# PTG Protein Depletion Rescues Malin-Deficient Lafora Disease in Mouse

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**Ubiquitin ligases regulate quantities and activities of target proteins, often pleiotropically. The malin ubiquitin E3 ligase is reported to regulate autophagy, the misfolded protein response, microRNA silencing, Wnt signaling, neuronatin-mediated endoplasmic reticulum stress, and the laforin glycogen phosphatase. Malin deficiency causes Lafora disease, pathologically characterized by neurodegeneration and accumulations of malformed glycogen (Lafora bodies). We show that reducing glycogen production in malin-deficient mice by genetically removing PTG, a glycogen synthesis activator protein, nearly completely eliminates Lafora bodies and rescues the neurodegeneration, myoclonus, seizure susceptibility, and behavioral abnormality. Glycogen synthesis downregulation is a potential therapy for the fatal adolescence onset epilepsy Lafora disease.**

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Lafora disease (LD) is a catastrophic cause of teenage onset progressive myoclonus epilepsy. The initial symptoms are headaches, decline in school performance, myoclonus, visual hallucinations, and generalized seizures. By 5 years, myoclonus is frequent, thoughts and speech are constantly interrupted by atypical and myoclonic absences, seizures are intractable, and a disinhibited dementia has set in. By 10 years, the patient is in a vegetative state, myoclonus is constant, and convulsive status epilepticus is frequent and soon fatal. Pathology reveals widespread intraneuronal Lafora bodies (LB), which consist of malformed glycogen molecules and a small amount (<10%) of protein.[1]

In ~50% of cases, LD is caused by mutations in the *EPM2A* gene,[1] which encodes a glycogen phosphatase, laforin. Glycogen possesses a minute but critical amount of phosphate, which is regulated by laforin. Absence of laforin results in glycogen hyperphosphorylation, which disturbs the construction of glycogen, preventing its elaborate spherical architecture essential to solubility.[2–4] The malformed glycogen (called polyglucosan) precipitates, aggregates, and accumulates into LB. Excess neuronal glycogen, normal or malstructured, is neurotoxic, and is the likely basis of the neurodegeneration and epilepsy in LD.[5,6] Although the role of phosphate and the mechanisms of generation of glycogen spheres are poorly understood, it is clear that glycogen synthase (GS), the enzyme that forms the vast majority of glycogen's interglucosidic (α1–4) bonds, is essential to glycogen synthesis, whether the final structure is normal or polyglucosan.[3] To treat LD by reducing polyglucosans, brain GS was recently genetically eliminated from laforin-lacking LD mice, which resulted in correction of the LD phenotype, including elimination of LB, neurodegeneration, and seizure predisposition.[7] Importantly, the same result was obtained through partial reduction of glycogen synthesis by genetically removing a protein, PTG, that activates GS.[8] These results indicated that LD is potentially treatable by glycogen synthesis downregulation.

In the remaining cases LD is caused by mutations in the *EPM2B* gene encoding a ubiquitin E3 ligase, malin,[1] which was shown to regulate laforin.[9–11] Malin was also suggested to regulate autophagy,[11] the misfolded protein response,[12] microRNA gene silencing,[13] Wnt signal transduction,[14] and neuronatin-mediated endoplasmic reticulum stress,[15] implying a possible complex causality in the *EPM2B* form of LD and therefore a lower likelihood of response to therapies targeting glycogen synthesis regulation.

We here removed PTG from the malin-deficient mouse model of LD and show normalization of the LD

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phenotype, including resolution of LB formation, neurodegeneration, myoclonus, seizure predisposition, and contextual fear response. LD due to *EPM2B* mutations is as responsive to glycogen synthesis downregulation as LD due to *EPM2A* mutations.

## Materials and Methods

### *Mice*

All animal procedures were approved by the Toronto Centre for Phenogenomics and the Hospital for Sick Children animal care committees. Both malin-deficient ($Epm2b^{-/-}$; malin knockout [MKO])[16] and PTG-deficient ($Ptg^{-/-}$; PTG knockout [PKO])[8] mouse lines have been previously described. To generate MKO mice lacking PTG, MKO and PKO mice were bred, and the resultant double heterozygous mice ($Epm2b^{+/-}/Ptg^{+/-}$) crossed together to generate the double-knockout (DKO) animals used in this work. All mice were studied at 7 to 10 months of age, unless otherwise indicated.

### *Pathology and Biochemistry*

Mice were sacrificed by cervical dislocation, and tissues were fixed in 10% formalin. Periodic acid-Schiff staining with diastase predigestion (PASD) to stain LB and glial fibrillary acidic protein staining to assess gliosis were as described previously.[8] For LB counts, images of PASD-stained brain sections at ×20 magnification were opened in Photoshop (Adobe, San Jose, CA) for color adjustments. Magenta was set to −200 and blue to +300, and images were inverted to show LB as white. Adjusted images were then opened in Volocity (PerkinElmer, Waltham, MA), and objects were found using intensity. Objects $<200\mu$m and $>20\mu$m were counted as LB.

For electron microscopy, mice were perfused with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), and tissues were processed as described.[8]

GS activity and glycogen measurements were performed as previously described.[10]

### *Myoclonus and Seizure Susceptibility*

Myoclonus measurements were performed as previously described, with slight modifications.[8] Briefly, mice were videotaped for 4-hour periods. Recordings were then analyzed and myoclonus was counted during 5-minute periods where the mouse was visible from head to tail. Data are reported as means ± standard error. Significance was evaluated using an unpaired Student *t* test.

Seizure susceptibility was assessed as the response to kainic acid, injected intraperitoneally (15mg/kg). Mice were classified susceptible if they developed convulsive seizures within 1 hour postinjection.

### *Contextual Fear Conditioning*

Prior to testing, mice were handled for 2 minutes per day for 3 days. On testing day, they were transported to the testing room and placed inside Plexiglas chambers with stainless steel grid floors (Colbourn Instruments, Whitehall, PA). Two minutes later a foot shock was delivered (1mA, 2-second duration). Mice remained in the chambers for an additional minute before being returned to their home cages. Twenty-four hours later, the mice were returned to the same conditioning chambers for 5 minutes, during which time no shock was delivered. The time spent freezing, a measure of fear memory, was calculated automatically using the FreezeFrame software package (Actimetrics, Wilmette, IL).

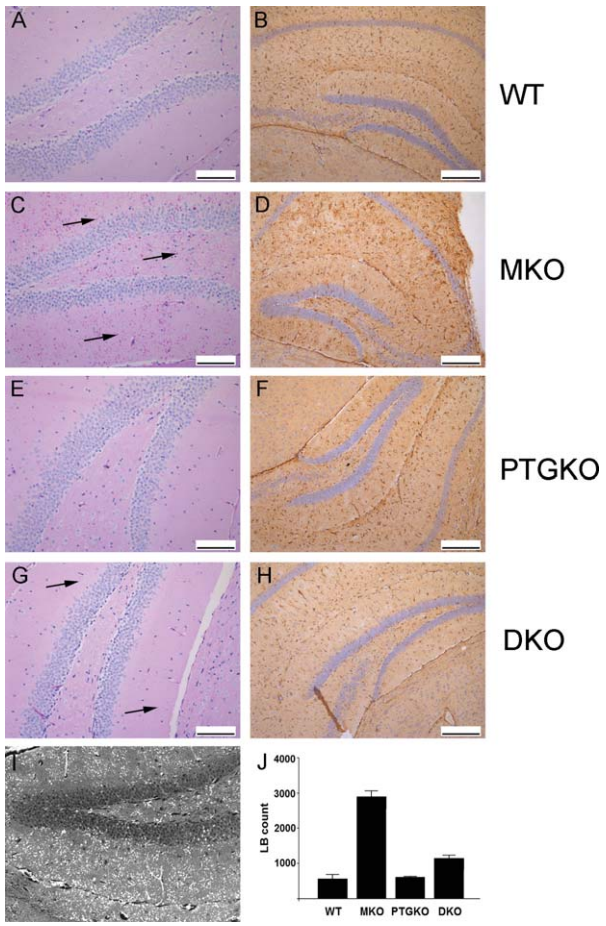


**FIGURE 1: Lafora bodies do not form and there is no gliosis in malin-deficient mice that lack PTG. (A, C, E, G) Periodic acid-Schiff staining with diastase predigestion stain with arrows pointing to Lafora bodies. (B, D, F, H) Glial fibrillary acidic protein immunostaining. (I, J) Lafora body counts in hippocampus. DKO = malin and PTG double knockout; LB = Lafora bodies; MKO = malin knockout; PTGKO = PTG knockout; WT = wild type. Bars = 100$\mu$m in A, C, E, and G; 200$\mu$m B, D, F, and H.**

## Results

### *MKO Mice Lacking PTG Have Drastic Reduction in LB and No Neurodegeneration*

MKO mice have profuse LB in multiple tissues including skeletal muscle and brain.[16] MKO mice lacking PTG (DKO mice) are similar to wild-type mice (Fig 1).

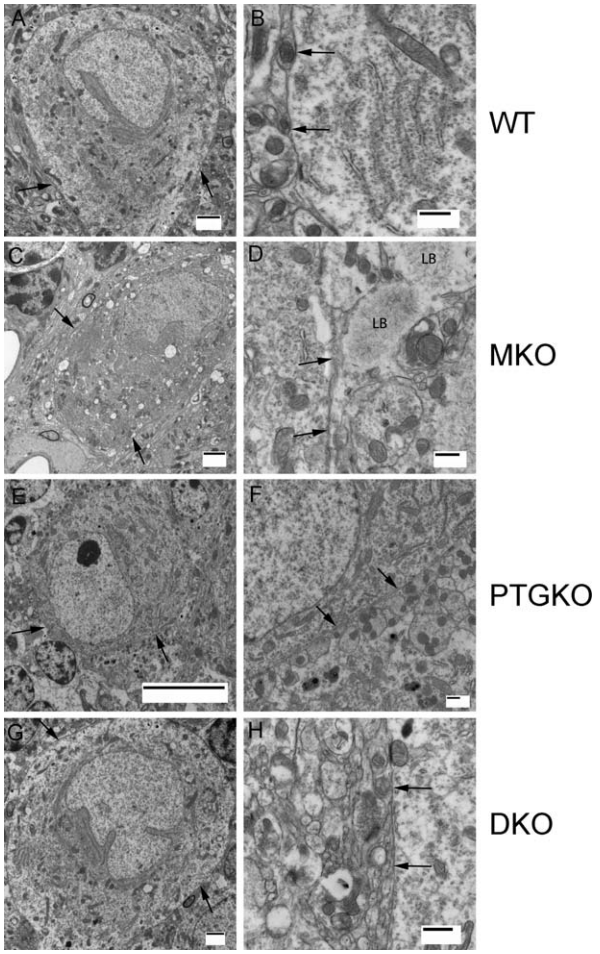


**FIGURE 2: Malin-deficient mice lacking PTG do not exhibit the neurodegeneration of Lafora disease. Cerebellar Purkinje cells are shown. (A, C, E, G) Low-power electron micrographs of representative cerebellar Purkinje cells; wild-type (WT), PTG knockout (PTGKO), and malin and PTG double knockout (DKO) neurons have normal full cytoplasms with smooth taut plasma membrane, whereas the malin knockout (MKO) cell has a dark, condensed cytoplasm and retracted plasma membrane; arrows indicate plasma membranes. (B, D, F, H) Electron micrographs of synaptic contacts *(arrows)* with Purkinje cell membranes; multiple normal synapses with smooth Purkinje cell plasma membrane are seen in WT, PTGKO, and DKO, and poor contacts with a distorted plasma membrane in MKO; note the Lafora bodies (LB) in the MKO cell. Bars = 2$\mu$m in A, C, and G; 500nm in B, D, F, and H; 10$\mu$m in E.**

Neurodegeneration in LD mouse models, laforin-deficient or MKO, is characterized by shrinkage and darkening of neurons, and retraction of neuronal nuclear and plasma membranes, the latter associated with loss of synaptic contacts. Gliosis, most prominently in the hippocampus, is also present.[7,8,17] DKO mice have no gliosis (see Fig 1) and have normal neuronal morphology (Fig 2).

The reduction in GS activity in the DKO mice compared to MKO was ~50% (Fig 3A). The reduction in glycogen was 30 to 50% (see Fig 3B, C).

### *DKO Mice Have No Abnormal Myoclonus and Dramatically Improved Seizure Susceptibility*

Although both laforin-deficient and MKO mice have spontaneous convulsive seizures, these are very infrequent and do not serve as a useful outcome measure. Spontaneous myoclonus and kainic acid seizure susceptibility, however, are readily quantifiable in these mice. Regarding myoclonus, wild-type mice have a certain amount of myoclonus, which is drastically increased in LD mice.[8,17]

Measurement of spontaneous myoclonus showed that DKO mice lack the abnormal increased myoclonus of their MKO littermates and are indistinguishable from wild-type mice (see Fig 3D). After kainic acid injection, no wild-type (0 of 4), all MKO (7 of 7), 1 of 6 PKO, and 1 of 6 DKO mice developed convulsive seizures.

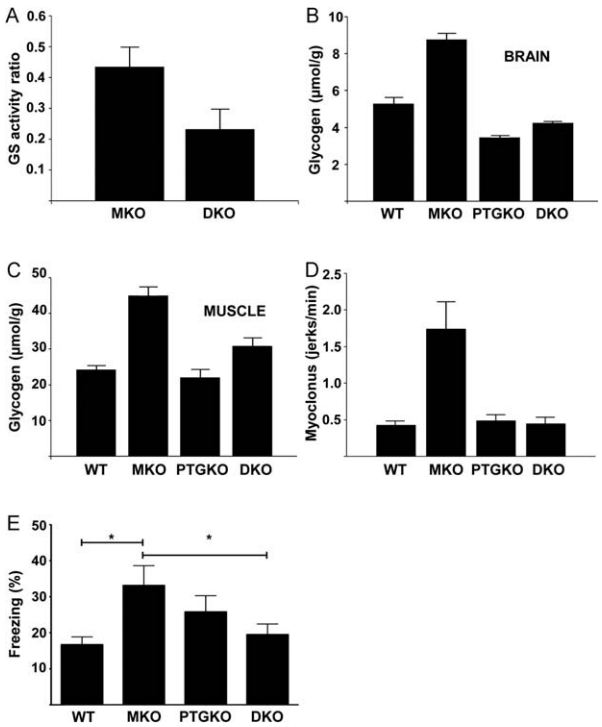


**FIGURE 3: Malin-deficient mice lacking PTG have decreased glycogen synthase (GS) activity (A) and glycogen (B, C), and do not have abnormal myoclonus (D) or contextual fear conditioning (E). Results in A are ratios: GS activity with no glucose 6-phosphate (G6P) added divided by GS activity in the presence of 8mM G6P. G6P is an allosteric activator and at 8mM leads to maximal GS activation; the ratios therefore reflect the extent of the enzyme's activation in each genotype. In A, *t* test statistics are: malin knockout (MKO) to malin and PTG double knockout (DKO), $p = 0.04$, n = 8–10 mice per genotype; in B and C *t* test statistics are: MKO to wild type (WT) and MKO to DKO, $p < 0.005$, n = 5–7 mice per genotype. In D, *t* test statistics are: MKO to WT, $p = 0.008$; MKO to DKO, $p = 0.003$; n = 6–8 mice per genotype. In E, analysis of variance statistics are: MKO to WT, $p = 0.008$; MKO to DKO, $p = 0.019$; n = 14–18 mice per genotype. *Statistically significant. PTGKO = PTG knockout.**

### *MKO Mice Have Abnormal Contextual Fear Conditioning, Which Is Corrected in DKO Mice*

Behavioral tests in LD mouse models are highly sensitive to unknown modifying effects in genetic background. Three separate MKO mouse lines, including the present mice, have been studied behaviorally. Ataxia, motor, and memory deficits were present in 1 (a C57/BL6 and 129Sv mix)[11] but not in another (pure C57/BL6),[17] and are not present in our MKO mice (a C57/BL6 and 129S6/SvEvTac mix; data not shown). Our mice do, however, have a clearly abnormal freezing fear response in contextual fear conditioning testing. This defect is fully corrected in their DKO littermates (see Fig 3E).

## Discussion

In silico analyses that identified laforin's carbohydrate binding domain were soon followed by experiments that unequivocally established that at least 1 function of laforin involves binding and acting on glycogen.[3,6] In laforin-deficient mice, preventing brain glycogen synthesis completely (by genetically removing GS) or partially (by removing the GS activator PTG) eliminated polyglucosan formation and LB. These results indicated that in LD caused by laforin deficiency, LB are due to a disturbance in glycogen metabolism, and that loss of laforin's glycogen-related function underlies this disturbance. Elimination of LB led to elimination of neurodegeneration, myoclonus, and seizure susceptibility, suggesting that LB underlie these neurological features of LD.[7,8]

Malin lacks a carbohydrate binding domain and has numerous attributed functions.[9,11–14,18] Here we show that interfering with glycogen production by removing PTG from MKO mice results in elimination of LB. This indicates that in LD caused by malin deficiency, LB are due to a disturbance in glycogen metabolism, and that of malin's many possible functions those that are glycogen-related underlie LB formation. As in laforin-deficient mice, removal of LB from MKO mice corrects neurodegeneration, myoclonus, and seizure susceptibility, confirming that LB are pathogenic in LD, and not mere epiphenomena. For the first time, our results show that removal of LB also corrects a behavioral phenotype, suggesting that the globality of the neurological disorder in LD, neurodegeneration, epilepsy, and behavioral disturbance, is secondary to the LD glycogenosis.

Fifty percent reduction of GS activity is harmless, as indicated by the finding that parents of patients who have no glycogen synthesis in the brain (glycogen storage disease type 0b) are healthy.[19] Absence of PTG reduces glycogen by approximately 30%[8] (30–50% in the present study). Our previous work showing that reducing glycogen synthesis by removing PTG prevents LD in mice with laforin deficiency suggested that interventions aimed at partially downregulating GS activity would be therapeutic in LD patients with *EPM2A* mutations. The present work shows that LD patients with *EPM2B* mutations, half of all LD patients, are equally likely to respond. Known GS inhibitors (eg, rapamycin used in tuberous sclerosis),[20] new small molecule inhibitors that could be identified through high-throughput screens, and genetic approaches such as antisense oligonucleotides against GS can all now be parts of the potential armamentarium that can be developed to treat LD, which is among the most severe of adolescence onset neurological diseases.

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## Authorship

J.T. and J.R.E. contributed equally to this work.

## Potential Conflicts of Interest

Nothing to report.

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