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Laforin, a protein with many faces: glucan phosphatase, adapter protein, et alii

Matthew S. Gentry[1,*], Carlos Romá-Mateo[2,*] and Pascual Sanz[2]

1 Department of Molecular and Cellular Biochemistry and Center for Structural Biology, University of Kentucky, Lexington, KY, USA
2 Instituto de Biomedicina de Valencia, CSIC and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Spain

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Correspondence
P. Sanz, Instituto de Biomedicina de Valencia, CSIC and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Jaime Roig 11, 46010 Valencia, Spain
Fax: +34 963690800
Tel: +34 963391779
E-mail: sanz@ibv.csic.es
M. S. Gentry, Department of Molecular and Cellular Biochemistry and Center for Structural Biology, University of Kentucky, Lexington, KY, USA
Fax: +1 8593235505
Tel: +1 8593238482
E-mail: matthew.gentry@uky.edu

*These authors contributed equally to this work.

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Lafora disease (LD) is a rare, fatal neurodegenerative disorder characterized by the accumulation of glycogen-like inclusions in the cytoplasm of cells from most tissues of affected patients. One hundred years after the first description of these inclusions, the molecular bases underlying the processes involved in LD physiopathology are finally being elucidated. The main cause of the disease is related to the activity of two proteins, the dual-specificity phosphatase laforin and the E3-ubiquitin ligase malin, which form a functional complex. Laforin is unique in humans, as it is composed of a carbohydrate-binding module attached to a cysteine-based catalytic dual-specificity phosphatase domain. Laforin directly dephosphorylates glycogen, but other proteinaceous substrates, if they exist, have remained elusive. Recently, an emerging set of laforin-binding partners apart from malin have been described, suggestive of laforin roles unrelated to its catalytic activity. Further investigations based on different transgenic mouse models have shown that the laforin–malin complex is also involved in other cellular processes, such as response to endoplasmic reticulum stress and misfolded protein clearance by the lysosomal pathway. However, controversial data and some missing links still make it difficult to assess the concrete relationship between glycogen deregulation and neuronal damage leading to the fatal symptoms observed in LD patients, such as myoclonic seizures and epilepsy. Consequently, clinical treatments are far from being achieved. In the present review, we focus on the knowledge of laforin biology, not only as a glucan phosphatase, but also as an adaptor protein involved in several physiological pathways.

Introduction

The gene encoding the glucan phosphatase laforin is mutated in Lafora progressive myoclonus epilepsy [Lafora disease (LD), OMIM 254780]. LD is a fatal autosomal recessive neurodegenerative disorder characterized by the presence of progressive neurological deterioration, myoclonus, and epilepsy (see [1] and [2] for reviews). LD initially manifests during adolescence, with generalized tonic–clonic seizures, myoclonus, absences, drop attacks, and visual hallucinations. As the disease proceeds, patients enter a vegetative state and eventually die, usually within the first decade from onset of the first symptoms [1,3].

Abbreviations
AMPK, AMP-activated protein kinase; CBM, carbohydrate-binding module; DSP, dual-specificity phosphatase; ER, endoplasmic reticulum; GDE, glycogen debranching enzyme; GSK, glycogen synthase kinase; LB, Lafora body; LD, Lafora disease; PTG, protein targeting to glycogen; PTP, protein tyrosine phosphatase; SEX4, starch excess 4.

A hallmark of LD is the accumulation of insoluble glucans (i.e. carbohydrates) called Lafora bodies (LBs) [4,5]. LBs form in the cytoplasm of cells from most tissues. LBs, like normal glycogen, are composed of glucose residues joined by α-1,4-glycosidic linkages with branches occurring via α-1,6-glycosidic linkages (reviewed in reference 2). However, branches in LBs occur less frequently than in glycogen, and LBs contain increased amounts of phosphate. These properties are shared with amylopectin, the major component of plant starch, and are the reason why LBs and plant starch are water-insoluble. LD patients exhibit increased neuronal cell death, number of seizures and LB accumulations as they age; thus, it is hypothesized that the LBs trigger these symptoms and ultimately the death of the patient [6].

Mutations causing LD have been identified in two genes, *EPM2A* [7,8] and *EPM2B* (*NHLRC1*) [9], and there is evidence for a third locus [10]. *EPM2A* encodes the glucan phosphatase laforin, a type of dual-specificity phosphatase (DSP), and *EPM2B* encodes malin, an E3-ubiquitin ligase [9,11,12]. Laforin prevents LD by at least two mechanisms: (a) it avoids hyperphosphorylation of glycogen by dephosphorylating it, probably thereby allowing proper glycogen formation; and (b) it is an adaptor protein, and targets proteins to be ubiquitinated by the E3-ubiquitin ligase activity of malin.

LD was described over 100 years ago [4]. It took almost 90 years to identify the two genes mutated in LD, and 96 years to define biologically relevant substrates of laforin and malin. Our understanding of laforin's multiple functions sheds light on the mechanisms causing LD. These advances now allow us to postulate ideas for the treatment of this devastating disease.

EPM2A gene

Laforin is encoded by the 130-kb four-exon gene *EPM2A* on chromosome 6q24 of the human genome. It is expressed in all tissues, although brain, skeletal muscle, heart and liver have higher levels of expression [8]. In the brain, laforin is expressed predominantly in the cerebellum, hippocampus, frontal cortex, and olfactory bulb [13]. Laforin expression increases after birth, reaching a maximum during adulthood [13].

EPM2A encodes a 331-residue bimodular protein with an N-terminal carbohydrate-binding module (CBM) (residues 1–124) and a C-terminal DSP domain (residues 157–326) (Fig. 1A). Loss-of-function point mutations in either domain result in LD, demonstrating the essential nature of a functional CBM and DSP domain (a comprehensive meta-analysis of reported mutations can be found in reference 14).

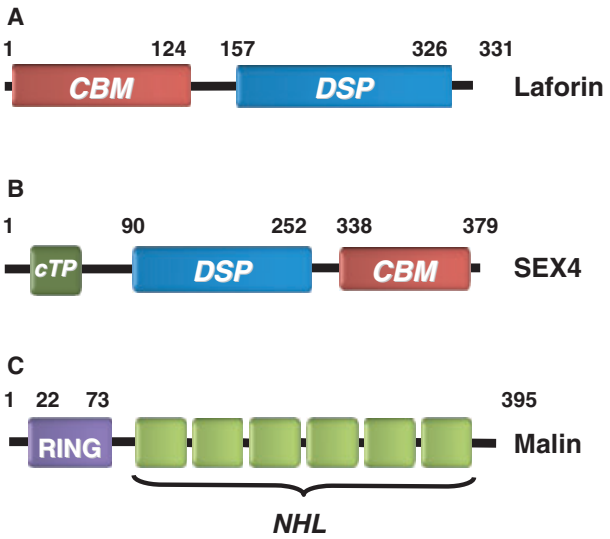

Fig. 1. Schematic depicting the domains present in human laforin (A), *Arabidopsis* SEX4 (B), and human malin (C). cTP, chloroplast-targeting peptide; RING, zinc-finger domain involved in E3-ubiquitin ligase activity; NHL, domains involved in protein–protein interaction; CBM, carbohydrate binding module; DSP, dual specificity phosphatase domain.

EPM2A alternative splicing results in two laforin isoforms that are identical from residues 1 to 309, but contain a divergent C-terminal domain. The isoform laforin-331 is the most abundant form, possesses phosphatase activity, and, when overexpressed in cell culture, localizes to the cytoplasm and endoplasmic reticulum (ER) [15]. The minor isoform, laforin-317, lacks phosphatase activity and localizes to the cytoplasm and nucleus [15]. Interestingly, it was found that the isoforms form heterodimers, and that the heterodimers also lack phosphatase activity [15]. These results suggest that laforin-317 may modulate laforin activity by binding laforin-331, and then functioning as a dominant negative. A recent study reported three additional isoforms of varying lengths, although the physiological role of these isoforms is still unclear [16].

Domains, biochemical properties, and phylogeny

CBM

CBMs are noncatalytic domains classified into 64 families on the basis of evolutionary relationships, polypeptide folds, and substrate preferences according to the Carbohydrate-Active Enzymes (CAZY) database [17]. Proteins containing a CBM utilize the domain to bind carbohydrates, and enzymatically modify the carbohydrates with a second domain (e.g. a hydrolase domain)

[18]. The laforin CBM belongs to the CBM20 family [19]. CBM20 domains are 90–130 residues long. They constitute one of the most well-characterized CBM families, and are characteristic of glucosylhydrolases and glucotransferases from bacteria, fungi, and plants [18,20,21]. CBM20 domains are highly heterogeneous at the amino acid level, and lack invariant residues, but contain moderately well-conserved aromatic residues that coordinate ligand binding. The CBM20 domain typically consists of seven β-strands that form an open-sided distorted β-barrel, with aromatic residues interacting with glucan chains rather than the starch crystalline surface, as seen with other CBM families [22]. In the case of laforin, the CBM allows laforin to bind glycogen and LBs as well as plant amylopectin [19,23,24]. It is of note that laforin is the only human phosphatase with a CBM present in the same polypeptide chain as the catalytic domain [25,26].

As no crystal structure of laforin is yet available, we generated a homology model of the CBM of laforin (residues 1–116), using the best available structure [*Geobacillus stearothermophilus* cyclodextrin glycosyltransferase (Protein Data Bank: 1CYG)] [27]. The homology model suggests that the laforin CBM folds into the characteristic two β-sheet fold, with the N-terminus and C-terminus pointing towards opposite ends of the longest axis of the molecule [28]. Conserved aromatic residues involved in carbohydrate binding are readily observable in the laforin CBM structure: Trp32, Trp85, and Trp99. In the model, these residues form a compact, rigid and surface-exposed hydrophobic site containing inter-ring spacing appropriate for binding to α-1,4-linked glucoses, as is the case for glycoamylase [28].

DSP domain

Laforin contains a C-terminal DSP domain. The DSPs belong to the larger protein tyrosine phosphatase (PTP) superfamily of cysteine-dependent phosphatases, which encompass ∼ 107 human genes [25,26]. PTPs utilize a conserved CX_5R motif to hydrolyze phosphoester bonds [26]. The DSP family includes phosphatases that dephosphorylate proteinaceous and/or nonproteinaceous substrates [26,29]. Like other DSPs, recombinant laforin dephosphorylates the artificial substrates *p*-nitrophenylphosphate and 3-*O*-methyl *fluorescein phosphate* [30–32]. As a cysteine-based enzyme, laforin requires a reduced environment to be active and is reversibly inactivated under oxidative conditions [33].

The endogenous substrate for laforin remained elusive for some time, with several laboratories searching for it by targeted approaches and unbiased screening methods. As laforin contains a CBM, and the hallmark of LD is aberrant glycogen, multiple laboratories systematically tested proteins involved in glycogen metabolism as possible laforin substrates. In the end, a multisystem approach revealed that laforin directly dephosphorylates glucans instead of proteins involved in glycogen metabolism, and these data established laforin as a glucan phosphatase [23,34,35] (see below).

Although the DSPs share significantly less amino acid conservation than the classical PTPs, they still retain the characteristic αβα PTP fold. In addition, the DSPs share many of the conserved elements first described for the classical PTPs. These enzymes utilize a cysteine at the base of the active site cleft within the PTP loop to perform nucleophilic attack on the phosphorus atom of the substrate. An aspartic acid ∼ 30 residues N-terminal of the catalytic cysteine acts as the general acid catalyst, enhancing catalysis. A key difference between the classical PTPs and DSPs is that the classical PTPs possess a deeper active site, allowing access to only phosphotyrosine, whereas the DSP active site is more shallow, to accommodate phosphoserine, phsophothreonine, and phosphotyrosine [26,29].

The laforin DSP domain was modeled by comparing it with that of the human DUSP22 phosphatase (Protein Data Bank: 1WRM [36]) [27]. This *in silico* approach suggests that the laforin DSP domain folds into the characteristic αβα PTP fold, consisting of four to five β-sheets surrounded by α-helices [37]. In this structure, the PTP loop, containing the catalytic cysteine (Cys266), and the conserved aspartic acid (Asp235) point towards the catalytic groove. Despite these efforts, a crystal structure of laforin is needed to determine how phosphoglucans are bound by CBM20 and positioned into the laforin DSP active site.

Laforin dimerization

Recombinant laforin purified from bacteria, laforin from cell culture and laforin from tissue all form dimers [38–40]. However, the domain(s) involved in this event, the mechanism(s) driving dimerization and the biological function of dimerization are poorly understood. One study reported that laforin forms SDS-resistant dimers both *in vitro* and *in vivo* [39]. In addition, it was reported that monomeric laforin is inactive, and that all of the phosphatase activity comes from dimeric laforin [39]. However, recent studies have challenged this finding by demonstrating that monomeric laforin is the most abundant form of the phosphatase under normal reduced conditions, and that laforin phosphatase activity is similar for both monomer and dimer species [33]. The discrepancy is probably attributable to the oxidative conditions in the

experimental methods, as an oxidative environment drives laforin oligomerization and abolishes laforin phosphatase activity. In the study performed by Liu *et al.* [39], little to no reducing agent was used, and for this reason it was concluded that monomeric laforin was inactive. Cumulatively, the new data establish that monomeric and dimeric laforin possess similar phosphatase activity and glucan-binding ability, and that dimerization is enhanced by increased oxidation [33]. Despite these findings, no biological role was identified for laforin dimers. Dimerization did not affect phosphatase activity, glucan binding, or binding to other known interacting partners. Thus, a role for laforin-331 homodimers is currently unknown.

Insights from phylogeny

The laforin gene is conserved in all vertebrate genomes, but it is absent from the genomes of most nonvertebrate organisms, including the standard model organisms yeast, flies, and worms [2,23,41] (Fig. 2). Surprisingly, laforin is conserved in the cephalochordate *Branchiostoma floridae* and in the cnidarian *Nematostella vectensis*, as well as in the following five protozoans: *Cyanidioschyzon merolae*, *Toxoplasma gondii*, *Eimeria tenella*, *Tetrahymena thermophila*, and *Plasmodium tetraurelia* [23,41]. Thus, laforin has an ancient and unique evolutionary lineage. Laforin conservation in these five protozoan species was a surprising and fortuitous finding. These five organisms all undergo a type of hibernation at some point in their life cycle, and when they 'hibernate' they form an insoluble glucan (floridean starch) that resembles LBs. This result provided an additional link to insoluble glucans, and thus offered an insight into the biological substrate of laforin.

A phylogenetic study of malin was also recently performed. It indicated that malin is present in all

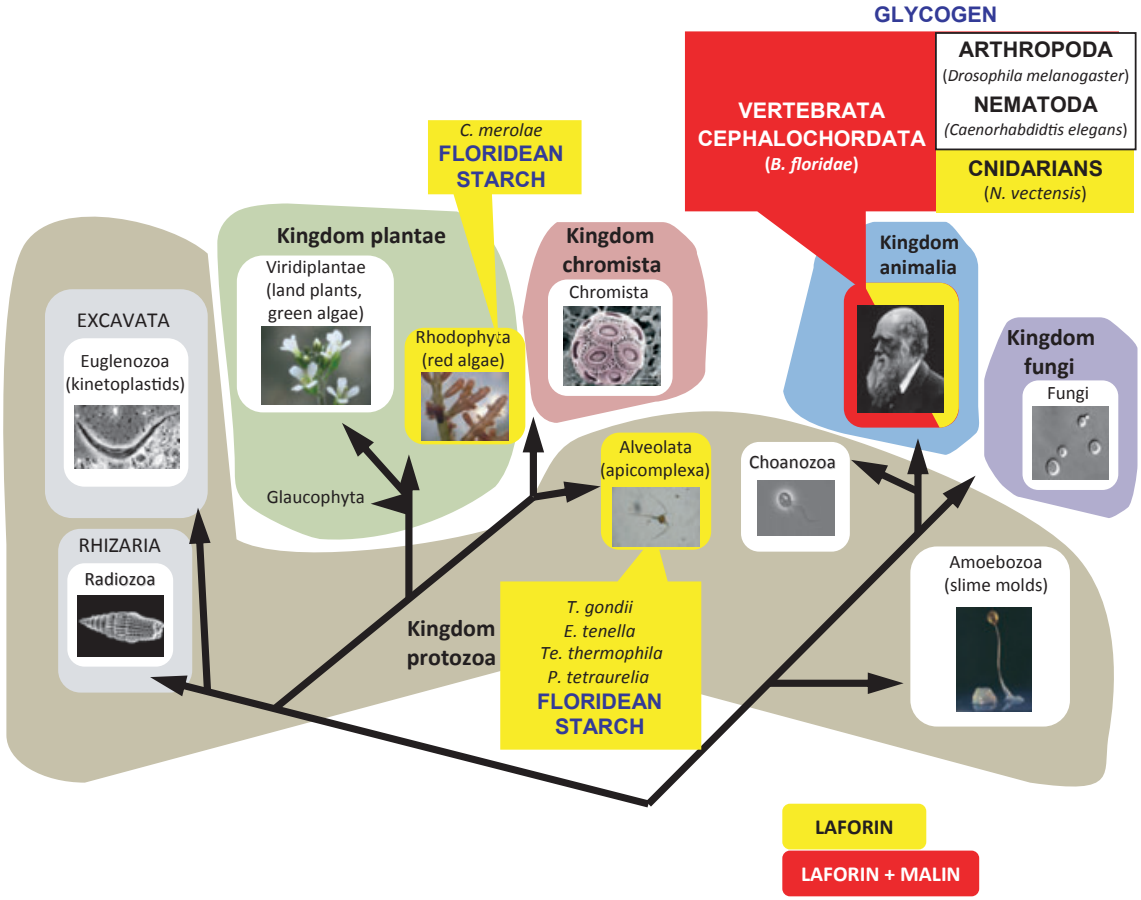

Fig. 2. Laforin and malin phylogeny. Schematic view of the presence of laforin (yellow background) or laforin and malin (red background) in the different kingdoms of the eukaryotes. Groups that do not contain either laforin or malin orthologs are displayed on a white background. In groups that contain laforin, malin, or both, the corresponding organisms are indicated, as well as the type of polyglucosan used as energy source. Note that malin orthologs are only present in organisms that also contain laforin. Modified from [88].

vertebrate species and a cephalochordate [42]. When the species distribution of malin was compared with that described for laforin [23,41], it was observed that laforin and malin do not correlate in species distribution (Fig. 2). As laforin is present in the genomes of more evolutionarily basal organisms than malin, these results suggest that laforin may perform additional functions independently of malin. It is possible that these functions are conserved from red algae to humans, but these results indicate that, at least in lower eukaryotes, laforin possesses malin-independent functions, probably glucan dephosphorylation [42].

Biological function

Glucan phosphatase activity

A single experiment based on unforeseen findings in the literature revealed that laforin is the founding member of a unique class of phosphatases that dephosphorylate phosphoglucans, the glucan phosphatases [34]. In the 1960s, Yokoi *et al.* and Sakai *et al.* [6,43] purified and biochemically characterized LBs from brains of LD patients. They utilized electron-probe microanalysis, by focusing a 1-μm beam of electrons on an LB, and they analyzed the wavelength of excited X-rays to determine specific elements within the LB. In a small table as part of a 33-page study, they reported that LBs possess two to threefold higher amounts of phosphate than glycogen, whereas other elements were equal in both samples [6,43]. Although they were unaware of laforin, they did postulate that ester-linked phosphate might explain why amylolytic enzymes are largely unable to degrade LBs [43]. In addition, they surmised that LBs are biochemically more like plant amylopectin than animal glycogen.

Work by Kerk *et al.* and Niittyla *et al.*, using *Arabidopsis*, also provided an intriguing clue to the function of laforin. These groups identified a protein similar to laforin in *Arabidopsis* that contains both a CBM and a DSP domain, but the domains are in the opposite orientation to that in laforin [44,45] (Fig. 1B). In addition, Niittyla *et al.* demonstrated that mutation of the gene results in an accumulation of starch, and designated the protein as starch excess 4 (SEX4) [45]. Prior to these data, multiple laboratories had identified glucan water dikinase and phosphoglucan water dikinase as two *Arabidopsis* dikinases that phosphorylate the C6 and C3 positions of glucose moieties on starch, respectively [46–49].

These findings, along with the finding of laforin in protozoan models, provided the impetus to test laforin as a glucan phosphatase. Initially, laforin was shown to dephosphorylate amylopectin from plant starch [23,34]. This result prompted the hypothesis that laforin removes phosphate monoesters from glycogen, allowing glycogen metabolism to proceed normally. In the absence of this activity, glycogen would accumulate more phosphate residues and longer unit chains, owing to inhibited branching by the phosphates, and would eventually form an insoluble LB that biochemically resembles amylopectin. The presence of phosphate groups in glycogen was demonstrated in the 1980s and 1990s [50,51], but, up to now, there has been no report on how these phosphates were removed. The Roach group [35] confirmed the *in vitro* dephosphorylation of amylopectin by laforin, and also showed that mammalian glycogen was a substrate of this phosphatase. In addition, they demonstrated that glycogen isolated from laforin knockout mice was hyperphosphorylated and developed an abnormal structure [35,52]. Cumulatively, these data established laforin as a glucan phosphatase, and provided one mechanism for LB formation. A recent study by Tagliabracci *et al.* completed the circle by identifying the source of glycogen phosphate. They found that glycogen synthase incorporates the β-phosphate of UDP-glucose (its substrate) at a rate of one phosphate per 10 000 glucose moieties as C2-linked and C3-linked monoesters [53]. Thus, one function of laforin is to prevent the enzymatic error mediated by glycogen synthase leading to the phosphorylation of glycogen.

Adaptor protein of enzymes involved in glycogen synthesis

Malin is an E3-ubiquitin ligase that contains an N-terminal RING domain and six C-terminal NHL domains that are predicted to form a β-propeller-type protein interaction domain [11,12] (Fig. 1C). Multiple labratories have demonstrated that laforin and malin form a complex, and that laforin recruits substrates to be ubiquitinated by malin. These substrates are ubiquitinated by malin in a laforin-dependent manner, and many of the substrates are enzymes involved in glycogen synthesis. The laforin–malin complex binds and ubiquitinates the muscle isoform of glycogen synthase [54] and protein targeting to glycogen (PTG), the glycogen-targeting subunit of protein phosphatase type 1 [55,56]. In these experiments, the laforin–malin complex ubiquitinated the substrate, decreased the substrate protein level, and decreased glycogen levels (Fig. 3). The role of laforin as an adaptor protein is uncoupled from its role as a glucan phosphatase, as a catalytically inactive phosphatase mutant (C266S) still recruits malin and targets it to glycogen-related

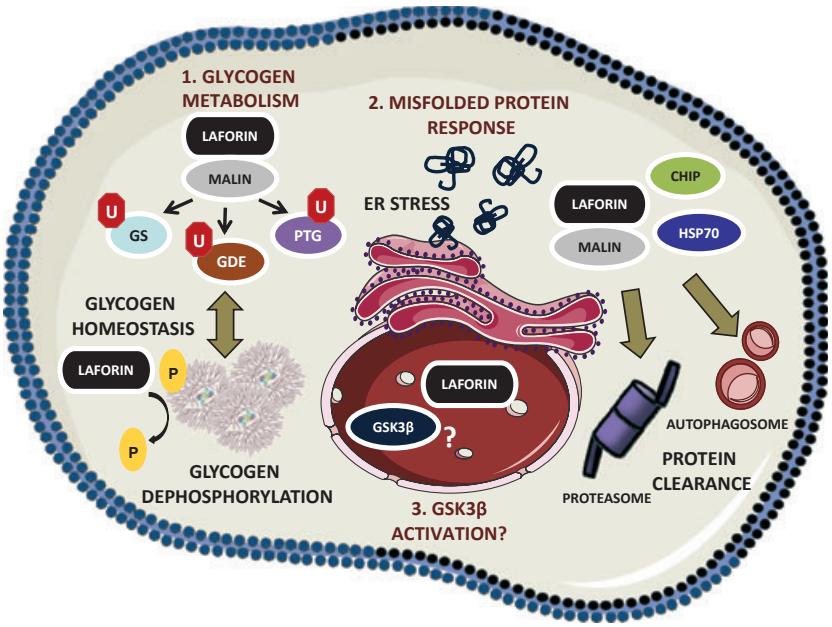

Fig. 3. Laforin functions. Schematic view of the different functions of laforin in cell physiology. GS, glycogen synthase; GDE, glycogen debranching enzyme; HSP70, heat shock protein 70; U, ubiquitin; P, phosphate. See text for details. The figure was produced with the Server Medical Art platform.

enzymes [55,56]. In addition to PTG and glycogen synthase, one report suggested that malin ubiquitinates glycogen debranching enzyme (GDE/AGL) [57]. This report showed that GDE ubiquitination is increased in a wild-type malin-dependent manner when both proteins are overexpressed. Additionally, these investigators demonstrated that increased levels of cAMP increased the interaction between malin and GDE as measured by coimmunoprecipitation and subcellular localization. New information on the contribution of the laforin–malin complex to glycogen regulation has been reported very recently. Jana *et al*. reported that the laforin–malin complex interacts with neuronatin, an 81-residue protein that stimulates glycogenesis. The laforin–malin complex ubiquitinates and promotes the proteasomal degradation of neuronatin; therefore, they proposed that, in the presence of an inactive laforin–malin complex, neuronatin accumulates and hyperstimulates glycogen synthesis [58].

Many of the conclusions regarding glycogen synthase, PTG and other glycogen-related proteins were based on cell culture experiments in which malin, laforin and/or the putative substrate were overexpressed. However, multiple laboratories have recently tested these initial findings under more biologically relevant conditions, and they have found conflicting results. In contrast to the cell culture data, 3-month-old mice lacking laforin did not show increased levels of glycogen synthase or PTG in muscle or brain extracts [52]. Additionally, two groups found no increase in glycogen synthase, PTG or GDE in malin-deficient mice aged of 3–6 months [59,60]. However, a third group found dramatically higher levels of glycogen synthase in brain extracts from 11-month-old malin-deficient mice than in those from wild-type mice [61].

Although protein ubiquitination was first described as a mechanism for targeting proteins for rapid proteasomal degradation, in recent years other functions of ubiquitination have been delineated that are driven by different types of ubiquitination, e.g. monoubiquitination, multiubiquitination versus polyubiquitination, and different chain topologies [62,63]. A recent study reported that the laforin–malin complex produces Lys63-linked polyubiquitin chains in PTG, AMP-activated protein kinase (AMPK)α, and AMPKβ [64]. These results suggest that the modification introduced by the laforin–malin complex may play a different role from targeting substrates for degradation by the proteasome.

It is possible that, under certain circumstances, the laforin–malin complex could also promote the formation of Lys48-linked ubiquitins. This possibility has also been described for parkin, an E3-ubiquitin ligase involved in Parkinson's disease that modifies synphilin-1 with both Lys63-linked and Lys48-linked ubiquitin chains [65]. Perhaps this is the reason why the overexpression of laforin and malin promotes the proteasomal degradation of PTG [55]. This possibility would reconcile the results obtained in mouse models lacking either laforin or malin, where it has been reported that, in spite of increased levels of glycogen in different tissues (skeletal muscle and brain), there are no differences in either the activity or the protein levels of glycogen synthase or PTG [35,59,60]. However, the most recent report on this matter indicates that, in

the brains of 11-month-old mice lacking malin, there is an increase in the levels of the muscle glycogen synthase [61]. These results indicate that the age of the mice may be important, as the previous studies examined younger mice. These latest results suggest that the laforin–malin complex does indeed have a role *in vivo* in downregulating proteins involved in glycogen homeostasis, but more work is needed to define the mechanism of these events. Supporting the possible role of the laforin–malin complex in the regulation of PTG levels, we found that PTG protein levels were also increased in primary fibroblasts from LD patients [66]. Further bolstering PTG as a genuine substrate is the ability to recapitulate *in vitro* ubiquitination of PTG by malin in a laforin-dependent manner, with purified components [56].

Adaptor protein in ER stress and protein clearance

The laforin–malin complex also plays a role in protecting cells from ER stress conditions. In cell culture models depleted of laforin, there is enhanced sensitivity to agents that trigger ER stress, e.g. thapsigargin and tunicamicin [67]. In laforin-depleted cells, there is a decrease in proteasome activity and an increase in apoptosis upon drug treatment, as compared with control cells [67]. Similar results regarding protein aggregation-induced cell death in malin-depleted cells have also been reported [68]. Therefore, in the absence of either laforin or malin, there is an increased ER stress response that eventually leads to decreased proteasome function and increased apoptosis, which could be important factors in the development of LD. Strong corroborating evidence for these cell models is that tissue from mice lacking laforin and human LD patients has increased levels of ER stress markers [67] (Fig. 3).

In addition to loss of laforin resulting in enhanced sensitivity to ER stress, laforin itself seems to contribute to ER stress. Overexpressed laforin is prone to aggregate, and these aggregates localize in perinuclear aggresome structures that colocalize with ubiquitin, ER chaperones, and proteasome subunits [69]. We have also observed colocalization of the autophagy marker p62 with these structures, suggesting that they might be labeled for degradation by autophagy (unpublished results). Laforin aggregation is enhanced when some LD mutant forms of laforin are overexpressed, and these aggregates also contribute to an increased ER stress response and increased apoptosis [70].

The laforin–malin complex has also been implicated in suppressing cytotoxicity produced by the accumulation of misfolded proteins. The Ganesh group [71] overexpressed aggregate-prone proteins, and demonstrated that the laforin–malin complex in conjunction with heat shock protein 70 degraded the aggregates and protected against cytotoxicity. The laforin–malin complex interacts with misfolded proteins, and targets them for degradation by the proteasome. Follow-up studies demonstrated that laforin and malin coimmunoprecipitate with the co-chaperone protein CHIP, and showed that CHIP stabilizes malin's tertiary structure [72,73].

Finally, laforin has also been described as a positive regulator of autophagy. In both cellular and mouse models lacking laforin, there is decreased autophagy. This decrease is a result of impaired formation of autophagosomes, which leads to decreased content of autophagic vesicles and lower levels of the LC3-II autophagic marker. The molecular basis of this defect is not known, although it seems that, in cells lacking laforin, the mammalian target of rapamycin pathway is overactivated. The changes in autophagy mediated by the absence of laforin may lead to the accumulation of diverse autophagy substrates that would contribute to cell stress and may contribute to cell death [74] (Fig. 3). Similar defects in autophagy have recently been described in a mouse model lacking malin (*Epm2b*$^{-/-}$) [75]. Therefore, autophagy dysfunction is observed in both mouse models of LD (*Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$).

Unexpected role – laforin as a tumor suppressor

An unexpected proposed function of laforin is as a tumor suppressor. One line of mice expressing SV40 large T antigen and a rearranged T-cell receptor developed T-cell lymphoma with almost 100% penetrance [76,77]. It was later discovered that, in these mice, the T-cell receptor disrupted exon 1 of one *EPM2A* locus and that the second locus underwent epigenetic silencing, and the authors concluded that laforin could act as a tumor suppressor [77]. The tumor suppression is associated with laforin phosphatase activity, as mice injected with a T-lymphoma cell line transduced with a wild-type laforin lentiviral vector displayed protection against tumor formation, whereas mice injected with a T-lymphoma cell line transduced with a catalytically inactive laforin (C266S) were not protected [77]. The authors proposed that laforin dephosphorylated p-Ser9 of glycogen synthase kinase (GSK)3β and, in the absence of laforin, GSK3β would accumulate in its inactive phosphorylated form. As GSK3β is a key regulator of Wnt signaling, the inactivation of GSK3β would lead to the accumulation of β-catenin inside the

nucleus, producing an increase in tumorigenesis [77]. In a follow-up study, the authors reported that laforin negatively regulates the cell cycle through dephosphorylation of GSK3β and its regulation of cyclin D1. Lack of laforin results in increased levels of cyclin D1, which promotes cell cycle progression [78] (Fig. 3). Despite convincing data demonstrating that laforin suppresses tumor growth in immunocompromised mice, which strongly links laforin with cell cycle progression, the data that support laforin as a direct GSK3β phosphatase are controversial. Other laboratories tested GSK3β as a laforin substrate by using *in vivo* and *in vitro* methods during targeted searches for a substrate, before the glucan phosphatase activity was discovered, and did not observe dephosphorylation of GSK3β by laforin [34,35,79]. Thus, the link between laforin and tumor suppression in immunocompromised mice remains to be elucidated.

Other interactions

Although laforin has been definitively shown to be a glucan phosphatase, multiple studies have also found that laforin directly and/or indirectly interacts with many proteins. Multiple techniques have been utilized to identify possible interaction partners and/or putative substrates, and these results are summarized in Table 1. Many of these interactions have been discussed above, but, apart from malin, it is unclear at this time what the physiological relevance of some of these interactions may be.

Controlling/regulating laforin

Although the list of interactive proteins and putative roles of laforin continues to expand, to date only four mechanisms have been described for the regulation of laforin. The first discovery of how laforin is regulated was both surprising and perplexing. Using cell culture models, we found that malin binds, ubiquitinates and targets laforin for degradation; and we were able to recapitulate the ubiquitination by using purified components *in vitro* [12]. This result is surprising, given that laforin and malin activity are both necessary to inhibit LB formation and LD. However, malin-directed degradation of laforin has been verified in multiple cell culture systems, mouse models, and LD patient tissue [54,55,59–61,67].

An additional mechanism regulating laforin levels is directly tied to glycogen stores. Roach and colleagues [80] examined mouse models that accumulate higher or lower levels of glycogen, and found that laforin levels directly correlate with the amount of glycogen. Although this link has been described, the mechanism regulating this fluctuation is currently unknown.

Recently, we demonstrated that laforin physically interacts with the AMPKα and AMPKβ subunits of the heterotrimeric AMPK, a key cellular energy sensor [55]. We found that AMPK is a positive regulator of the laforin–malin complex, as the interaction between laforin and malin is enhanced under conditions of AMPK activation [55]. In a follow-up study, we demonstrated that AMPK phosphorylates laforin at Ser25 both *in vivo* and *in vitro* [27]. We found that Ser25 is critical for both laforin phosphatase activity and for its ability to interact with established binding partners, e.g. dimerization with itself, malin, and PTG [27]. These results suggest that laforin-Ser25 phosphorylation by AMPK modulates the laforin–malin interaction and provides a means to regulate the role of laforin and malin in glycogen metabolism. However, these data, as with many in the laforin field, are also

Table 1. Laforin interaction partners. Proteins reported to interact with laforin are indicated, along with their biological function and the corresponding identification method. Co-IP, coimmunoprecipitation.

Protein	Function	Identification method	Reference
Malin	E3-ubiquitin ligase	Yeast two-hybrid screening	38
PTG	PPP1R3C regulatory subunit	Yeast two-hybrid screening	38
GL	PPP1R3B regulatory subunit	Co-IP	34
R6	PPP1R3D regulatory subunit	Functional interaction	56
HIRIP5	Possibly involved in iron homeostasis	Yeast two-hybrid screening	85
EPM2AIP1	Unknown	Yeast two-hybrid screening	86
GS	Glycogen synthase	Co-IP	34
GSK3β	Involved in Wnt pathway regulation	Mammalian two-hybrid; Co-IP	11
AMPKα/β	Cell energy sensor	Yeast two-hybrid; Co-IP	55
TAU	Microtubule-associated protein	Pulldown	87

controversial. Roach and co-workers [59] investigated the levels of PTG in exercised mice, in which AMPK is activated, and they saw no change in PTG levels. Thus, additional work must be performed to determine the role of AMPK in controlling the laforin–malin complex.

The last reported means of regulating laforin activity is via heterodimerization of different splice variants. Ganesh and colleagues [15,16] characterized different laforin isoforms, and found that they display distinct subcellular localizations in cell culture. In addition, they reported that heterodimerization between truncated isoforms and full-length laforin results in a phosphatase-inactive complex [15], thus offering a mechanism for the regulation of laforin function.

LD causes and possible therapeutics

Given the ever-expanding reports of putative laforin functions and laforin-interacting proteins, it is clear that multiple mechanisms drive the progression of LD. The different lines of data strongly suggest that glycogen phosphorylation, interactions with glycogen metabolism enzymes and cellular stresses are all intimately involved in disease progression. However, deciphering the intercalated pathways driving these mechanisms will probably take many more years.

One definitive function of laforin is to remove phosphate from glycogen. Failure to remove covalently attached phosphate from glycogen disrupts glycogen organization, and results in LB formation. Laforin clearly functions as a glucan phosphatase, but there is some controversy regarding all of the other proposed functions. It seems likely that laforin participates in other aspects of glycogen metabolism by functioning as an adaptor protein for malin-directed ubiquitination of some glycogen metabolism enzymes. If both of these functions are correct, then LB formation would result from either lack of laforin glucan phosphatase activity or lack of laforin's scaffolding ability, with either of these resulting in LB formation. Once an LB begins to nucleate, it seems probable that the cell will sense a disturbance in its homeostasis and respond with an increased unfolded protein response, ubiquitination, and autophagy (Fig. 3). As the recurring theme in LD is glycogen, it is not surprising that a link between laforin, energy metabolism and cell cycle progression has been uncovered. How these pathways impact on LD remains to be determined. Despite a lack of consensus regarding many of the proposed pathways that laforin impacts on and the proposed laforin-interacting proteins, laforin and LD researchers have made significant advances. These results are allowing researchers to propose and test putative therapeutic paths.

Both the glucan phosphatase activity of laforin and the ability of laforin to act as a scaffold impinge on glycogen metabolism. In addition, if either of these functions falters, then one would predict that inhibiting glycogen synthesis might prevent LB formation, and if LBs are the causative agents of LD, then preventing glycogen synthesis should relieve neuronal cell death and epilepsy. An elegant collaborative study found that depletion of PTG in mice lacking laforin resulted in downregulation of glycogen synthesis, with near-complete disappearance of LBs as well as decreased neuronal cell death and myoclonic epilepsy [81], supporting a role for glycogen dysregulation in LD pathogenesis. Thus, removing PTG, an activator of glycogen synthase and inhibitor of glycogen phosphorylase, dramatically reduced the hallmark features of LD and caused no obvious harm to the mice. This study opens the possibility of utilizing chemical inhibitors to disrupt the PTG–glycogen synthase interaction and/or the PTG–glycogen phosphorylase interaction as a means to inhibit glycogen accumulation and disease progression.

A similar line of thinking could be utilized to explore therapeutic options focused on the unfolded protein response and autophagy. As autophagy is impaired in the absence of laforin, the use of different strategies aimed at enhancing autophagy could be an interesting therapeutic possibility. Similarly, one could attempt to enhance protein folding by upregulating chaperone proteins and/or increasing proteasomal activity as a means to turn over misfolded proteins.

Additional experimental therapeutics are still in their infancy. The use of gene therapy to express *EPM2A* or treatment with Trojan horse liposomes (also called PEGylated immunoliposomes) containing the gene of interest are both putative options [82]. In the cases where the disease is produced by nonsense mutations in *EPM2A* (e.g. R241X, the most frequent mutation in Mediterranean countries), treatment with gentamicin or other aminoglycoside antibiotics that produce readthrough of stop codons could be potentially relevant. The use of these antibiotics would be clinically justified for this fatal disorder, as in cystic fibrosis patients [1,83,84].

In summary, 100 years after the first clinical description of LD, the molecular bases of the disease are beginning to be understood. However, more work is still needed to fully decipher the functions of the two main players in the disease, laforin and malin. With this knowledge, rational therapeutic designs will be

proposed that could offer a window of hope to patients suffering from this devastating disease.

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