Glycogen Hyperphosphorylation Underlies Lafora Body Formation

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Objective: Glycogen, the largest cytosolic macromolecule, acquires solubility, essential to its function, through extreme branching. Lafora bodies are aggregates of polyglucosan, a long, linear, poorly branched, and insoluble form of glycogen. Lafora bodies occupy vast numbers of neuronal dendrites and perikarya in Lafora disease in time-dependent fashion, leading to intractable and fatal progressive myoclonus epilepsy. Lafora disease is caused by deficiency of either the laforin glycogen phosphatase or the malin E3 ubiquitin ligase. The 2 leading hypotheses of Lafora body formation are: (1) increased glycogen synthase activity extends glycogen strands too rapidly to allow adequate branching, resulting in polyglucosans; and (2) increased glycogen phosphate leads to glycogen conformational change, unfolding, precipitation, and conversion to polyglucosan. Recently, it was shown that in the laforin phosphatase-deficient form of Lafora disease, there is no increase in glycogen synthase, but there is a dramatic increase in glycogen phosphate, with subsequent conversion of glycogen to polyglucosan. Here, we determine whether Lafora bodies in the malin ubiquitin ligase-deficient form of the disease are due to increased glycogen synthase or increased glycogen phosphate.
Methods: We generated malin-deficient mice and tested the 2 hypotheses.
Results: Malin-deficient mice precisely replicate the pathology of Lafora disease with Lafora body formation in skeletal muscle, liver, and brain, and in the latter in the pathognomonic perikaryal and dendritic locations. Glycogen synthase quantity and activity are unchanged. There is a highly significant increase in glycogen phosphate.
Interpretation: We identify a single common modification, glycogen hyperphosphorylation, as the root cause of Lafora body pathogenesis.

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The average glycogen molecule packs a formidable 13,000 glucose units into a 30nm sphere. This shape of glycogen is essential to its solubility. Glycogen synthesis proceeds with glycogen synthase (GS) adding 6 consecutive glucose units through α1–4 linkages to the end of an oligosaccharide. These are then detached as a hexamer by glycogen branching enzyme (BE), and reattached upstream through an α1–6 linkage. The resultant fork now has 2 ends on which GS can act and elongate by 6 units, each new hexamer again to be removed and internally reattached by BE, and so on. This organized elongation and branching generates the spherical structure of the macromolecule.[1] Imbalance in this process, that is, insufficient branching or excessive GS activity, would result in poorly branched and insoluble glycogen.

Lafora disease (LD) is an autosomal recessive teenage-onset fatal epilepsy associated with time-dependent moderate increase in glycogen, but more importantly with gradual accumulation of poorly branched and insoluble glycogen, termed polyglucosan, in many tissues. In brain, polyglucosan bodies (Lafora bodies) form in neuronal somata, and polyglucosan masses gradually replace the cytoplasms of large numbers of dendrites, likely underlying onset, progression, and intractability of this epilepsy.[2,3]

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In addition to LD, 2 other diseases are associated with polyglucosan accumulation. One is type IV glycogen storage disease, caused by BE deficiency.[4] The polyglucosans in this disease are structurally identical to those of LD, but for reasons not understood accumulate in neuronal axons and not in the somatodendritic compartment, causing an axonopathy with motor, sensory, and autonomic deficits but no epilepsy.[5,6] Humans have a single BE gene, *GBE1*, expressed ubiquitously. BE activity is normal in LD.[7] This, along with the fact that BE deficiency causes type IV glycogenosis and not LD, rules out decreased branching enzyme activity as the mechanism of polyglucosan generation in LD.

The final polyglucosan disease, type VII glycogen storage disease, is due to muscle-specific phosphofructokinase deficiency and affects only skeletal muscle.[8] Deficiency of this early glycolytic enzyme results in accumulation of its precursor, glucose 6-phosphate (G6P), a potent allosteric activator of GS (GS is inhibited by phosphorylation and activated by dephosphorylation and by G6P). The resultant increased GS activity disturbs the glycogen extension:-branching balance in favor of extension, leading to muscle polyglucosan.[9] Humans possess several phosphofructokinase genes, and the 1 expressed in brain is the platelet isoform (*PFKP*).[10] The ubiquitous presence of polyglucosans in LD, including in skeletal muscle, rules out *PFKP* as a LD gene. However, raised GS activity due to some alternate cause as the mechanism of polyglucosan generation in LD remains an important possibility.

We identified the first LD gene, *EPM2A*, using a positional cloning approach and showed that it encodes a phosphatase, laforin, possessing a carbohydrate-binding domain.[11,12] We and others then sought to determine whether laforin regulates GS and found substantial indirect evidence supporting this hypothesis.[13–17] With the availability of an *Epm2a*$^{-/-}$ mouse model, which faithfully replicates the disease,[18] it became possible to test this directly. Roach and colleagues measured GS quantity and activity in *Epm2a*$^{-/-}$ mice and found no significant difference from wild type (WT) in skeletal muscle, liver, and brain. There were also no differences in proteins that regulate GS (GSK3, PTG, AMPK).[19,20,23] At the same time, they and others showed that laforin is a glycogen phosphatase,[19–21] that in *Epm2a*$^{-/-}$ mice glycogen becomes gradually hyperphosphorylated in an age-dependent fashion, and that hyperphosphorylated glycogen unfolds and precipitates. They proposed that the primary abnormality in LD is not dysregulation of glycogen-synthesizing enzymes, but of glycogen itself, namely of its phosphorylation state. Unfolded insoluble glycogen would accumulate, explaining the increased glycogen in LD, and over time, in ways not understood, would convert into polyglucosans,[19,20]

We identified the second LD gene, *EPM2B (NHLRC1)*, in similar fashion and showed that it encodes an E3 ubiquitin ligase, malin.[22] Again, substantial indirect evidence suggested that malin might regulate GS and its regulators through ubiquitination.[13–17] Several groups aimed to generate *Epm2b*$^{-/-}$ mice to test: (1), whether this form of LD is due to GS upregulation; and (2), whether pathogenesis of this form of LD, where the deficient protein is not a glycogen phosphatase, is also through glycogen hyperphosphorylation. Roach and colleagues reported on their *Epm2b*$^{-/-}$ mice first, at 3 months of age, and showed that there is no dysregulation of GS or of its regulators.[23] They did not address the second question, because glycogen phosphorylation changes in *Epm2b*$^{-/-}$ mice only reach significance after the age of 3 months (see below). Here we describe the generation of our *Epm2b*$^{-/-}$ mice and the results of glycogen phosphorylation studies at 6 months of age.

Materials and Methods

Generation of the Epm2b$^{-/-}$ Construct

The targeting vector used to generate the *Epm2b*$^{-/-}$ mouse line replaced the entire single-exon *Epm2b* coding region with a floxed neomycin transferase (NeoR) gene. First, a bacterial artificial chromosome (BAC) containing the *Epm2b* gene was obtained from Geneservices (bMQ-274C23, designated *Epm2b*-BAC), which was electroporated into bacterial strain DY380. Standard recombineering was used to amplify the floxed NeoR gene from plasmid PL452.[24] Fifty base-pair linkers corresponding to the 5′ and 3′ regions of the *Epm2b* coding sequence were introduced to facilitate recombineering. The product was electroporated into *Epm2b*-BAC, and recombinant clones were selected (*Epm2b*-KO-BAC). A 9.9kb fragment containing the 1.9kb floxed NeoR (flanked by 3.5kb 5′ and 4.5kb 3′ arms) was amplified and transferred to the shuttle vector pMDSsv (kind gift from Knut Woltjen). The final construct contained a floxed NeoR gene replacing the coding region of *Epm2b* for positive selection, along with diphtheria toxin sequence for negative selection (Fig 1A).

Generation of the Epm2b$^{-/-}$ Mouse Line

G4 embryonic stem (ES) cells were maintained, expanded, and electroporated as described previously.[25,26] The *Epm2b*$^{-/-}$ construct was linearized and electroporated into ES cells grown in the presence of G418 (Sigma, St Louis, MO) to select for recombinants. Recombinant ES cells containing the targeted construct were confirmed by long-range polymerase chain reaction (PCR). Positive clones were aggregated with diploid ICR (Institute of Cancer Research) host embryos as previously described.[27] Resultant chimeras were crossed with C57/BL6 mice, and progeny was screened for positive transmission of the targeted allele. Heterozygous mice were bred together to obtain *Epm2b*$^{-/-}$ mice.

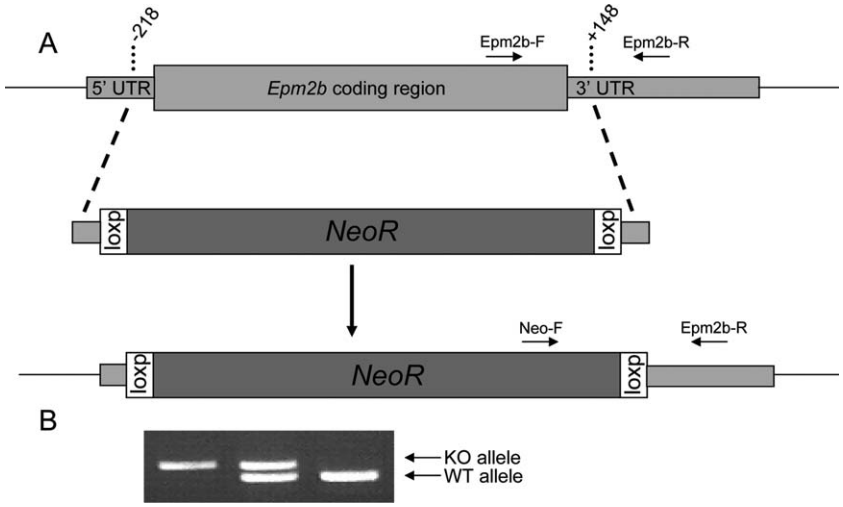

FIGURE 1: Generation of the *Epm2b*$^{-/-}$ mice. (A) Schematic diagram of the generation of the knockout (KO) construct. Top panel depicts the single *Epm2b* exon; UTR = untranslated regions. The region between nucleotides −218 and +148 was replaced by the targeting construct *(middle panel)*, which contains loxP sites flanking a neomycin resistance gene. (B) Confirmation of the *Epm2b*$^{-/-}$ null allele. The knockout (KO) allele is amplified using Neo-F and Epm2b-R, and the wild type (WT) allele with primers Epm2b-F and Epm2b-R. NeoR = neomycin transferase.

Histology

Animals were sacrificed by cervical dislocation, and tissues were immediately fixed in either 10% formalin for 24 hours or minced into 1 mm^3 pieces and fixed in 2.5% glutaraldehyde overnight. For periodic acid-Schiff (PAS)-diastase (PAS-D) staining, sections were incubated with diastase prior to staining with PAS reagent for detection of Lafora bodies.[28] Electron microscopy methods were as previously published.[14]

Western Blotting

Lysates were prepared from tissues using RIPA buffer and protease inhibitor cocktail (Roche, Indianapolis, IN). Forty micrograms of protein was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Membranes were probed using laforin (Abnova, Taipei, Taiwan), GS (Cell Signaling, Beverly, MA), or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Secondary antibodies used were from Santa Cruz Biotechnology.

GS Activity, Glycogen Quantity, and Glycogen Phosphate Measurements

GS activity assays were performed as previously described.[29–31] Activity was measured in the presence of 0.17 and 8mM G6P, the allosteric activator of GS. Fractional velocity was calculated as the activity with 0.17mM G6P divided by that with 8mM G6P. Glycogen isolation, measurement, and covalent phosphate determination were as previously described.[20]

Tissue Fractionations

Fractionations were as reported.[19] For brain, the mouse's head was guillotined into liquid nitrogen after cervical dislocation. Brain was removed on dry ice, ground in liquid nitrogen, lysed with homogenization buffer, and centrifuged at 10,000*g* to separate soluble from insoluble fractions.

Statistical Analysis

The data is shown as means ± standard error of the mean. Statistical significance was evaluated using an unpaired Student *t* test. Values were considered significant at $p < 0.05$.

Results

Epm2b$^{-/-}$ *Mice Have Lafora Disease*

Mice were born at the expected Mendelian frequencies, and the 3 genotypes (WT, *Epm2b*$^{+/-}$, and *Epm2b*$^{-/-}$) could be distinguished by PCR (see Fig 1B). In the previously described *Epm2a*$^{-/-}$ mouse, glycogen phosphate was already increased at 3 months of age, and continued to rise over time.[20] Significant and progressive increase in glycogen quantity and Lafora bodies occurred after this age.[18,19] The progressive epilepsy started at around 9 months.[18] For purposes of the present study, we analyzed our *Epm2b*$^{-/-}$ mice at 6 months. Detailed behavioral, neurophysiologic, and pathologic studies will be reported subsequently.

The 6-month-old mice exhibited numerous Lafora bodies in skeletal muscle (Fig 2), liver (not shown), and brain (Fig. 3), the commonly studied organs. In the brain, Lafora bodies were in their pathognomonic locations in neuronal perikarya and dendrites (see Fig 3C, D), confirming that we had indeed generated LD.

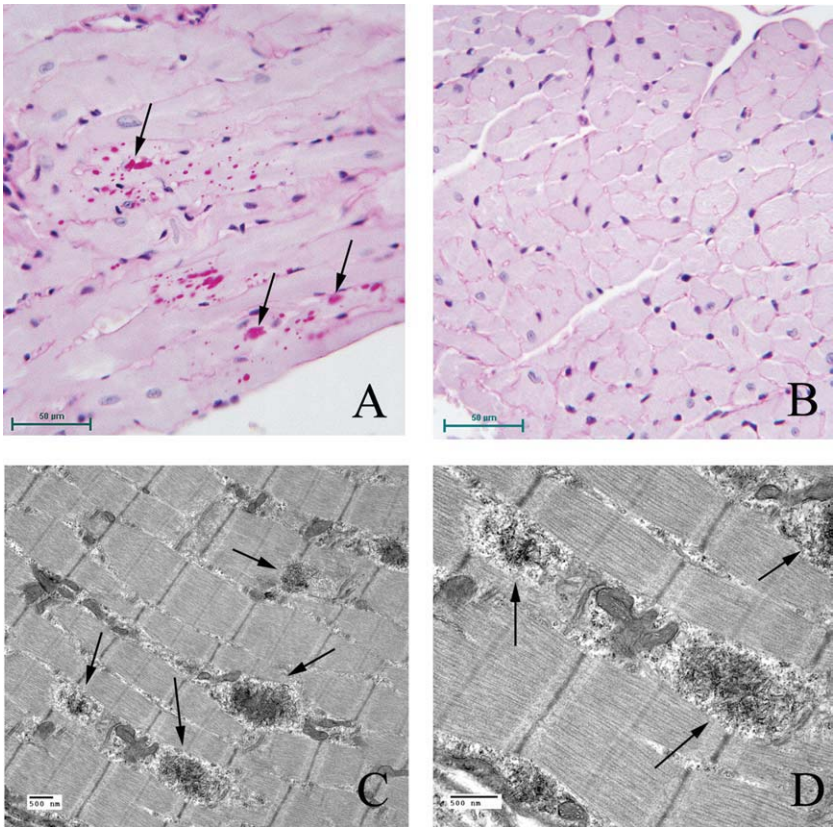

FIGURE 2: Lafora bodies in skeletal muscle. (A) Muscle from an *Epm2b*$^{-/-}$ mouse stained using periodic acid-Schiff-diastase. Note the numerous Lafora bodies *(arrows)* throughout the sarcoplasm of many muscle fibers. (B) Comparable field from a wild type animal. (C) Low-power transmission electron micrograph of a muscle cell containing several Lafora bodies *(arrows)*. (D) Higher-power view of C. Note the characteristic filamentous polyglucosans.

Glycogen Levels Are Elevated in the Epm2b$^{-/-}$ *Mice*

Studies in this and the next sections are in the glycogen-rich polyglucosan-forming organs skeletal muscle and liver. Brain glycogen quantities are very low, and their measurement is often unreliable, because brain glycogen immediately breaks down with hypoxia, even with rapid sacrificing.

In *Epm2a*$^{-/-}$ mice, liver and muscle glycogen levels were not increased at 3 months of age. At 9 to 12 months, muscle glycogen was increased 3-fold (liver not studied).[19] In our 6-month-old *Epm2b*$^{-/-}$ mice, compared to their WT littermates, glycogen levels were 1.6- and 1.2-fold increased in skeletal muscle and liver, respectively (Fig 4A).

Glycogen Synthase Quantity and Activity Are Not Increased in Epm2b$^{-/-}$ *Mice*

Analysis of protein levels by Western blot using whole tissue lysates showed no difference in GS quantity between the *Epm2b*$^{-/-}$ mice and WT (see Fig 4B). GS activity was measured in the presence of low G6P (0.17mM), which reflects the actual GS activity in the tissue, and in the presence of high G6P (8mM), which is such a potent activator of the enzyme that it eliminates all inhibition and thus reflects total GS activity. Fractional velocity (activation state) is the ratio of activity with 0.17mM over that with 8mM and measures the actually active portion of total GS activity. We found no increase in GS activity with 0.17 or 8mM G6P. Activities in both conditions were significantly reduced (see Fig 4C). The activation state of the enzyme was equal to WT (see Fig 4D).

Glycogen Phosphate Content Is Significantly Increased in Epm2b$^{-/-}$ *Mice*

In 3-month-old *Epm2a*$^{-/-}$ mice, liver and muscle glycogen phosphate are increased 40% and 4-fold, respectively.[19,20] At 9 to 12 months, muscle glycogen phosphate is increased 6-fold (liver not studied).[19] In our 6-month-old *Epm2b*$^{-/-}$ mice, glycogen phosphate is increased 1.5-fold in both skeletal muscle and liver. The increases in both organs are highly statistically significant ($p < 0.005$) (Fig 5). It is to be noted, however, that the 1.5-fold increase in muscle in our *Epm2b*$^{-/-}$ mice is

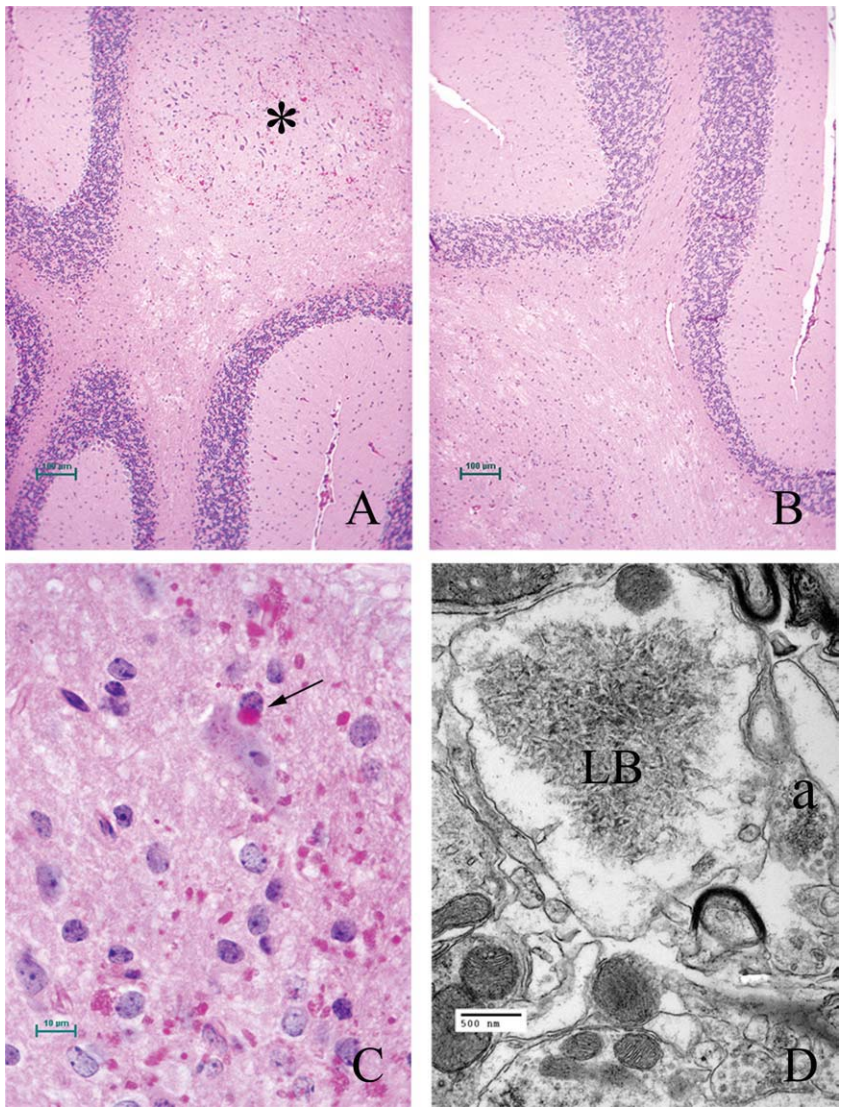

FIGURE 3: Lafora bodies in brain. (A) Low-power view of mouse cerebellum and cortex from an *Epm2b*$^{-/-}$ animal stained with periodic acid-Schiff-diastase. Numerous Lafora bodies are seen in the Purkinje cell layer and cortex *(asterisk)*. (B) Similar area from a wild type mouse. (C) Higher-power view of cerebellar cortex. Note the presence of perikaryal Lafora bodies *(arrow)* as well as numerous ground glass-like Lafora bodies (which under the electron microscope are shown to be in dendrites). (D) Electron micrograph of a dendritic Lafora body. Note the filamentous polyglucosans (LB). a = axon.

substantially less than the increase in 3-month-old *Epm2a*$^{-/-}$ mice.

Laforin Is Increased in the Insoluble Fraction of Epm2b$^{-/-}$ *Mouse Brain*

In their study of 3-month-old *Epm2b*$^{-/-}$ mice, Roach and colleagues reported a 90% decrease of laforin from the soluble fraction of brain homogenates accompanied by an equal increase in the insoluble fraction, indicating a major redistribution of laforin out of the cytosol. They theorized that this decrease in soluble laforin may explain a potential glycogen hyperphosphorylation in *Epm2b*$^{-/-}$ mice.[23] We were unable to confirm this result. In our mice, there is an increase of total brain laforin, an increase of laforin in the insoluble fraction, and no decrease in the amount of laforin in the soluble fraction (Fig 6), that is, we do not see a redistribution of laforin, but do find that its amount in the insoluble fraction is increased.

Discussion

We generated *Epm2b*$^{-/-}$ mice and tested the 2 leading theories of Lafora body generation: increased GS activity and increased glycogen phosphate. We found no evidence of increased GS activity and found a highly significant increase in glycogen phosphate. How could malin regulate glycogen phosphate? Existence of a glycogen phosphatase, laforin, strongly suggests that there exists its

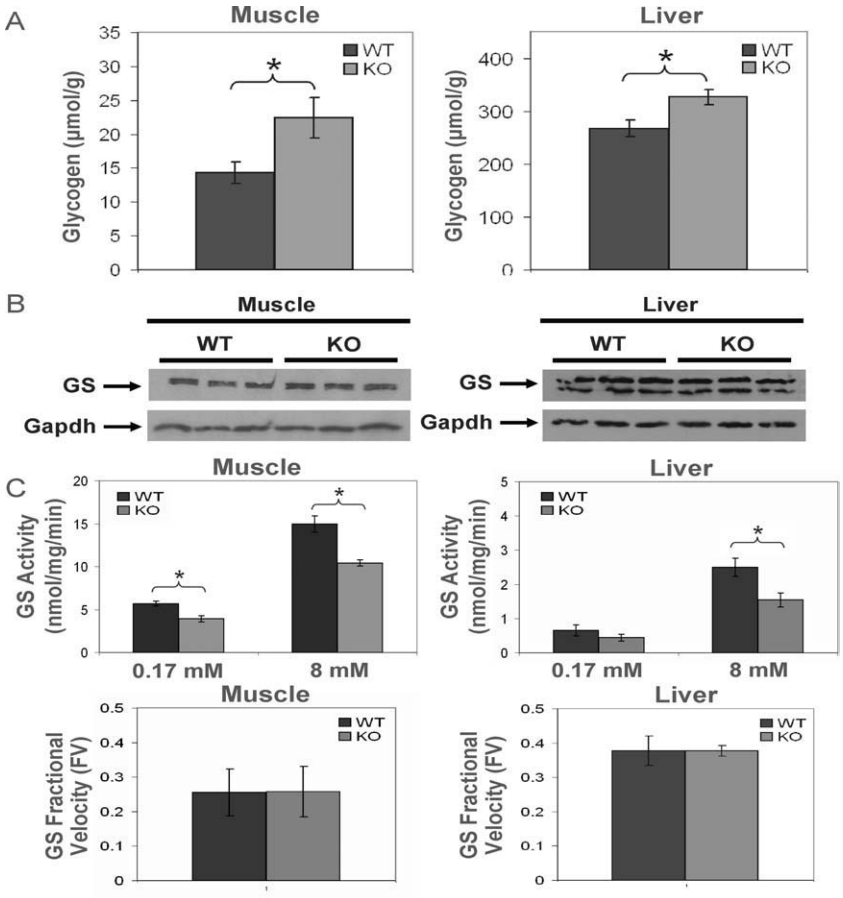

FIGURE 4: Glycogen and glycogen synthase (GS) levels, and GS activity in *Epm2b*$^{-/-}$ mice; n = 5 to 6 mice per group for all biochemical measurements. (A) Glycogen (µmol glucose/g tissue). (B) GS Western blots; glyceraldehyde-3-phosphate dehydrogenase (Gapdh) is shown to confirm equal protein loading. (C) GS total activity (nmol glucose incorporated/mg protein/min); $p < 0.005$ for total GS activity in the presence of saturating 8mM G6P concentration. (D) GS fractional velocity (activation state of the enzyme). WT = wild type; KO = knockout; FV = flow velocity.

counterpart kinase that phosphorylates glycogen. Malin could downregulate this unknown kinase. Alternatively, malin could upregulate laforin, and could do so directly, given that the 2 proteins interact.[14,32]

Malin could upregulate laforin by increasing its quantity. In our malin-lacking mice, the amount of active (soluble) laforin, that is, laforin on normal soluble glycogen and free laforin, is not decreased, indicating that malin does not increase the amount of functional laforin. The mice do exhibit increased laforin in the insoluble fraction. We previously showed in human *EPM2B*$^{-/-}$ LD patients and *Epm2b*$^{-/-}$ LD dogs that laforin tightly binds

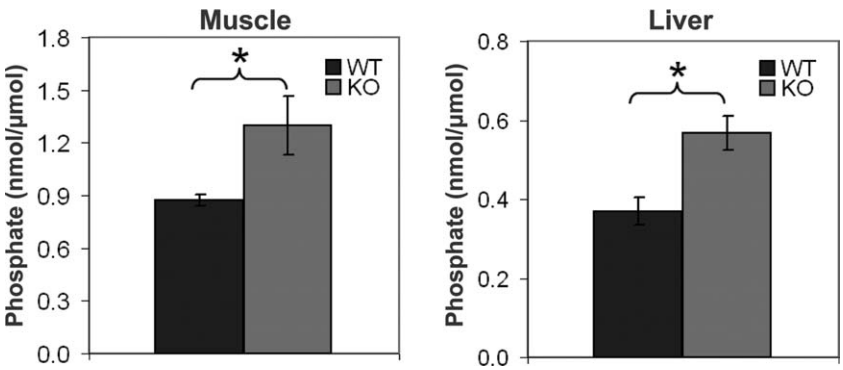

FIGURE 5: Glycogen phosphate levels (nmol phosphate/µmol glucose); n = 5 to 6 mice per group. WT = wild type; KO = knockout.

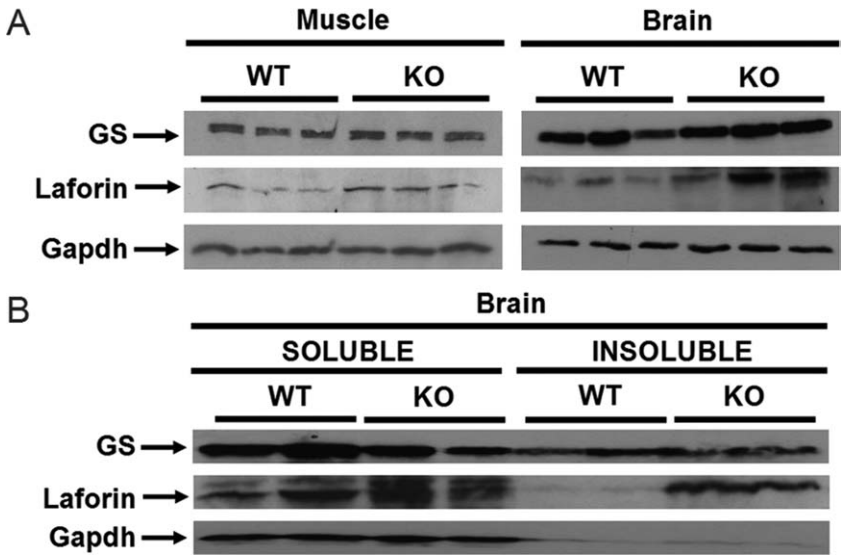

FIGURE 6: Laforin levels in skeletal muscle and brain in *Epm2b*$^{-/-}$ mice. (A) Laforin levels from whole tissue lysates. (B) Laforin levels in the soluble and insoluble fractions from the *Epm2b*$^{-/-}$ brain. WT = wild type; KO = knockout; GS = glycogen synthase; Gapdh = glyceraldehyde-3-phosphate dehydrogenase.

polyglucosans through its carbohydrate-binding domain, precipitates with them, and accumulates in large amounts in Lafora bodies.[14,28] The increased laforin in the insoluble fraction in the *Epm2b*$^{-/-}$ mice is likely likewise laforin trapped on inert insoluble glycogen, polyglucosans, and Lafora bodies. In sum, malin does not upregulate laforin by increasing its quantity, because in its absence laforin is not decreased. If anything, laforin is increased, likely due to its accumulation with polyglucosans.

Malin could upregulate laforin by increasing its activity, possibly through ubiquitination. It has been previously shown that when laforin and malin are co-overexpressed in cell lines, malin ubiquitinates laforin.[32] Ubiquitination has long been known to be a modification that targets proteins to proteasomal degradation, but in recent years it has been implicated in multiple other functions, including functional regulation.[33]

Whether malin regulates glycogen phosphate through laforin, through its prospective counterpart kinase, or otherwise, the hyperphosphorylated glycogen that forms in the absence of malin likely precipitates in the same way as it does in laforin-deficient mice.[19] How does this insoluble glycogen convert to polyglucosans? In the laforin-deficient mice, it has been shown that GS remains bound to the insoluble glycogen and precipitates with it, but BE does not.[19] We theorize that this results in extension of the strands of this insoluble glycogen, unaccompanied by branching, thus converting it to polyglucosans and from there to Lafora bodies.

Finally, does malin deficiency result in polyglucosan formation exclusively through glycogen hyperphosphorylation? Although the increase in glycogen phosphate in our malin-deficient mice is highly significant, the extent of increase at 6 months, 1.5-fold, is significantly less than the increase in laforin-deficient mice at 3 months. It could be considered that this might explain the often quoted slightly lesser severity of the *EPM2B* form of LD compared to the *EPM2A* form.[34–37] However, this statistical lesser severity is due to the presence of several benign missense mutations in *EPM2B* patient cohorts.[34,35] In our clinical experience following many LD patients, the clinical features and severities of the 2 forms of the disease are identical when gene-null cases are compared. We expect that behavioral and epileptic phenotypes of malin and laforin-lacking mice will be identical. The lesser extent of glycogen overphosphorylation in *Epm2b*$^{-/-}$ mice is also likely not due to methodological or mouse strain differences, because our methods are identical to those used in the *Epm2a*$^{-/-}$ study, and the mouse strains are the same. In any case, the extent of Lafora body formation in 6-month-old malin-deficient mice is equal to that of laforin-deficient mice of the same age (unpublished observation). Considering these points, we tentatively conclude that in malin-deficient mice a lesser degree of glycogen overphosphorylation than in laforin-deficient mice is associated with Lafora body formation. We therefore theorize that malin deficiency results in polyglucosan formation only in part through glycogen overphosphorylation, and that malin has an additional function in regulating glycogen structural integrity beyond its regulation of glycogen phosphate.

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Authorship

J.T. and P.W. contributed equally to this work.

Potential Conflicts of Interest

Nothing to report.

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