doi:10.1093/brain/awt353 Brain 2014: 137; 806–818 | 806

BRAIN
A JOURNAL OF NEUROLOGY

The phosphatase activity of laforin is dispensable to rescue *Epm2a*$^{-/-}$ mice from Lafora disease

Javier Gayarre,[1,2,*] Lara Duran-Trío,[1,2,3,*] Olga Criado Garcia,[1,2,*] Carmen Aguado,[2,4] Lucia Juana-López,[1] Inmaculada Crespo,[2,3] Erwin Knecht,[2,4] Paola Bovolenta[2,3] and Santiago Rodríguez de Córdoba[1,2]

1 Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones, Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain
2 Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Álvaro de Bazán, 10 Bajo, 46010 Valencia, Spain
3 Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Universidad Autónoma de Madrid, 28049 Madrid, Spain
4 Laboratorio de Biología Celular, Centro de Investigación Principe Felipe, Avda, Autopista de Saler 16, 46012 Valencia, Spain

*These authors contributed equally to this work.

Correspondence to: Dr. Santiago Rodríguez de Córdoba,
Centro de Investigaciones Biológicas,
Consejo Superior de Investigaciones Científicas,
Ramiro de Maeztu 9, 28040 Madrid, Spain
E-mail: srdecordoba@cib.csic.es

Lafora progressive myoclonus epilepsy (Lafora disease) is a fatal autosomal recessive neurodegenerative disorder characterized by the presence of glycogen-like intracellular inclusions called Lafora bodies. The vast majority of patients carry mutations in either the *EPM2A* or *EPM2B* genes, encoding laforin, a glucan phosphatase, and malin, an E3 ubiquitin ligase, respectively. Although the precise physiological role of these proteins is not fully understood, work in past years has established a link between glycogen synthesis, Lafora bodies formation and Lafora disease development. To determine the role of the phosphatase activity of laforin in disease development we generated two *Epm2a*$^{-/-}$ mouse lines expressing either wild-type laforin or a mutant (C265S) laforin lacking only the phosphatase activity. Our results demonstrate that expression of either transgene blocks formation of Lafora bodies and restores the impairment in macroautophagy, preventing the development of Lafora bodies in *Epm2a*$^{-/-}$ mice. These data indicate that the critical pathogenic process is the control of abnormal glycogen accumulation through intracellular proteolytic systems by the laforin–malin complex, and not glycogen dephosphorylation by laforin. Understanding which is the essential process leading to Lafora disease pathogenesis represents a critical conceptual advance that should facilitate development of appropriate therapeutics.

Keywords: Lafora disease; phosphatase activity; glycogen phosphorylation; autophagy; Lafora bodies
Abbreviation: PTG = protein targeting to glycogen

Introduction

Lafora progressive myoclonus epilepsy (Lafora disease) is a fatal neurodegenerative disorder characterized by the presence of glycogen-like intracellular inclusions called Lafora bodies (Lafora, 1911; Lafora and Glueck, 1911; Harriman *et al.*, 1955; Yokoi *et al.*, 1968). Lafora disease manifests during adolescence with generalized tonic–clonic seizures, myoclonus, absences, drop attacks or partial visual seizures. The disease advances to a progressive dementia, leading patients to a vegetative state and death, usually within the first decade from onset of the disease (Berkovic *et al.*, 1986). Lafora disease is an autosomal recessive disorder

Received July 18, 2013. Revised October 27, 2013. Accepted November 2, 2013. Advance Access publication January 14, 2014

with most of the patients carrying mutations in either the *EPM2A* (Minassian *et al.*, 1998; Serratosa *et al.*, 1999) or the *EPM2B* genes (Chan *et al.*, 2003*a*), encoding laforin and malin, respectively. Laforin is a protein containing a dual-specificity protein phosphatase catalytic domain in the C-terminus and a carbohydrate-binding domain in the N-terminus (Ganesh *et al.*, 2000; Wang *et al.*, 2002). Malin is an E3 ubiquitin ligase, with a typical RING domain in the N-terminus and a protein–protein interacting C-terminal region composed of six NHL domains (Chan *et al.*, 2003*b*; Gentry *et al.*, 2005; Lohi *et al.*, 2005).

Understanding the physiological role of laforin and malin and why their absence causes Lafora disease has been subject to intense investigation for more than a decade. Defining steps were the discovery that laforin and malin organize into a functional complex, the formation of which is regulated by activated AMPK (Gentry *et al.*, 2005; Solaz-Fuster *et al.*, 2008), and that laforin interacts with proteins related to glycogen biosynthesis, including the protein targeting to glycogen (PTG) protein phosphatase (Fernández-Sánchez *et al.*, 2003). Crucially, subsequent studies showed that the laforin–malin complex downregulates glycogen accumulation in different tissues and that this associates with a malin-dependent ubiquitination and proteasome degradation of muscle glycogen synthase and PTG (Vilchez *et al.*, 2007; Solaz-Fuster *et al.*, 2008; Worby *et al.*, 2008). The link between glycogen synthesis, Lafora body formation and Lafora disease development is further supported by the observation that lowering glycogen synthesis by PTG or glycogen synthase depletion reduces the presence of Lafora bodies and ameliorates the pathological manifestations that characterize Lafora disease (Turnbull *et al.*, 2011; Pederson *et al.*, 2013). Remarkably, both *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mice show Lafora bodies and elevated glycogen levels in different tissues with no detected differences in levels or activity of the proteins implicated in glycogen metabolism, including PTG (DePaoli-Roach *et al.*, 2010; Turnbull *et al.*, 2010).

Studies in cultured mammalian cells, or in Lafora disease mouse models have illustrated defects in the main intracellular proteolytic systems affecting protein clearance, macroautophagy (hereafter referred as autophagy) and the ubiquitin–proteasome system, suggesting that these processes are positively regulated by the laforin-malin complex and contribute to prevent Lafora disease development (Mittal *et al.*, 2007; Aguado *et al.*, 2010; Puri and Ganesh, 2010; Criado *et al.*, 2012; Knecht *et al.*, 2012; Puri *et al.*, 2012; Sharma *et al.*, 2012). Among these findings, we have recently shown that lack of the laforin-malin complex in both *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mouse models of Lafora disease associates with reduced LC3-II levels and, thus, reduced autophagosome formation, resulting in impaired autophagy (Aguado *et al.*, 2010; Criado *et al.*, 2012). However, defects in the ubiquitin–proteasome system were only observed in the *Epm2a*$^{-/-}$ mice (Vernia *et al.*, 2009; Aguado *et al.*, 2010).

Apart from the activities of laforin involving the formation of complexes with malin and the interaction with other proteins, a remarkable finding was to demonstrate that laforin is a glucan phosphatase that removes phosphates from phosphate-labelled amylopectin, isolated muscle glycogen and muscle glycogen synthesized by muscle glycogen synthase (Wang *et al.*, 2002; Ganesh *et al.*, 2004; Worby *et al.*, 2006; Gentry *et al.*, 2007; Tagliabracci *et al.*, 2007). Consequently, it was proposed that laforin, by suppressing excessive glycogen phosphorylation, prevents the formation of Lafora bodies. However, the fact that the glucan phosphatase activity of laforin does not require malin and is inhibited when laforin binds to glycogen is difficult to accommodate with the proposal that this laforin activity plays a significant role in Lafora disease pathogenesis. In this respect, it is noteworthy that in malin knockout mice there are significantly elevated levels of soluble, phosphatase-active, laforin, coincident with the onset of appearance of Lafora bodies, which strongly suggests that the glucan phosphatase activity of laforin does not suffice to prevent Lafora body formation (Criado *et al.*, 2012).

In the present work, we have asked whether the specific loss of the glucan phosphatase activity of laforin is sufficient to cause the appearance of Lafora bodies. To this end we have generated and characterized two transgenic mouse lines, expressing either laforin wild-type or a mutant laforin lacking phosphatase activity (C265S) in the *Epm2a*$^{-/-}$ background. We show that both transgenes rescue the Lafora disease signs of the *Epm2a*$^{-/-}$ mice, indicating that the glucan phosphatase activity of laforin does not play a primary role in Lafora disease pathogenesis.

Materials and methods

Generation of transgenic mice

The laforin transgene constructs were generated cloning the mouse complementary DNA for laforin in a modified version of the pCAGGs plasmid bearing the cytomegalovirus-immediate early enhancer, the chicken beta-actin promoter, and intron 1 and the simian virus 40 poly(A) signal. In the transgene construct coding a laforin lacking phosphatase activity, the codon Cys265 was substituted with Ser using site-directed mutagenesis (QuickChange®; Stratagen). These constructs were excised from the vector by digestion with KpnI and SalI and purified using a gel extraction kit (QIAEX® II; QIAGEN) followed by Elutip® purification (Scheleicher and Schuell). DNAs were injected into fertilized FVB mouse eggs, and these were transplantated into C57BL6 foster females. Progeny were screened for transgene integration by PCR, and overexpression of laforin was determined by western blotting. Heterozygous laforin-deficient mice expressing the laforin transgenes were generated by intercrossing the transgenic animals with C57BL6 *Epm2a*$^{-/-}$ mice (Ganesh *et al.*, 2002). The presence of the transgenes was detected by PCR using genomic DNA and oligonucleotides located within exon 1 (TRANSLAFW: CTGTGGCTCGCC GAGGTG) and exon 3 (TRANSLAFRV: CAGGGTAGCGGTTGCAGC) of the mouse *Epm2a* exonic sequence. The oligonucleotides for the genotyping of the *Epm2a*$^{-/-}$ mice were located within the neomycin cassette used to delete part of the fourth exon (pgkneo-F2: CAGTTTCATAGCCTGAAGAACG) and within the exon 4 (EPM2A-KO-R: TTCCTTAACTAAGCGTGAGGTC). The presence of endogenous laforin was detected with the oligonucleotides LGPCf (GCCACAGTTC AAGAGAGGAAGG) and LGPCr (CATCAGAGGTAGGGGAGCAAC) situated in the 3′ UTR region of the *Epm2a* gene.

Oligonucleotides for GAPDH product were located within exon 6 (GAPDHFW: GACAACCTGGTCCTCAGTGTA) and in the previous intron (GAPDHRV: ACTGGCGCTGCCAAGGCTGTGGGCA).

Quantitative polymerase chain reaction

The number of transgene copies inserted in genomic DNA of the LAFWT and LAFC265S lines was determined by quantitative PCR analysis that was carried out in iQ^{TM}5 system (Bio-Rad). The oligonucleotides for the *Epm2a* gene were located within exon 4 (LAFEX4: ATGCTGC CACAGGCTGTGTG) and in the 3′ UTR region (LAF 3′UTR: CCACTAA CCACACCCCCAGG). The oligonucleotides for GAPDH as a reference gene were located within exon 6 (GAPDHFW: GACAACCTGGTCCTC AGTGTA) and in the previous intron (GAPDHRV: ACTGGCGCTG CCAAGGCTGTGGGCA). Experiments were performed in triplicate using 2500, 5000 and 10 000 copies of genomic DNA per reaction with Bio-Rad SYBR® Green. The conditions used for quantitative PCR consisted of denaturation for 30 s at 94°C, annealing for 30 s at 66°C and amplification for 1 min at 72°C for 40 cycles, with an initial step of denaturation at 94°C for 7 min. After each cycle, accumulation of PCR products was detected by monitoring the increase of fluorescence of the double stranded DNA-binding SYBR® Green reporter dye. The analysis was carried out both heterocygotic and homocygotic mice, referring the results to the copies of wild-type mouse. The relative copy number of target sequences in DNA samples was determined using a comparative C_T ($\Delta\Delta C_T$) method. The relative copy number (fold change) of the transgenic DNA is given by $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_T^{\text{transgenic mouse}} - \Delta C_T^{\text{C57BL6}}$ and each $C_T = C_T^{\text{transgen}} - CT^{\text{GAPDH}}$, using *Gadph* as a housekeeeping gene.

Antibodies and other reagents

Primary antibodies used in this study were the following: anti-actin (1:4000, 1:5000, respectively; Sigma); anti-LC3 (1:1000, Nanotools); mouse monoclonal anti-laforin 3.5.5, which recognizes human and mouse laforin (generated in our laboratory immunizing *Epm2a*$^{-/-}$ mice with recombinant human laforin). Primary antibodies were detected with anti-mouse or anti-rabbit IgG-HRP secondary antibodies (1:5000, Dako). Other reagents were foetal calf serum (Invitrogen), bovine serum albumin (Sigma), Triton^{TM} X-100 (Sigma), Tween-20 (Sigma), periodic acid (Panreac), Schiff reagent (Merk), haematoxylin (Merk), DePeX (BDH), and enhanced chemiluminesence substrate (ECL, GE Healthcare).

Histological procedures

Animals were transcardiacally perfused with 4% paraformaldehyde in phosphate buffer 0.2 M pH 7.3 and thereafter post-fixed for 3 h at room temperature. Tissue was embedded either in 4% gelatin/15% sucrose solution in PBS or in paraffin and sectioned at 10–12 μm with either a cryostat or a microtome (Leica). Sections were either de-paraffinized and rehydrated or washed in PBS and then and incubated with 1% periodic acid for 15 min, before incubation with Schiff reagent for 15 min (periodic acid–Schiff staining). Sections were counterstained with haematoxylin, dehydrated and mounted in DePeX.

Western blotting

Tissue homogenates and cell lysates were prepared in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton^{TM} X-100, 1% sodium deoxycholate, 1 mM PMSF) containing 1 mM sodium orthovanadate, 50 mM NaF, aprotinin and benzamidine, 2 μg/ml each. Protein content was determined by Bradford analysis (Bio-Rad). Comparable amounts of proteins were analysed by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were blocked with 5% non-fat milk for 1 h at room temperature. Blots were probed with primary antibodies in TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.5 plus 0.1% Tween 20) containing 5% non-fat milk. Anti-mouse or anti-rabbit IgG-horseradish peroxidase secondary antibodies were visualized using the ECL detection kit.

Object recognition task

The novel object recognition test was performed as described (Scullion *et al.*, 2011) with minor changes. Briefly, sets of 12–18 animals of 7 months of age were subjected to habituation, acquisition and trial test. During habituation, animals were allowed to explore an empty open field arena (36 cm × 56 cm × 30 cm) for 20 min. The day after, the animals could explore the same area containing two identical objects (object A) positioned in two opposite corners for 10 min. After 3 h, animals were probed for their capacity to recognize the familiar object (object A) or the novel object (object B). The total time dedicated to explore each of the two objects within a time frame of 10 min was recorded. The recognition index was defined as the amount of time spent exploring the novel object over the total time spent exploring both objects (TA / (TA + TB)).

Statistics

Normality of the distribution was calculated with the Kolmogorov–Smirnov test. When the distribution was normal, statistically significant differences were detected using parametric tests (Student *t*-test), otherwise non-parametric tests (Median test) were applied. *Post hoc* analysis was performed to assess specific group comparisons (Mann-Whitney U test), when the χ^2 value was significant. Values are represented by box-plots. Analysis was performed with the SPSS statistical package version 20.0, using a significance level of $P \leqslant 0.05$.

Results

Generation of *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic mice

To inactivate the glucan phosphatase activity in murine laforin, the codon encoding Cys265 in the dual-specificity protein phosphatase catalytic domain was substituted by a Ser codon using site-directed mutagenesis. Then, a modified version of the pCAGGS plasmid bearing the CMV-EI enhancer and the chicken β-actin promoter was used to construct the wild-type and C265S mutant laforin-encoding transgenes LAFWT and LAFC265S, respectively (Fig. 1A). The construct was excised from the vector and injected into fertilized FVB mouse eggs, which were transplanted into foster C57BL6 females. Founder mice, demonstrating correct and ubiquitous expression of either the LAFWT or the LAFC265S transgene were identified by PCR and western blot analyses (Fig. 1B) and bred to generate the FVB *Epm2a*$^{+/+}$.LAFWT and *Epm2a*$^{+/+}$.LAFC265S mouse lines.

Importantly, LAFWT and LAFC265S FVB transgenic mice do not show presence of Lafora bodies at any age, which is in contrast with early data in which transgenic overexpression of a human C266S mutant laforin transgene in Svj129 mice resulted in focal expression of characteristic Lafora bodies in the hippocampus and other areas of the brain, as well as in liver, heart and skeletal muscle of 12-month-old animals (Chan *et al.*, 2004). Despite these differences neither the Svj129 (hu)LAFC266S transgenic

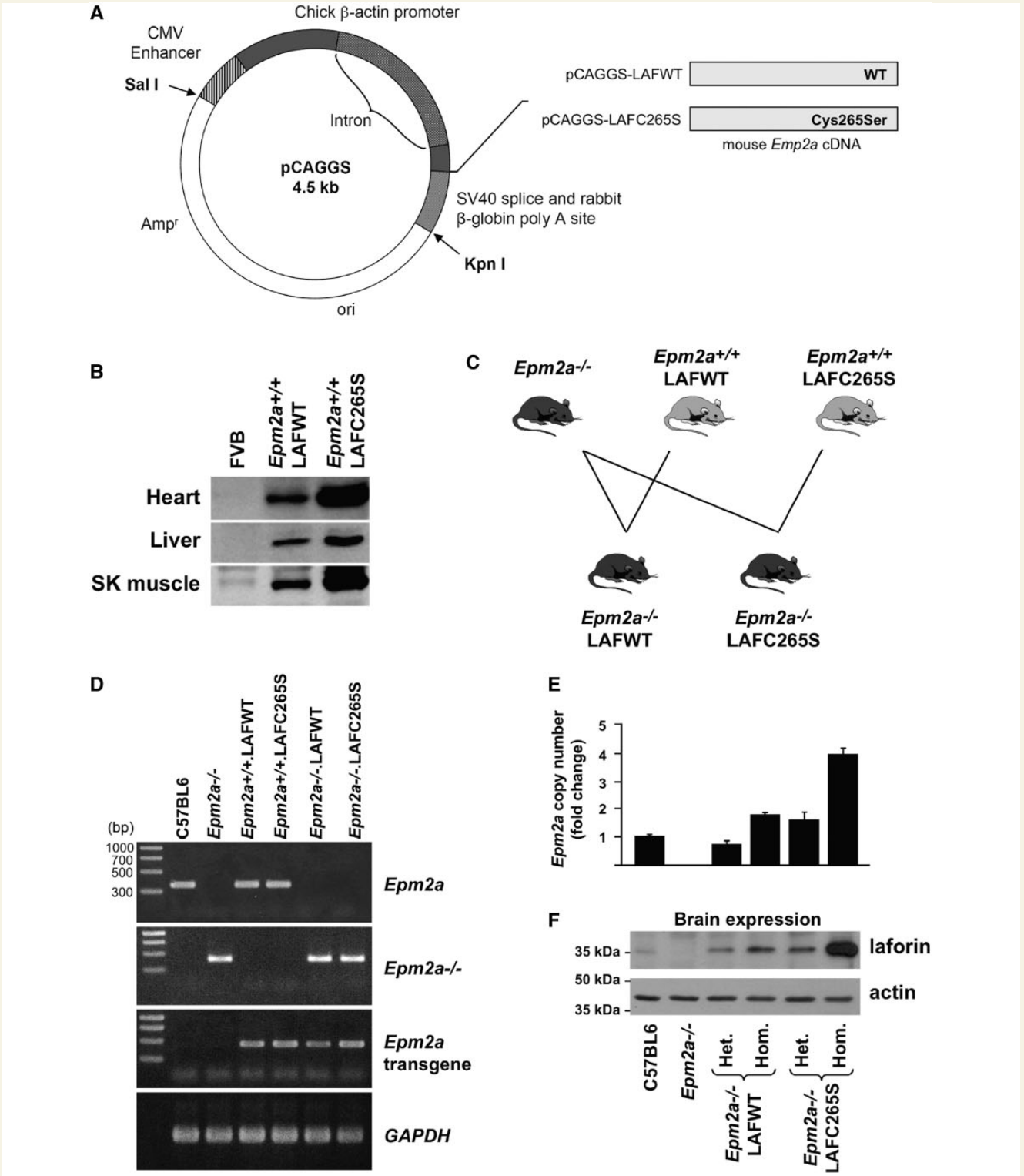

Figure 1 Generation and characterization of the *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic mice. (**A**) Schematic representation of the construct used to generate the transgenic lines. (**B**) Representative western blot of laforin expression in the heart, liver and skeletal muscle in control, *Epm2a*$^{+/+}$.LAFWT and *Epm2a*$^{+/+}$.LAFC265S of 3 month-old mice. (**C**) Schematic representation of the crosses used to generate the *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice. (**D**) PCR analysis to determine the presence of the wild-type (lane 1), or transgenic laforin (lane 2, 3) and the GAPDH gene product (lane 4). (**E**) PCR analysis of the number of copies of the *Epm2a* gene present in the different mouse lines. The *y*-axis represents means ± standard error of the mean (SEM) of copy number (fold change) in transgenic mice relative to C57BL6 mice. *Epm2a* gene was normalized with *Gadph* as a reference gene. The experiment was performed three times with three independent animals. Mouse lines are ordered as in **F**. (**F**) Representative western blot of laforin expression in the brain of control, *Epm2a*$^{-/-}$, *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice.

mice nor our FVB LAFWT and LAFC265S transgenic mice exhibited neurological alterations or signs of neurodegeneration.

These contrasting results regarding the appearance of Lafora bodies between the Svj129 and FVB transgenic expressing mutant laforin could perhaps be explained by the different genetic background of the transgenic mice strains (Svj129 versus FVB), by the levels of expression of the transgene, which seem to be much higher in the Svj129 (hu)LAFC266S transgenic line than in our transgenic mice (see below), or by the myc tag used to label the exogenous laforin in the Svj129 (hu)LAFC266S transgenics, which is not present in our LAFC265S transgenic FVB mice. Furthermore, the differences could also relate to the fact that we used a homologous laforin transgene, whereas Chan *et al*. (2004) used a heterologous human laforin transgene. We backcrossed our FVB transgenic mice with Svj129 wild-type mice, but this did not result in the appearance of Lafora bodies. Interestingly, the quantity and distribution of Lafora bodies in the Svj129 (hu)LAFC266S transgenic mice (Chan *et al.*, 2004) are dramatically distinct from the massive presence of Lafora bodies that can be observed at very early ages in most tissues of all Lafora disease mouse models (Ganesh *et al.*, 2002; Aguado *et al.*, 2010; DePaoli-Roach *et al.*, 2010; Valles-Ortega *et al.*, 2011; Criado *et al.*, 2012). In fact Lafora bodies in Svj129 (hu)LAFC266S transgenic mice are scarce and resemble the patchy accumulation of polyglucosans that are sometimes observed in >12-month-old wild-type mice, suggesting that the reported massive expression of the (hu)LAFC266S transgene (>100-fold compared with endogenous laforin) has a dominant negative effect in these Svj129 mice and accelerates the polyglucosan accumulation that frequently associates with a normal ageing process (see below). Importantly, Tiberia *et al*. (2012) have shown that overexpression of a LAFWT transgene has a similar dominant negative effect, which is likely the result of the competition between an excess of free laforin and the laforin-malin complex for the laforin ligands.

After extensive observation over >2 years, we concluded that the expression of transgenic laforin in our FVB LAFWT and LAFC265S transgenic mice did not interfere with the function of the endogenous laforin, as no evidence of Lafora bodies or manifestations of a Lafora disease-related phenotype were ever observed in these mice.

To test whether the transgenic laforins could substitute the lack of endogenous laforin we backcrossed our FVB LAFWT and LAFC265S transgenic mice with C57BL6 *Epm2a*$^{-/-}$ mice (Fig. 1C). The identification of *Epm2a*$^{-/-}$ mice overexpressing transgenic wild-type or mutant C265S laforin was performed by PCR and western blot (Fig. 1D and F). It should be noted that our C57BL6 *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic mice, similar to FVB transgenics, express different levels of laforin, which were both higher than the levels of expression of the endogenous laforin in the C57BL6 control mice (Fig. 1E). Using quantitative PCR on genomic DNA we concluded that the different laforin expression of the LAFWT and LAFC265S transgene in these animals was only because of the different number of copies of the transgene; the *Epm2a*$^{-/-}$. LAFWT mouse line carries two copies of the transgene per haploid genome (0.7-fold change), whereas the *Epm2a*$^{-/-}$.LAFC265S has four copies (1.6-fold change) (Fig. 1E). As expected, our western blot analysis showed that the levels of laforin in the *Epm2a*$^{-/-}$.LAFWT homozygote were identical to those in *Epm2a*$^{-/-}$.LAFC265S heterozygote and that the levels of expression of laforin from the transgene were higher than those resulting from the endogenous promoter. Most importantly, our western blot analysis showed that in the *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S heterozygotes the expression of the transgenic laforin did not exceed 2- to 4-fold the expression of the endogenous laforin, respectively (Fig. 1F). Most of the experiments described in this report were performed in animals from the F5 or higher backcross with C57BL6 *Epm2a*$^{-/-}$ mice. Littermates without the LAFWT or the LAFC265S transgene were used as *Epm2a*$^{-/-}$ control mice. Extensive sequencing analysis in our transgenic animals did not provide evidence of Ser265 to Cys265 revertants in the LAFC265S transgene.

Transgenic laforin from the LAFWT transgene is functionally active whereas that encoded by LAFC265S lacks phosphatase activity

Once homozygous *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S were generated and the activity of the transgenic laforins could be tested without the interference of the endogenous laforin, it was critical to confirm that the transgenic wild-type laforin was functionally active and the mutant C265S laforin lacked phosphatase activity. We used an amylose-Sepharose column to purify the transgenic laforin from the soluble fraction from identical amounts of whole brain tissue homogenates from *Epm2a*$^{-/-}$, *Epm2a*$^{-/-}$.LAFWT, *Epm2a*$^{-/-}$.LAFC265S and C57BL6 control mice of 3 months of age. Western blot analyses demonstrated that the levels of soluble laforin in the brain of the transgenic mice and the C57BL6 controls were those expected from the expression of the endogenous and transgenic laforin (Fig. 2A). Then, identical volumes of eluates from the amylose–Sepharose column were used to assess the phosphatase activity of laforin using 3-*O*-methylfluorescein phosphate (Sigma) as a substrate (Hill *et al.*, 1968; Ganesh *et al.*, 2000; Girard *et al.*, 2006).

The results confirmed that the LAFWT transgene produces an active laforin, the activity of which correlates with its level of expression in the brain of the *Epm2a*$^{-/-}$.LAFWT transgenic. Notably, we could confirm using high levels of C265S mutant laforin purified from the brain of *Epm2a*$^{-/-}$.LAFC265S transgenic mice that the transgenic C265S laforin is indeed completely devoid of phosphatase activity (Fig. 2B).

The glucan phosphatase activity of laforin is not required to prevent the formation of Lafora bodies in *Epm2a*$^{-/-}$ mice

Several studies have documented the distribution of Lafora bodies in *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mice (Ganesh *et al.*, 2002; Aguado *et al.*, 2010; DePaoli-Roach *et al.*, 2010; Turnbull *et al.*, 2010;

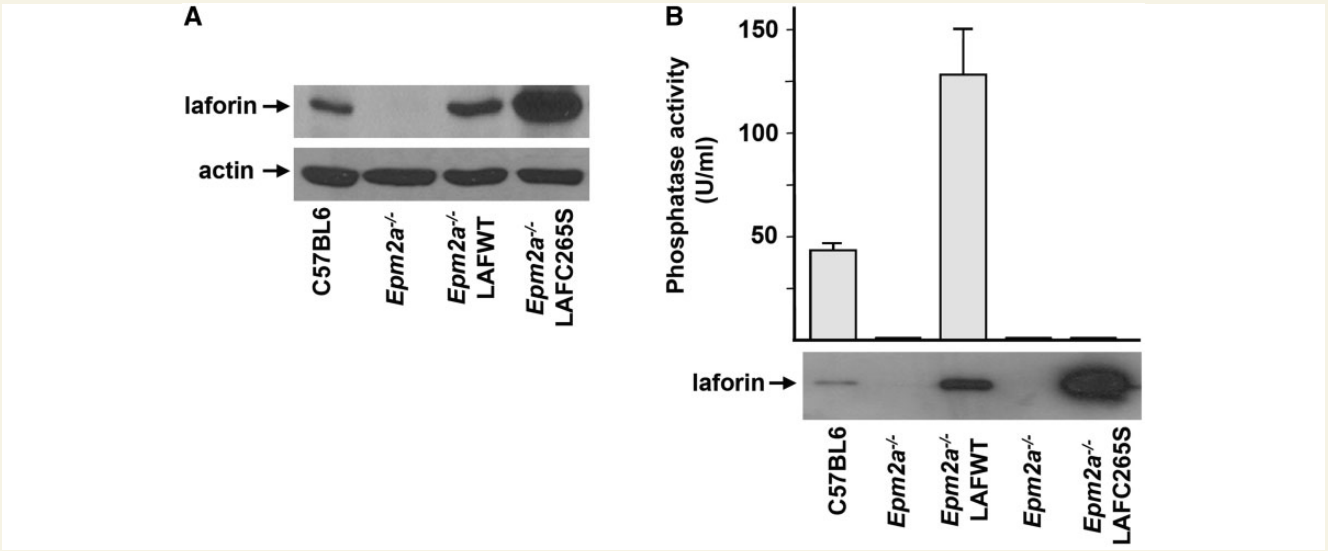

Figure 2 Phosphatase activity of soluble laforin in *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice. (**A**) Western blot experiment to show laforin expression in the brain homogenates from control, *Epm2a*$^{-/-}$, *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice used in the laforin purification experiments. (**B**) Soluble fractions from brain homogenates (total protein: 25 mg) of *Epm2a*$^{-/-}$, *Epm2a*$^{-/-}$.LAFWT, *Epm2a*$^{-/-}$.LAFC265S and control mice at 3 months of age, were subjected to affinity chromatography using amylose–Sepharose, as described in the 'Materials and methods' section. Identical volumes (225 μl) of the eluted fractions were assayed for phosphatase activity using 3-*O*-methylfluorescein phosphate as substrate (75 μl). Activity is expressed as U/ml. Values are the average ± SEM of three independent experiments. Western blot analysis of laforin in whole brain. Identical volumes (20 μl) of the different eluted fractions were analysed by western blot.

Valles-Ortega *et al.*, 2011; Criado *et al.*, 2012). Lafora bodies begin to appear at 1.5–2 months of age initially in the pontine nuclei and hippocampus and thereafter are found in several other brain regions, including the cerebellum, amygdala, pyriform cortex, olfactory bulbs, pre-optic region and the superior and inferior colliculi. Lafora bodies increase with age and become progressively more evident, for example, in the CA1 and CA2 areas of the hippocampus (Fig. 3D–F) and the granule cell layer of the cerebellum (Fig. 4D–F).

To evaluate the distribution of Lafora bodies in the *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic mice, sections of different brains from 3-, 7- and 12-month-old mice were stained with periodic acid–Schiff, with and without treatment with diastase. Periodic acid–Schiff is a histochemical staining used to detect structures containing a high proportion of carbohydrate macromolecules, such as glycogen, glycoproteins or proteoglycans. Diastase is an enzyme that digests free glycogen from histological sections. Lafora bodies formed by abnormally branched glycogen and polyglucosans are stained by periodic acid–Schiff and are diastase resistant.

In contrast with their *Epm2a*$^{-/-}$ transgenic negative littermates, the brains of both *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic animals were completely devoid of Lafora bodies up to 7 months of age (Figs 3 and 4; Table 1). In most of the older analysed animals (>1 year of age) occasional Lafora bodies were observed in the hippocampus, which incidentally is one of the brain regions with higher glucose consumption (Magistretti and Pellerin, 1999) and in which Lafora bodies first appear (Criado *et al.*, 2012). Of note, clusters of tiny Lafora bodies were detected also in the hippocampus of age matched wild-type animals, suggesting that these accumulations likely reflect a normal ageing process (Gertz *et al.*, 1985) as they were never detected in the brain of younger wild-type animals.

The heart is characteristically overloaded with Lafora bodies in patients with Lafora disease and also in mouse models of Lafora disease (Fig. 5D–F). In agreement with the data obtained in brain, we confirmed that in other tissues such as the heart, no Lafora bodies were detected in both *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic animals (Fig. 5).

The efficacy of the LAFC265S transgene in preventing the generation of Lafora bodies is evident from the data presented in this report, which also shows that both the LAFWT and the LAFC265S transgenes are, in this respect, equivalent (Table 1).

Transgenic expression of either wild-type or mutant C265S laforin increases the levels of LC3-II in *Epm2a*$^{-/-}$ mice

We have described defects in protein clearance, in autophagy and in the ubiquitin-proteasome system in *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mice, suggesting that the laforin–malin complex is a positive regulator of these processes and that their impairment contributes to Lafora disease pathogenesis (Aguado *et al.*, 2010; Criado *et al.*, 2012; Knecht *et al.*, 2012; Puri and Ganesh, 2012; Puri *et al.*, 2012). We also reported that overexpression of laforin in cultured cells increases LC3-II levels (Aguado *et al.*, 2010). LC3-II is the

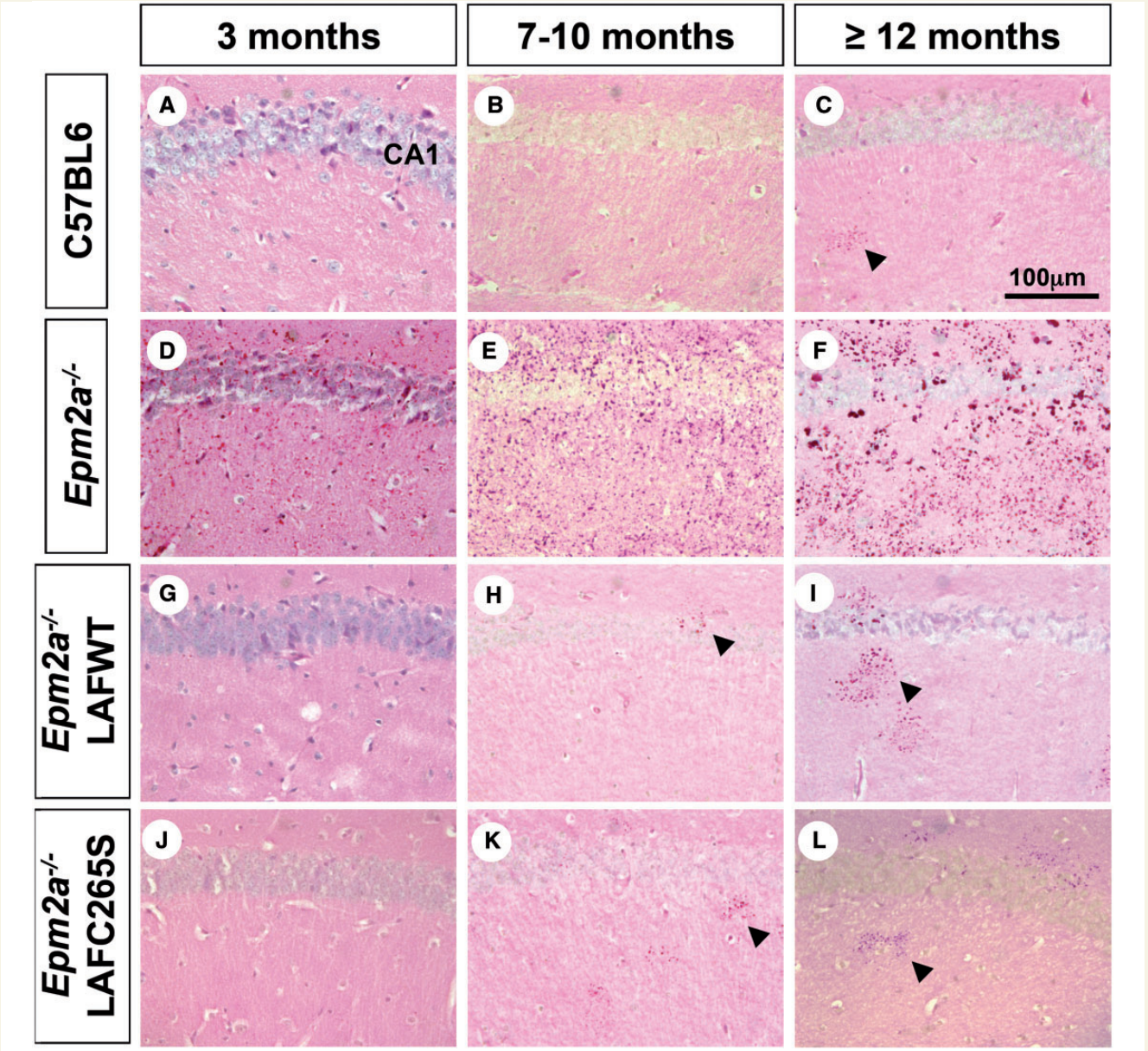

Figure 3 Transgenic expression of LAFWT and LAFC265S suppresses the formation of Lafora bodies in the hippocampus of *Epm2a*$^{-/-}$ mice. Representative frontal sections of the hippocampus from C57BL6 (**A–C**), *Epm2a*$^{-/-}$ (**D–F**), *Epm2a*$^{-/-}$.LAFWT (**G–I**) and *Epm2a*$^{-/-}$.LAFC265S (**J–L**) mice at different ages as indicated were stained with periodic acid-Schiff and counterstained with haematoxylin. Lafora bodies are abundantly localized in the hippocampus of *Epm2a*$^{-/-}$ mice, but almost absent from those of *Epm2a*$^{-/-}$.LAFWT, *Epm2a*$^{-/-}$.LAFC265S <1 year of age. Sparse Lafora bodies were observed at 1 year of age in both control and transgenic animals. CA1 = cornus ammonis 1. Scale bar = 100 μm.

only known protein to specifically associate with autophagosomes, and thus, it is widely accepted as a marker for monitoring autophagy. Here, using an anti-LC3 antibody, we show that transgenic expression of either wild-type or mutant C265S laforin increases the levels of LC3-II in *Epm2a*$^{-/-}$ mice, indicating that the regulation of autophagy by the laforin-malin complex does not require the phosphatase activity of laforin (Fig. 6A).

To confirm these findings we measured the endogenous LC3-II levels in mouse embryonic fibroblasts derived from *Epm2a*$^{-/-}$, *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic mice. In agreement with the results obtained with brain tissue, transgenic expression of either wild-type or mutant C265S laforin increases the levels of LC3-II in mouse embryonic fibroblasts, under high (Krebs-Henseleit medium) and low (full medium) proteolysis (Fig. 6B). These increased levels of LC3-II were also observed in experiments performed in the presence of bafilomycin A1, a lysosomal inhibitor. Taken together, these data demonstrate that the phosphatase activity of laforin is not required for restoring the impartment in autophagosome formation that characterizes *Epm2a*$^{-/-}$ mice.

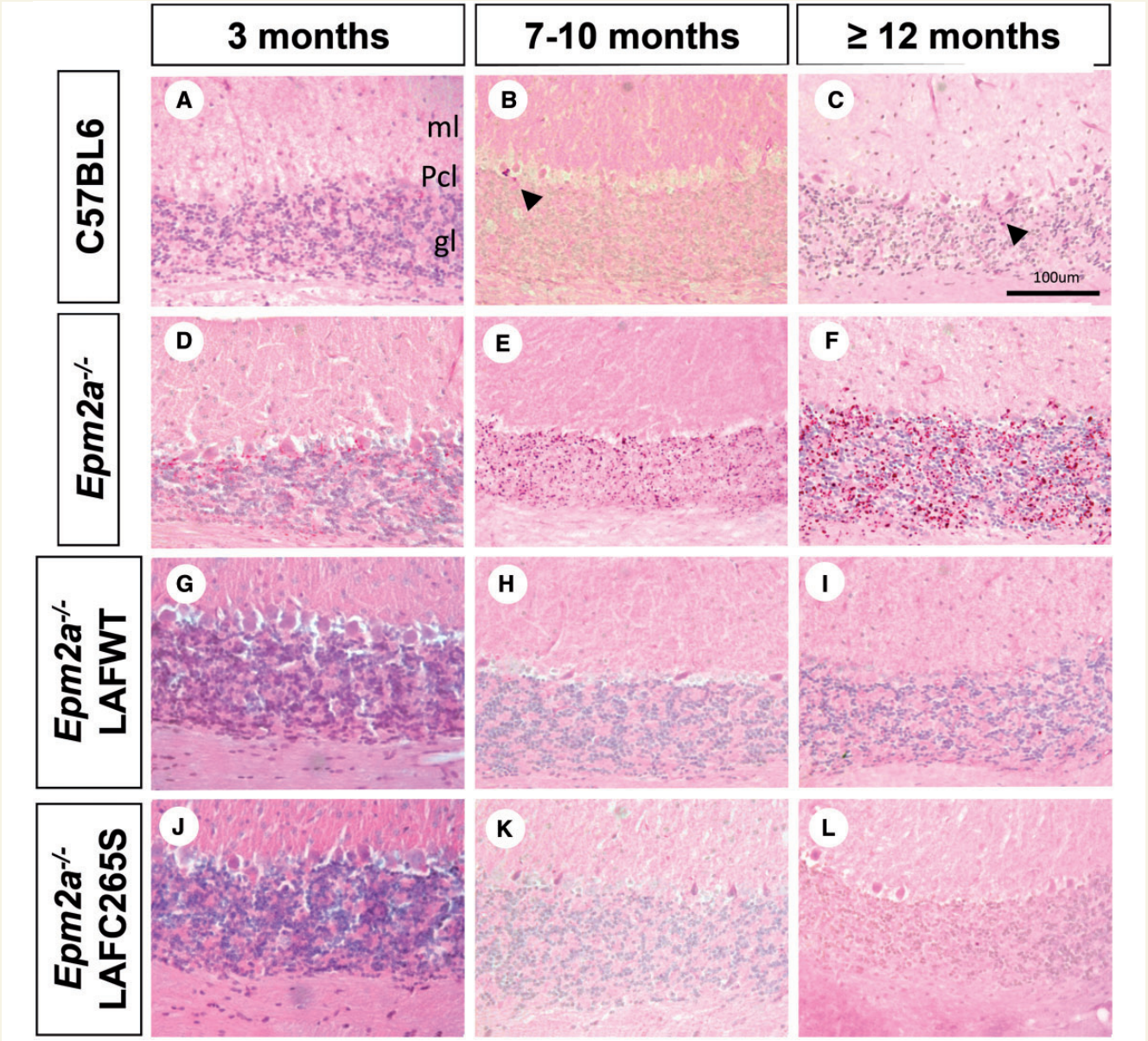

Figure 4 Transgenic expression of LAFWT and LAFC265S suppresses the formation of Lafora bodies in the cerebellum of *Epm2a*$^{-/-}$ mice. Representative frontal sections of the cerebellum from C57BL6 (**A–C**), *Epm2a*$^{-/-}$ (**D–F**), *Epm2a*$^{-/-}$.LAFWT (**G–I**) and *Epm2a*$^{-/-}$.LAFC265S (J–L) mice at different ages as indicated were stained with periodic acid–Schiff and counterstained with haematoxylin. Note the absence of Lafora bodies in the presence of the transgenes. Pcl = Purkinje cell layer; ml = molecular layer; gl = granule cell layer. Scale bar = 100 μm.

Table 1 Presence of Lafora bodies in the hippocampus

	C57BL6	*Epm2a*$^{-/-}$	*Epm2a*$^{-/-}$ LAFWT	*Epm2a*$^{-/-}$ LAFC265S
1.5 months	5^{-}	1^{+}	1^{-}	1^{-}
3 months	3^{-}	4^{++}	1^{-}	5^{-}
7–10 months	2^{+}	7^{+++}	6^{+}	9^{+}
⩾12 months	3^{+}	5^{+++}	8^{+}	6^{+}

(-) no Lafora bodies, (+) sparse Lafora bodies, (++) intermediate Lafora bodies, and (+++) dense accumulation of Lafora bodies.
The number of analysed animals is indicated.

Assessment of memory performance in *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic mice

To determine the effect that transgenic expression of wild-type or mutant C265S laforin has on memory performance, groups of different animals at 7 months of age were evaluated with the object recognition test (Fig. 7). The recognition index among the four groups was significantly different as determined by the Median test ($P = 0.029$). The *Epm2a*$^{-/-}$ group presented a

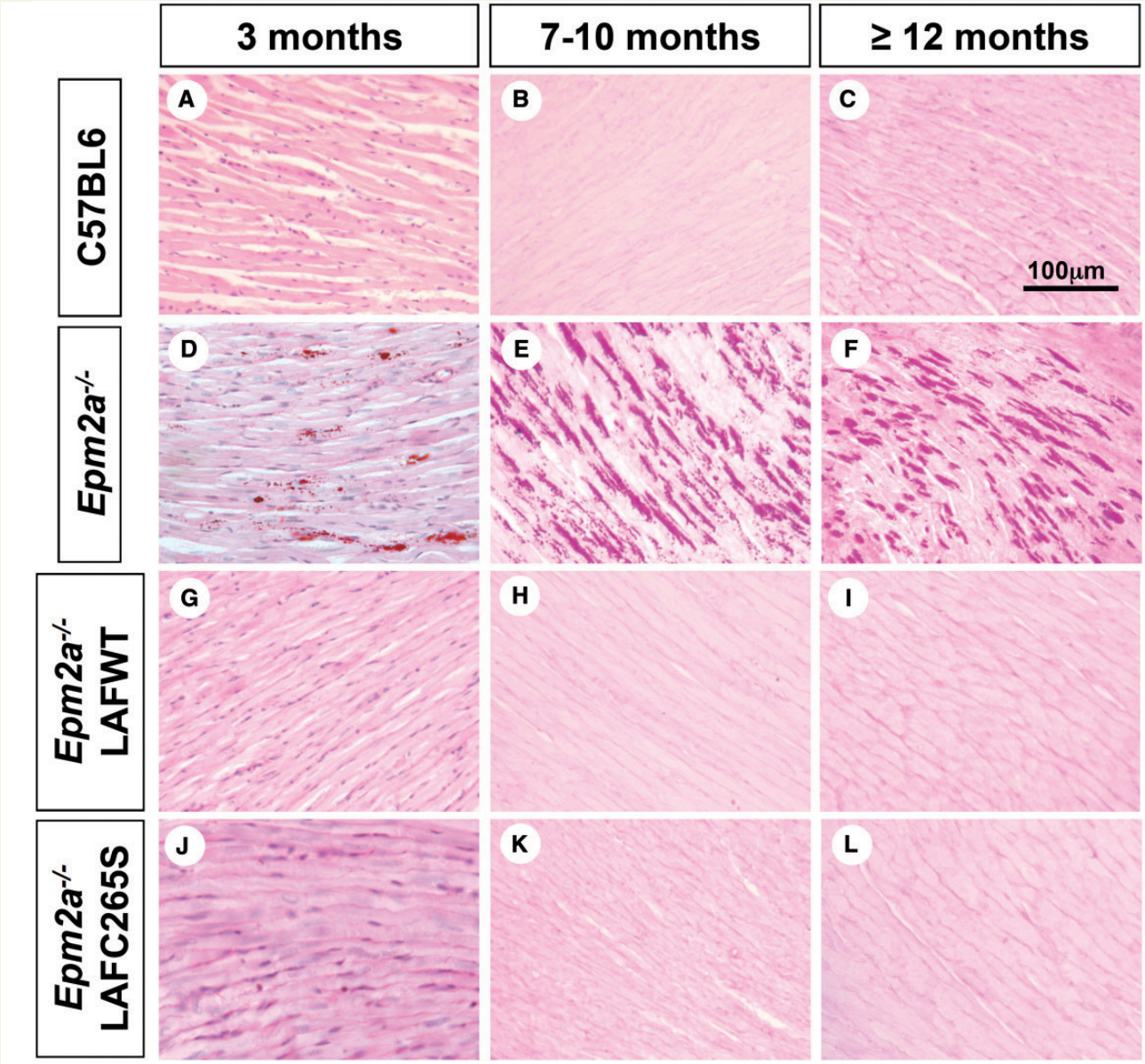

Figure 5 Cardiac tissue from of *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice lacks Lafora bodies. Representative sections of the heart from C57BL6 (**A–C**), *Epm2a*$^{-/-}$ (**D–F**), *Epm2a*$^{-/-}$.LAFWT (**G–I**) and *Epm2a*$^{-/-}$.LAFC265S (**J–L**) mice at different ages as indicated were stained with periodic acid–Schiff and counterstained with haematoxylin. The cardiac muscle of trasgenic mice lacks Lafora bodies, which are instead abundant in that of *Epm2a*$^{-/-}$ mice. Scale bar = 100 μm.

statistically significant lower recognition index than C57BL6 control animals (Mann-Whitney *U* test; $P = 0.018$). In contrast, the recognition index of *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S transgenic mice was not significantly different to that of control animals, although their performance did not reach a statistically significant difference when compared with *Epm2a*$^{-/-}$ mice ($P = 0.189$ and $P = 0.254$ for LAFC265S and LAFWT, respectively).

Discussion

Two, not necessarily incompatible, roles for laforin have been proposed to explain why the lack of laforin ends in Lafora disease. One relates to the finding that laforin is a glucan phosphatase and argues that this phosphatase activity of laforin maintains glycogen dephosphorylated, blocking the formation of Lafora bodies (Wang *et al.*, 2002; Chan *et al.*, 2004; Ganesh *et al.*, 2004; Wang and Roach, 2004; Tagliabracci *et al.*, 2008, 2011; Turnbull *et al.*, 2010; Roach, 2011; Gentry *et al.*, 2013). The second proposed role of laforin in Lafora disease is based on the discovery that laforin and malin form a complex in which laforin recruits substrates to be ubiquitinated by malin. Under this second situation the laforin-malin complex is visualized as a positive regulator of the intracellular proteolytic systems, controlling abnormal glycogen accumulation and Lafora body formation and preventing endoplasmic reticulum stress caused by unfolded protein accumulation

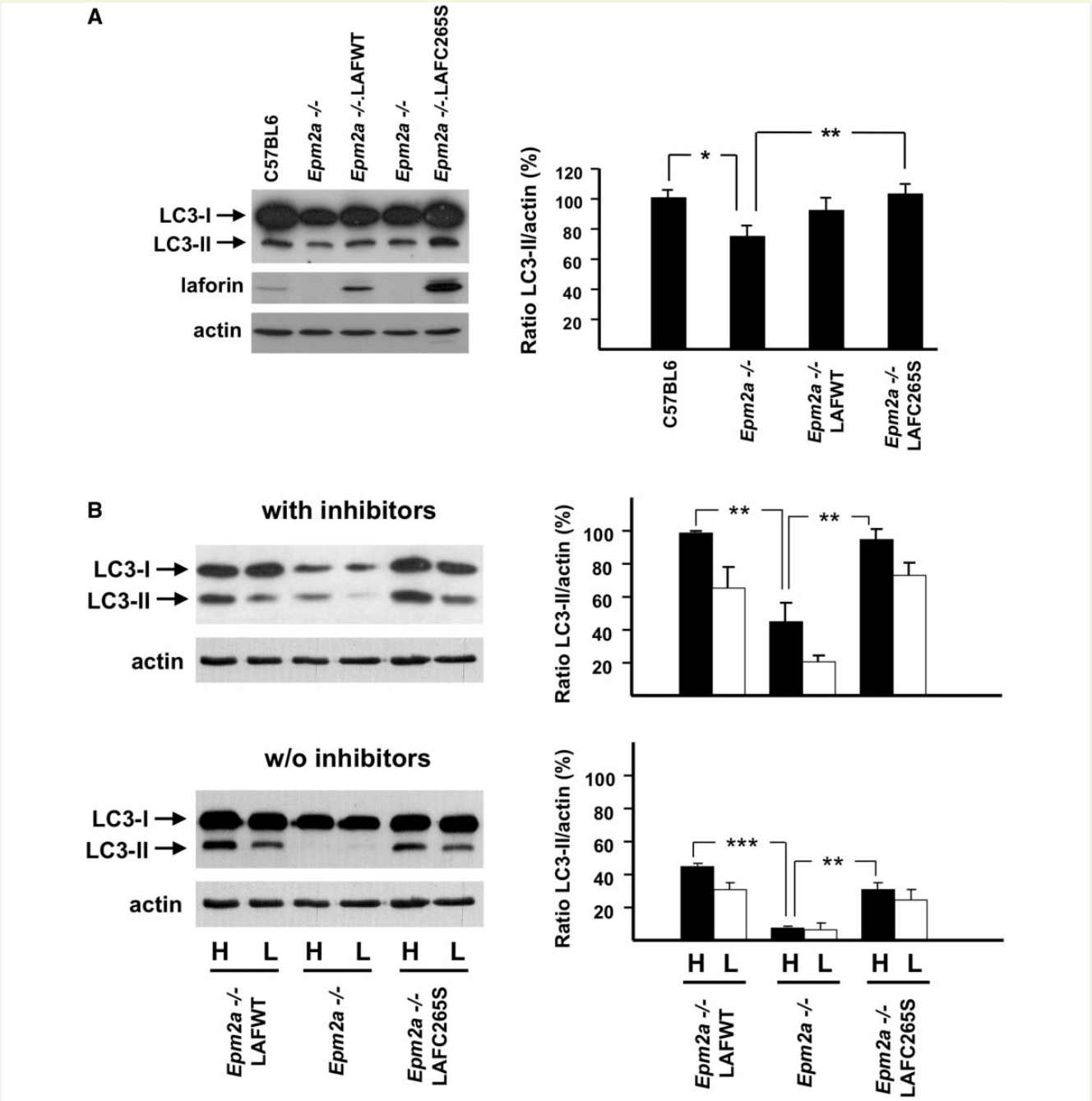

Figure 6 LAFWT and LAFC265S expression in *Epm2a*$^{-/-}$ mice recover autophagy impairment. (**A**) Representative blots, using anti-LC3, anti-laforin and anti-actin, of lysates from total brain (40 μg protein) of control, *Epm2a*$^{-/-}$, *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice of 3 months of age. LC3-II bands from seven different experiments similar to those shown in the blot were analysed by densitometry and normalized to the corresponding actin bands. Data are expressed in % relative to control mice. Values are means ± SEM. (**B**) Representative blots, using anti-LC3 or anti-actin, of mouse embryonic fibroblasts lysates from *Epm2a*$^{-/-}$, *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice, incubated for 2 h with (*top*) or without (*bottom*) bafilomycin A1 (400 nM) under conditions of high (H, Krebs-Henseleit medium) and low (L, full medium) proteolysis. The histograms show the densitometric quantification of LC3-II bands from three different experiments normalized to the corresponding actin bands. Values are expressed in % relative to mouse embryonic fibroblasts from control mice, incubated under conditions of high proteolysis with bafilomycin A1, and are means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

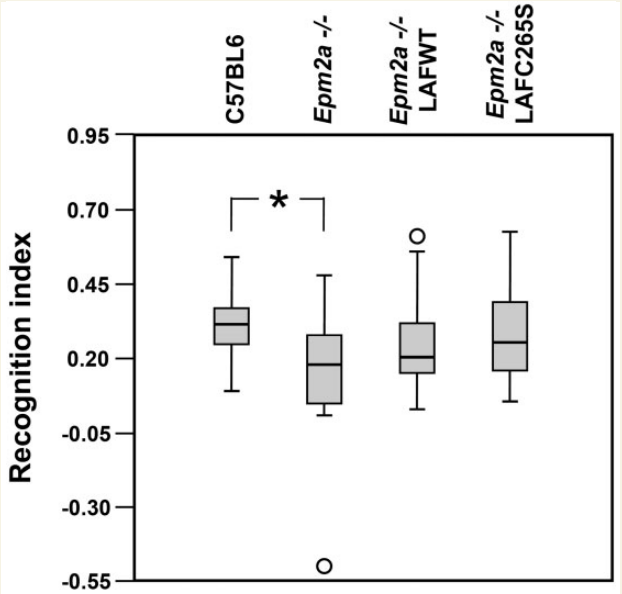

Figure 7 The discrimination ability of transgenic *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S mice is normal. Animals of 7 months of age were subjected to the object recognition test. Note that *Epm2a*$^{-/-}$ mice present a lower recognition index than wild-type, whereas this effect is partially reverted in the *Epm2a*$^{-/-}$.LAFWT and *Epm2a*$^{-/-}$.LAFC265S groups. Data are represented using box-plot that shows minimal and maximal values as well as lower quartile (Q1), median (Q2), upper quartile (Q3) and outliers. *$P \leqslant 0.05$ versus wild-type mice.

(Mittal *et al.*, 2007; Vilchez *et al.*, 2007; Solaz-Fuster *et al.*, 2008; Liu *et al.*, 2009; Vernia *et al.*, 2009; Zeng *et al.*, 2012; Gentry *et al.*, 2013; Sharma *et al.*, 2013). These activities of laforin are independent, as an inactive phosphatase mutant of human laforin (C266S) still interacts *in vitro* with malin and regulates glycogen accumulation (Solaz-Fuster *et al.*, 2008; Worby *et al.*, 2008). It is, however, unclear whether both activities of laforin are relevant in Lafora disease pathogenesis.

Data from patients with Lafora disease and *Epm2b*$^{-/-}$ (malin knockout) mice suggest that the glucan phosphatase activity of laforin is not sufficient to prevent formation of Lafora bodies. In fact, *Epm2b*$^{-/-}$ mice have elevated levels of a phosphatase active functional laforin and develop Lafora bodies even earlier than *Epm2a*$^{-/-}$ mice, lacking laforin (Criado *et al.*, 2012). Similarly, genetic analysis and subsequent functional characterization of Lafora disease-associated *EPM2A* and *EPM2B* mutations in our laboratory have identified patients in whom the phosphatase activity of laforin is not altered, but have affected the formation of laforin-malin complexes (Vilchez *et al.*, 2007; Solaz-Fuster *et al.*, 2008) or the interaction between laforin and PTG (Fernández-Sánchez *et al.*, 2003). Altogether, these findings suggest that the contributions to Lafora disease pathogenesis of the laforin-glycogen and protein-protein interactions of laforin are overall more critical than its glucan phosphatase activity.

Unfortunately, *EPM2A* mutations affecting exclusively the glucan phosphatase activity of laforin have not been found in patients with Lafora disease and therefore it has not been possible to interrogate whether the lack of the glucan phosphatase activity of laforin is sufficient to generate Lafora bodies and cause Lafora disease. To answer this question, and to advance further our understanding of the pathogenic mechanisms underlying Lafora disease, we have expressed transgenically wild-type mouse laforin or a mutant (C265S) mouse laforin lacking exclusively the phosphatase activity in *Epm2a*$^{-/-}$ mice and tested whether these transgenes were able to rescue the Lafora disease phenotype that characterize these mice. In agreement with our early *in vitro* data showing that laforin C265S still interacts with malin and regulates glycogen accumulation (Solaz-Fuster *et al.*, 2008; Worby *et al.*, 2008), the analysis of our transgenic mice demonstrates that expression of either the wild-type laforin or the mutant laforin (C265S) lacking phosphatase activity, prevents the formation of Lafora bodies in *Epm2a*$^{-/-}$ mice. These data do not challenge that laforin is a glucan phosphatase or argue against this activity of laforin contributing to regulate abnormal glycogen accumulation. However, they add to early genetic data to demonstrate that the glucan activity of laforin is neither necessary nor sufficient to prevent Lafora body formation.

The removal of phosphate from glycogen by the glucan phosphatase activity of laforin preserves normal glycogen branching and it is likely to be a relevant process for glycogen metabolism to proceed normally. In addition, it is also likely that the glucan phosphatase activity slows down the accumulation of abnormally-structured glycogen that may eventually develop into Lafora bodies. Our data, however, suggest that the generation of polyglucosan accumulations (we will refer to them as pre-Lafora bodies) are constantly formed despite the presence of laforin glucan phosphatase activity. As these pre-Lafora bodies are likely as implicit to glycogen synthesis as misfolded proteins are to protein synthesis, a removal mechanism must exist to remove these potentially dangerous structures. It seems probable that when these pre-Lafora bodies start to nucleate, the cell will sense a disturbance in its homeostasis and respond, activating the intracellular proteolytic systems. We believe that the evidence accumulated during past years suggest that the role of the laforin-malin complex is to sense the generation of these potentially dangerous structures and respond to them, acting as a positive regulator of the autophagy and proteasomal activities of the intracellular proteolytic systems. In support of this possibility, polyglucosan accumulations resembling Lafora bodies are also observed in regions of the brain with particularly active glucose consumption, even in wild-type animals of 1 year of age, when metabolic processes begin to slow down as a consequence of normal ageing (Baron and Marchal, 1992; Borrás *et al.*, 2009). In addition, ageing has been frequently associated with an impaired autophagy (Rubinsztein *et al.*, 2011) and a defective ubiquitin proteasome system (Baraibar and Friguet, 2012).

Previously we and others have shown that the laforin-malin complex causes specific ubiquitination and proteasome-dependent degradation of proteins involved in glycogen biosynthesis and interpreted this as a regulatory mechanism to avoid abnormal glycogen accumulation (Vilchez *et al.*, 2007; Solaz-Fuster *et al.*, 2008; Worby *et al.*, 2008). We suggest now that this regulatory mechanism may involve the removal of pre-Lafora bodies and the proteins attached to them, including muscle glycogen synthase

(MGS), targeting to glycogen (PTG), protein phosphatase 1 regulatory subunit R6 (R6), laforin and malin. Further experiments are, however, needed to confirm this hypothesis and to explain the contrasting *in vitro* and *in vivo* data regarding whether there are differences in the levels of the proteins implicated in glycogen metabolism in the presence or absence of the laforin–malin complex (Vilchez *et al.*, 2007; Solaz-Fuster *et al.*, 2008; Worby *et al.*, 2008; DePaoli-Roach *et al.*, 2010; Turnbull *et al.*, 2010).

Laforin presents a carbohydrate-binding module that is disrupted by some of the mutations found in patients with Lafora disease (Fernández-Sánchez *et al*., 2003). It is likely that this carbohydrate-binding module plays a role in recruiting the laforin-malin complex to the glycogen particles and that one role of laforin in the laforin-malin complex is, therefore, sensing the generation of pre-Lafora bodies. In theory, the glycogen phosphatase active site of laforin, binding to phosphorylated glycogen, may also contribute to sense pre-Lafora bodies by increasing the avidity of the complex for hyperphosphorylated glycogen.

If generation of pre-Lafora bodies associates with glycogen synthesis, then it should be expected that in the absence of the appropriate mechanisms to remove these potentially dangerous structures, the Lafora bodies will be formed, and if they are causative agents of Lafora disease, the disease will develop. On the contrary, inhibiting glycogen synthesis might prevent Lafora body formation and the neurological abnormalities that characterize Lafora disease. Consistent with this interpretation, it has been found that depletion of PTG or glycogen synthase in mice lacking laforin resulted in down-regulation of glycogen synthesis, with near-complete disappearance of Lafora bodies as well as decreased neuronal cell death and myoclonic epilepsy (Turnbull *et al.*, 2011; Pederson *et al.*, 2013).

Importantly, experiments in the *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mouse models of Lafora disease have illustrated defects in the intracellular proteolytic systems affecting protein clearance and autophagy and in the *Epm2a*$^{-/-}$ mice also impairment of the ubiquitin–proteasome system, suggesting that these processes are positively regulated by the laforin-malin complex and contribute to prevent Lafora disease development (Aguado *et al.*, 2010; Criado *et al.*, 2012). Here we also show that expression of both transgenes equally restored the impairment in autophagy that characterizes the *Epm2a*$^{-/-}$ mice, suggesting that the glucan phosphatase activity of laforin is not required for the positive regulation of the intracellular proteolytic system by the laforin–malin complex. Noteworthy, restoration of macroautophagy in our transgenic mice correlates with the absence of Lafora bodies. The data presented in this report provide an important advance in understanding the pathogenic mechanism in Lafora disease, illustrating that regulation of the intracellular proteolytic system by the laforin-malin complex has a critical role in this process. Further work is needed to dissect the molecular mechanisms involved in the activities of the laforin–malin complex and to uncover the physiological situations that require its activity.

Funding

J.G. holds a postdoctoral fellowship from the Program "Junta para la Ampliación de Estudios" (JAE-Doc) co-funded by the Fondo Social Europeo (FSE) and L.D-T. enjoys a FPU fellowship (AP2009-2698). This work was funded by the Spanish Ministerio de Economia y Competitividad (SAF2011-26583, BFU2011-22630 and BFU2010-16031), the Fundación Marato TV3 (Ref. 100132), the Consejo Superior de Investigaciones Cientificas (PIE 201020E043) and the Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER).

References

Aguado C, Sarkar S, Korolchuk VI, Criado O, Vernia S, Boya P, et al. Laforin, the most common protein mutated in Lafora disease, regulates autophagy. Hum Mol Genet 2010; 19: 2867–76.

Baraibar MA, Friguet B. Changes of the proteasomal system during the aging process. Prog Mol Biol Transl Sci 2012; 109: 249–75.

Baron JC, Marchal G. Cerebral and cardiovascular aging and brain energy metabolism. Studies with positron-emission tomography in man [in French]. Presse Med 1992; 21: 1231–7.

Berkovic SF, Andermann F, Carpenter S, Wolfe LS. Progressive myoclonus epilepsies: specific causes and diagnosis. N Engl J Med 1986; 315: 296–305.

Borrás C, Stvolinsky S, López-Grueso R, Fedorova T, Gambini J, Boldyrev A, et al. Low *in vivo* brain glucose consumption and high oxidative stress in accelerated aging. FEBS Lett 2009; 583: 2287–93.

Criado O, Aguado C, Gayarre J, Duran-Trio L, Garcia-Cabrero AM, Vernia S, et al. Lafora bodies and neurological defects in malin-deficient mice correlate with impaired autophagy. Hum Mol Genet 2012; 21: 1521–33.

Chan EM, Ackerley CA, Lohi H, Ianzano L, Cortez MA, Shannon P, et al. Laforin preferentially binds the neurotoxic starch-like polyglucosans, which form in its absence in progressive myoclonus epilepsy. Hum Mol Genet 2004; 13: 1117–29.

Chan EM, Bulman DE, Paterson AD, Turnbull J, Andermann E, Andermann F, et al. Genetic mapping of a new Lafora progressive myoclonus epilepsy locus (EPM2B) on 6p22. J Med Genet 2003a; 40: 671–5.

Chan EM, Young EJ, Ianzano L, Munteanu I, Zhao X, Christopoulos CC, et al. Mutations in NHLRC1 cause progressive myoclonus epilepsy. Nat Genet 2003b; 35: 125–7.

DePaoli-Roach AA, Tagliabracci VS, Segvich DM, Meyer CM, Irimia JM, Roach PJ. Genetic depletion of the malin E3 ubiquitin ligase in mice leads to Lafora bodies and the accumulation of insoluble laforin. J Biol Chem 2010; 285: 25372–81.

Fernández-Sánchez ME, Criado-García O, Heath Karen E, García-Fojeda B, Medrano-Fernández I, Gomez-Garre P, et al. Laforin, the dual-phosphatase responsible for Lafora disease, interacts with R5 (PTG), a regulatory subunit of protein phosphatase-1 that enhances glycogen accumulation. Hum Mol Genet 2003; 12: 3161–71.

Ganesh S, Agarwala KL, Ueda K, Akagi T, Shoda K, Usui T, et al. Laforin, defective in the progressive myoclonus epilepsy of Lafora type, is a dual-specificity phosphatase associated with polyribosomes. Hum Mol Genet 2000; 9: 2251–61.

Ganesh S, Delgado-Escueta AV, Sakamoto T, Avila MR, Machado-Salas J, Hoshii Y, et al. Targeted disruption of the Epm2a gene causes formation of Lafora inclusion bodies, neurodegeneration, ataxia, myoclonus epilepsy and impaired behavioral response in mice. Hum Mol Genet 2002; 11: 1251–62.

Ganesh S, Tsurutani N, Suzuki T, Hoshii Y, Ishihara T, Delgado-Escueta AV, et al. The carbohydrate-binding domain of Lafora disease protein targets Lafora polyglucosan bodies. Biochem Biophys Res Commun 2004; 313: 1101–9.

Gentry M, Worby C, Dixon J. Insights into Lafora disease: Malin is an E3 ubiquitin ligase that ubiquitinates and promotes the degradation of laforin. Proc Natl Acad Sci USA 2005; 102: 8501–6.

Gentry MS, Dowen RH, Worby CA, Mattoo S, Ecker JR, Dixon JE. The phosphatase laforin crosses evolutionary boundaries and links carbohydrate metabolism to neuronal disease. J Cell Biol 2007; 178: 477–88.

Gentry MS, Romá-Mateo C, Sanz P. Laforin, a protein with many faces: glucan phosphatase, adapter protein, et alii. FEBS J 2013; 280: 525–37.

Gertz HJ, Cervos-Navarro J, Frydl V, Schultz F. Glycogen accumulation of the aging human brain. Mech Ageing Dev 1985; 31: 25–35.

Girard JM, Lê KH, Lederer F. Molecular characterization of laforin, a dual-specificity protein phosphatase implicated in Lafora disease. Biochimie 2006; 88: 1961–71.

Harriman DGF, Millar JHD, Stevenson AC. Progressive familial myoclonic epilepsy in three families: its clinical features and pathological basis. Brain 1955; 78: 325–49.

Hill D, Summer GK, Waters MD. An automated fluorometric assay for alkaline phosphatase using 3-O-methylfluorescein phosphate. Anal Biochem 1968; 24: 9–17.

Knecht E, Criado-García O, Aguado C, Gayarre J, Duran-Trio L, Garcia-Cabrero AM, et al. Malin knockout mice support a primary role of autophagy in the pathogenesis of Lafora disease. Autophagy 2012; 8: 701–3.

Lafora G, Glueck B. Beitrag zur Histopathologie der myoklonischen epilepsie. Z Gesamte Neurol Psych 1911; 6: 1–14.

Lafora GR. Über das Vorkommen amyloider Körperchen im Innern der Ganglienzellen: zugleich en Beitrag zum Studium der amyloiden Substanz im Nervensystem. Virchows Arch Pathol 1911; 205: 295–303.

Liu Y, Wang Y, Wu C, Liu Y, Zheng P. Deletions and missense mutations of EPM2A exacerbate unfolded protein response and apoptosis of neuronal cells induced by endoplasm reticulum stress. Hum Mol Genet 2009; 18: 2622–31.

Lohi H, Ianzano L, Zhao X-C, Chan EM, Turnbull J, Scherer SW, et al. Novel glycogen synthase kinase 3 and ubiquitination pathways in progressive myoclonus epilepsy. Hum Mol Genet 2005; 14: 2727–36.

Magistretti PJ, Pellerin L. Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. Philos Trans R Soc Lond B Biol Sci 1999; 354: 1155–63.

Minassian BA, Lee JR, Herbrick JA, Huizenga J, Soder S, Mungall AJ, et al. Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. Nat Genet 1998; 20: 171–4.

Mittal S, Dubey D, Yamakawa K, Ganesh S. Lafora disease proteins malin and laforin are recruited to aggresomes in response to proteasomal impairment. Hum Mol Genet 2007; 16: 753–62.

Pederson BA, Turnbull J, Epp JR, Weaver SA, Zhao X, Pencea N, et al. Inhibiting glycogen synthesis prevents Lafora disease in a mouse model. Ann Neurol 2013; 74: 297–300.

Puri R, Ganesh S. Laforin in autophagy: a possible link between carbohydrate and protein in Lafora disease? Autophagy 2010; 6: 1229–31.

Puri R, Ganesh S. Autophagy defects in Lafora disease: cause or consequence? Autophagy 2012; 8: 289–90.

Puri R, Suzuki T, Yamakawa K, Ganesh S. Dysfunctions in endosomal–lysosomal and autophagy pathways underlie neuropathology in a mouse model for Lafora disease. Hum Mol Genet 2012; 21: 175–84.

Roach PJ. Are there errors in glycogen biosynthesis and is laforin a repair enzyme? FEBS Lett 2011; 585: 3216–8.

Rubinsztein DC, Mariño G, Kroemer G. Autophagy and aging. Cell 2011; 146: 682–95.

Scullion GA, Kendall DA, Marsden CA, Sunter D, Pardon MC. Chronic treatment with the α2-adrenoceptor antagonist fluparoxan prevents age-related deficits in spatial working memory in APP $\times$ PS1 transgenic mice without altering β-amyloid plaque load or astrocytosis. Neuropharmacology 2011; 60: 223–34.

Serratosa JM, Gómez-Garre P, Gallardo ME, Anta B, de Bernabé DB, Lindhout D, et al. A novel protein tyrosine phosphatase gene is mutated in progressive myoclonus epilepsy of the Lafora type (EPM2). Hum Mol Genet 1999; 8: 345–52.

Sharma J, Mukherjee D, Rao SN, Iyengar S, Shankar SK, Satishchandra P, et al. Neuronatin-mediated aberrant calcium signaling and endoplasmic reticulum stress underlie neuropathology in Lafora disease. J Biol Chem 2013; 288: 9482–90.

Sharma J, Mulherkar S, Mukherjee D, Jana NR. Malin regulates Wnt signaling pathway through degradation of Dishevelled2. J Biol Chem 2012; 287: 6830–9.

Solaz-Fuster MC, Gimeno-Alcañiz JV, Ros S, Fernandez-Sanchez ME, Garcia-Fojeda B, Criado-Garcia O, et al. Regulation of glycogen synthesis by the laforin-malin complex is modulated by the AMP-activated protein kinase pathway. Hum Mol Genet 2008; 17: 667–78.

Tagliabracci VS, Girard JM, Segvich D, Meyer C, Turnbull J, Zhao X, et al. Abnormal metabolism of glycogen phosphate as a cause for Lafora disease. J Biol Chem 2008; 283: 33816–25.

Tagliabracci VS, Heiss C, Karthik C, Contreras CJ, Glushka J, Ishihara M, et al. Phosphate incorporation during glycogen synthesis and Lafora disease. Cell Metab 2011; 13: 274–82.

Tagliabracci VS, Turnbull J, Wang W, Girard JM, Zhao X, Skurat AV, et al. Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen *in vivo*. Proc Natl Acad Sci USA 2007; 104: 19262–6.

Tiberia E, Turnbull J, Wang T, Ruggieri A, Zhao XC, Pencea N, et al. Increased laforin and laforin binding to glycogen underlie Lafora body formation in malin-deficient Lafora disease. J Biol Chem 2012; 287: 25650–9.

Turnbull J, DePaoli-Roach AA, Zhao X, Cortez MA, Pencea N, Tiberia E, et al. PTG depletion removes Lafora bodies and rescues the fatal epilepsy of Lafora disease. Plos Genet 2011; 7: e1002037.

Turnbull J, Wang P, Girard JM, Ruggieri A, Wang TJ, Draginov AG, et al. Glycogen hyperphosphorylation underlies Lafora body formation. Ann Neurol 2010; 68: 925–33.

Valles-Ortega J, Duran J, Garcia-Rocha M, Bosch C, Saez I, Pujadas L, et al. Neurodegeneration and functional impairments associated with glycogen synthase accumulation in a mouse model of Lafora disease. EMBO Mol Med 2011; 3: 667–81.

Vernia S, Rubio T, Heredia M, Rodríguez de Córdoba S, Sanz P. Increased endoplasmic reticulum stress and Decreased proteasomal function in Lafora disease models lacking the phosphatase laforin. PLoS One 2009; 4: e5907.

Vilchez D, Ros S, Cifuentes D, Pujadas L, Valles J, Garcia-Fojeda B, et al. Mechanism suppressing glycogen synthesis in neurons and its demise in progressive myoclonus epilepsy. Nat Neurosci 2007; 10: 1407–13.

Wang J, Stuckey JA, Wishart MJ, Dixon JE. A Unique carbohydrate binding domain targets the Lafora disease phosphatase to glycogen. J Biol Chem 2002; 277: 2377–80.

Wang W, Roach PJ. Glycogen and related polysaccharides inhibit the laforin dual-specificity protein phosphatase. Biochem Biophys Res Commun 2004; 325: 726–30.

Worby CA, Gentry MS, Dixon JE. Laforin, a dual specificity phosphatase that dephosphorylates complex carbohydrates. J Biol Chem 2006; 281: 30412–8.

Worby CA, Gentry MS, Dixon JE. Malin decreases glycogen accumulation by promoting the degradation of protein targeting to glycogen (PTG). J Biol Chem 2008; 283: 4069–76.

Yokoi S, Austin J, Witmer F, Sakai M. Studies in myoclonus epilepsy (Lafora body form) I. Isolation and preliminary characterization of Lafora bodies in two cases. Arch Neurol 1968; 19: 15–33.

Zeng L, Wang Y, Baba O, Zheng P, Liu Y, Liu Y. Laforin is required for the functional activation of malin in endoplasmic reticulum stress resistance in neuronal cells. FEBS J 2012; 279: 2467–78.