healthy and various brain regions of wild type, mlc1 KO and mlc1 glialcam AKO mutant zebrafish. Region of interest (ROI) selected for T2 relaxation time measurements are shown in left images. ROI: (1) ventral telencephalon, (2) lesion in telencephalon, (3) lesion in mesencephalon, (4) ventricle.

Additional file 3: Table S1. Statistical comparison of wild type and mutant groups for percentage of vacuolation of Telencephalon with respect to whole brain (related to Fig. 2).

Abbreviations

MLC: Megalencephalic leukoencephalopathy with subcortical cysts; MRI: Magnetic resonance imaging; mRNA: messenger RNA; PCR: Polymerase chain reaction; VRAC: Volume regulated anion channel

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Authors’ contributions

CP-R, MF, AA, MNE, MLD and AB performed zebrafish studies. XEV, MBHB and VN performed mice studies. RE directed the project and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article (and its Additional files).

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the European Community Guidelines on Animal Care and Experimentation and were approved by the institutional animal care and use committees.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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