Review

Lafora disease: insights into neurodegeneration from plant metabolism

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Reversible phosphorylation modulates nearly every step of glycogenesis and glycogenolysis. Multiple metabolic disorders are the result of defective enzymes that control these phosphorylation events, enzymes that were identified biochemically before the advent of the molecular biology era. Lafora disease is a metabolic disorder resulting in accumulation of water-insoluble glucan in the cytoplasm, and manifests as a debilitating neurodegeneration that ends with the death of the patient. Unlike most metabolic disorders, the link between Lafora disease and metabolism has not been defined in almost 100 years. The results of recent studies with mammalian cells, mouse models, eukaryotic algae, and plants have begun to define the molecular mechanisms that cause Lafora disease. The emerging theme identifies a new phosphorylation substrate in glycogen metabolism, the glucan itself.

Nearly a century of Lafora disease history

In 1911, Dr. Gonzalo Lafora, a student of Dr. Alois Alzheimer, reported autopsy results from patients with 'teenage-onset myoclonus epilepsy with dementia' and described 'amyloid bodies in the protoplasm of the ganglion cells' [1,2]. Although amyloid was later shown to be proteinaceous, the term originally referred to any material that stained in a manner similar to that of starch, which is a mixture of amylose and amylopectin [3]. The deposit that Dr. Lafora described was later shown to be an accumulation of water-insoluble glucans, i.e. polymers of glucose linked by glycosidic bonds, and named a Lafora body (LB) [4–6]. Like the 'amyloid deposits', the disease that Dr. Lafora described now bears his name, and is called Lafora disease (LD) (OMIM 254780).

LD is an autosomal recessive neurodegenerative disorder resulting in severe epilepsy and death. It is one of five major progressive myoclonus epilepsies (PMEs) [7–10]. Unlike most other forms of epilepsy, LD is only moderately managed by medication for a brief period of time. LD commonly presents as a single seizure in the second decade of the patient's life; this single event is followed by progressive central nervous system degeneration and ends with the death of the patient within ten years of the first seizure [8,11–13].

LD is unique among the PMEs because of the patient's rapid neurological deterioration and the accumulation of cytoplasmic LBs, which contain 80–93% polyglucans [1,5]. LD is unique among neurodegenerative diseases in that it involves formation of an inclusion body that is largely non-proteinaceous. Whereas LBs are found in the cytoplasm of cells from most tissues, clinical features of LD are confined to the CNS and non-neurologic symptoms are rare [12]. LD patients exhibit increased neuronal cell death, numerous seizures, and LB accumulation as they age; thus, it is hypothesized that LBs trigger these symptoms and ultimately the death of the patient [5].

Two groups independently identified epilepsy, progressive myoclonus 2A (*EPM2A*) as a gene mutated in approximately 48% of LD cases [14,15]. *EPM2A* encodes the bimodular protein laforin that contains a canonical dual-specificity phosphatase (DSP) active site motif, HC*XX*G*XX*RS/T (Cx_5R), and a carbohydrate-binding

Glossary

CBM: a carbohydrate-binding module. CBMs are defined by their tertiary fold, which allows them to bind to one or many types of carbohydrates. They are classified into one of 53 families on the basis of amino acid similarity, substrate binding preferences, polypeptide folds, and evolutionary relationships.
CX_5R: the catalytic active site motif of the protein tyrosine phosphatase (PTP) superfamily.
DSP: the dual specificity phosphatases are a heterogeneous group of phosphatases that are more evolutionarily diverse than the classical PTPs. They dephosphorylate pTyr, pSer, pThr, and non-proteinaceous substrates (e.g. phosphoinositols, RNA, and glucans).
Glucan: a polymer of glucose monomers linked by glycosidic bonds, e.g. starch, glycogen, amylopectin, cellulose, Lafora body.
GWD: α-glucan, water dikinase. A plant kinase that transfers the β-phosphate of ATP to the C6 position of glucose in starch.
Lafora body: insoluble glucan that closely resembles plant amylopectin and accumulates in the cytoplasm of most cells in LD patients.
Protist: a diverse group of eukaryotic organisms with a unicellular level of organization.
PTP: the protein tyrosine phosphatase superfamily, which is encoded by the largest family of phosphatase genes. PTPs are defined by the active-site motif CX_5R, in which the cysteine functions as a nucleophile and is essential for activity. They are divided into the classical PTPs that dephosphorylate pTyr, and the DSPs that dephosphorylate pTyr, pSer, pThr, and non-proteinaceous substrates.
PWD: phosphoglucan, water dikinase. A plant kinase that transfers the β-phosphate of ATP to the C3 position of glucose in starch subsequent to phosphorylation of the C6 position.

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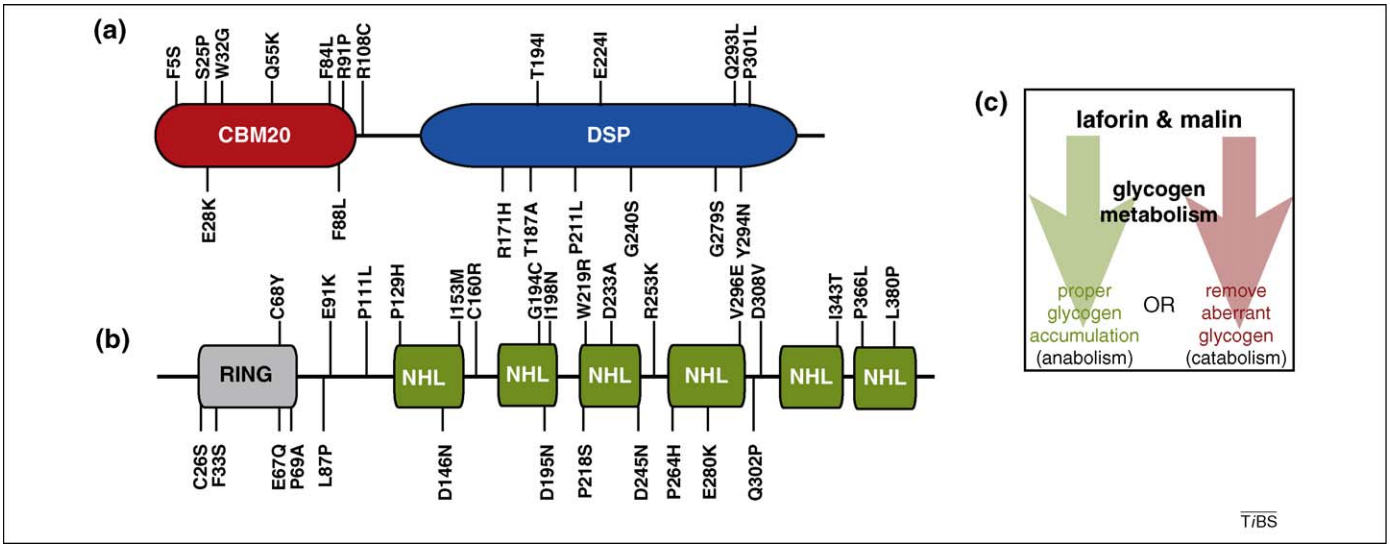

Figure 1. A schematic of laforin and malin. Amino acid substitutions stemming from Lafora disease missense mutations are shown for laforin and malin. (**a**) Laforin contains a carbohydrate-binding module (CBM) and a dual-specificity phosphatase domain (DSP). (**b**) Malin contains a RING domain followed by six NHL repeats. (**c**) Malin and laforin are involved in one of the two branches of glycogen metabolism.

module (CBM) (Figure 1a). Accordingly, recombinant laforin is able to hydrolyse phosphotyrosine and phosphoserine/threonine substrates *in vitro* [16,17]. The laforin amino-terminus contains a CBM belonging to family 20 (CBM20), which targets laforin to subcellular sites of glycogen synthesis and promotes the binding of laforin to glycogen both *in vitro* and *in vivo* [17]. Intriguingly, out of the 107 human protein tyrosine phosphatases (PTP superfamily), 15 human phosphoprotein phosphatases (PPP family), and 16 human phosphoprotein metallo-dependent phosphatases (PPM family) [18,19], only laforin possesses a CBM of any type. Greater than 70% of proteins that contain a CBM are amylases, glucohydrolases, or cellulases (i.e. enzymes that act on the carbohydrate itself) of plant, fungal, bacterial, or parasitic origins [20–22]. Of the LD cases without mutations in *EPM2A*, 40% are the result of mutations in epilepsy, progressive myoclonus 2B (*EPM2B*), and 12% might have mutations in non-coding regions of *EPM2A* or *EPM2B*, which could be the result of a copy number variant, or could be associated with mutations in an unidentified gene [23].

EPM2B encodes the 395 amino acid protein malin [24], which contains a consensus RING domain and six NHL domains (Figure 1b). RING domains are characteristic of one class of E3 ubiquitin ligases [25]. NHL domains form a six-bladed β-propeller and are involved in protein–protein interactions, similar to WD40 repeats [26,27]. We demonstrated that malin functions as an E3 ubiquitin ligase *in vitro* and *in vivo* [28]. We found that malin binds, ubiquitinates, and promotes laforin degradation [28]. This result was initially counter-intuitive because mutation of either malin or laforin results in LD. Why then would malin degrade laforin if they both inhibit Lafora disease? More recent data have allowed us to propose a model that better explains this perplexing result, and this work is discussed in detail below. In addition, malin regulates glycogen synthesis by ubiquitination and promoting the degradation of enzymes that orchestrate glycogen synthesis: glycogen debranching enzyme (AGL/GDE), protein targeting to glycogen (PTG), and the muscle isoform of glycogen synthase, which is also expressed in neurons [29–33].

Two transgenic mouse models have been developed for LD. One disrupted *EPM2A* to generate null mice [34], and the other generated transgenic mice by over-expressing inactivated laforin [35] in all tissues. Both mouse models mimicked the human disease, in that LBs are present and the mice develop epilepsy, but differ in the respect that the life span of the transgenic mice is not shortened. Neither study determined a molecular role for laforin in LD.

Although the mouse models did not determine the molecular etiology of LD, the data cumulatively placed laforin in the context of being involved in regulating glycogen metabolism. As laforin inhibits the formation of LBs, we and others have proposed that laforin functions to either actively promote proper glycogen accumulation or to actively remove aberrant glycogen (Figure 1c). Lafora initially proposed that the disease was a result of 'abnormal metabolism' [1]. However, many studies have reported that all known enzymes involved in glycogen metabolism from LD patients display normal activities [11,36,37]. Thus, it seems probable that LD is the result of a defect in a previously uncovered aspect of glycogen metabolism.

What is a Lafora body?

A glucan is one of a variety of complex carbohydrates composed of glucose moieties linked together by glycosidic bonds. One such glucan is glycogen. Glycogen is a branched polymer of glucose produced in the cytoplasm of the majority of archaebacterial, bacterial, fungal, and animal species and is an energy storage molecule. Most non-photosynthetic eukaryotes produce glycogen from UDPglucose, whereas most bacteria synthesize glycogen from ADPglucose. Glycogen is composed of glucose residues joined by α-1,4-glycosidic linkages, formed by glycogen synthase, with branches occurring in a continuous pattern every 12–14 residues via α-1,6-glycosidic linkages, formed by the branching enzyme [38]. The branches are referred to as

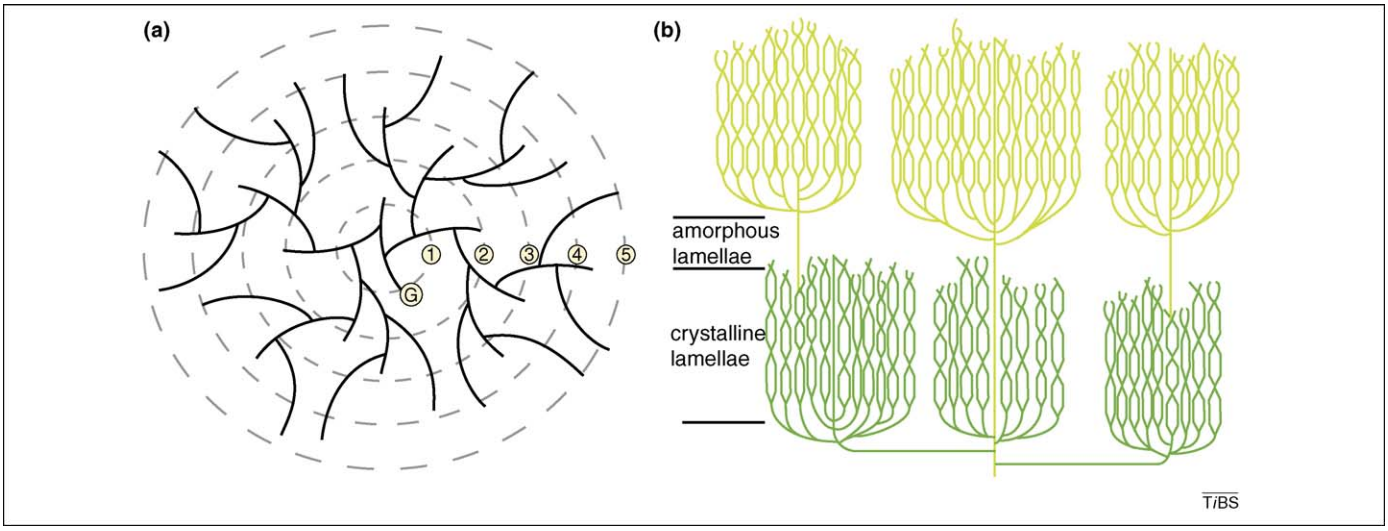

Figure 2. Models of glycogen and amylopectin. The three-dimensional structure of glycogen and starch cannot be determined experimentally due to their polydispersity, but these are the widely accepted models [98–100]. In each model, unbroken lines represent glucan chains. (**a**) Glycogen production is initiated when glycogenin (G) covalently attaches glucose to itself at Tyr194 and continues with the autocatalytic addition of about ten glucosyl residues. This protein–glucosyl complex serves as the starting point that glycogen synthase and branching enzyme utilize to link glucose by α-1,4-glycosidic linkages with branches linked by α-1,6-glycosidic linkages every 12–14 residues. Glycogen synthase and branching enzyme construct up to 12 tiers of branches, five of which are depicted here. These tiers are organized in a continuous manner, rendering glycogen water soluble. (**b**) Amylopectin is also composed of α-1,4-glycosidic linkages with α-1,6-glycosidic branches, but with branches arranged in clusters at regular intervals. The glucan chains within the clusters interact and this is represented by intersection of the adjacent chains, which form double helices and organize into crystalline lamellae. Between each cluster is a non-branched region that makes up the amorphous lamellae. The decreased branching and the crystalline lamellae render amylopectin and starch insoluble in water.

tiers, with a single glycogen molecule being composed of up to 12 tiers [39,40]. These characteristics make glycogen a water-soluble polymer (Figure 2a and Table 1). Interestingly, two groups reported that glycogen contains small amounts of phosphate, but the purpose of the phosphate or the enzymes responsible for the phosphate have not been determined [41–43].

Starch is the functional equivalent of glycogen for photosynthetic eukaryotes. In green algae and higher plants, starch is produced in a plastid, one type of which is a chloroplast [44]. In contrast to glycogen, starch is a water-insoluble, semi-crystalline mixture of <10% w/w amylose and >80% w/w amylopectin produced in diurnal cycles in *Arabidopsis thaliana* leaves [45]. Amylose is a linear molecule with very few α-1,6-glycosidic linkages. Amylopectin, like glycogen, is composed of α-1,4-glycosidic linkages with α-1,6-glycosidic branches, but with branches arranged in clusters at regular intervals (Figure 2b and Table 1). Within the clusters, adjacent chains form double helices and the clusters organize into crystalline lamellae. The decreased branching and the crystalline lamellae render amylopectin, and thus starch, insoluble in water.

Although glycogen is the normal glucan storage molecule for animals, LBs are accumulations of poorly branched, insoluble glucans and are not defined as glycogen. In fact, studies from the 1960s defined the biochemical composition of LBs and characterized them as more closely resembling plant amylopectin than glycogen [5,46,47]. Therefore, a LB is an aberrantly formed glucan. In this sense, LBs are similar to the misfolded proteinaceous accumulations seen in multiple neurodegenerative diseases. The biochemical characterization of LBs in the 1960s was largely overlooked, but this work demonstrated clearly and convincingly that LBs are more similar to plant amylopectin than animal glycogen [5,46,47]. This work spurred us on to examine the literature on the composition of another insoluble glucan called floridean starch.

Floridean starch is synthesized from UDPglucose in the cytoplasm of a group of eukaryotic, non-photosynthetic organisms and photosynthetic red algae, all of which are derivatives of kingdom Plantae/Archaeplastida [48–52]. Floridean starch was isolated originally from the multicellular red alga Florideophycidae and is composed of amylopectin and amylose [50,53]. The major differences between floridean starch and other plant starches are (1) that floridean starch is generated in the cytoplasm and starch is generated in plastids in other plants and (2) floridean starch is generated from UDP-glucose and starch from ADP-glucose [44,52]. Upon probing the genome of organisms that generate floridean starch, we found that laforin is conserved in a subset of protozoans, *Toxoplasma gondii, Eimeria tenella, Tetrahymena thermophila, Paramecium tetraurelia,* and *Cyanidioschyzon merolae* [54]. These organisms synthesize floridean starch and utilize

Table 1. Biochemical and physical properties of glucans

Glucan	Residues/branch	Branching pattern	Water soluble	Phosphate content
Eukaryotic glycogen	12–14[106,99]	Continuous[91,105,106]	Yes	0.064–0.25% w/w[41–43,66]
Bacterial glycogen	10–15[111,44]	Continuous[111,44]	Yes	N.D.
Amylopectin	12–25[98,109]	Discontinuous[98,109]	No	0.1–0.5% w/w[107,108,84]
Floridean starch	12–20[110,77]	Discontinuous[110,77]	No	N.D.
Lafora body	12–30+[47,66]	Discontinuous[65]	No	0.35–1.0% w/w[66,47]

it during the hibernation stage of their life-cycle. Thus, laforin is conserved in all vertebrates and in a small, defined group of protists. This finding prompted us to re-examine the molecular role of laforin in Lafora disease and to investigate unique possibilities.

The substrate is the key

Although mouse models existed that faithfully mimicked Lafora disease, the molecular etiology of LD remained a mystery largely because the function of laforin was unknown; i.e. the substrate was not identified. Two very plausible hypotheses dominated the LD field.

As glycogen metabolism is driven by the coordinated activity of glycogen synthase (GS) and branching enzyme (BE), one hypothesis suggested that LBs formed as a result of misregulation of one of these enzymes [12,34,55–57]. This hypothesis postulated that GS and BE were at opposite ends of a fulcrum and that misregulation of either one would lead to an accumulation of a glucan with decreased branching and decreased solubility, the biochemical hallmarks of LBs. The best evidence for this model came from the surprising finding that glycogen synthase over-expression in mouse muscle resulted in aberrant glycogen that resembled an LB [56,57]. However, Roach and colleagues later demonstrated definitively that both arms of this pathway (GS and BE) are normal in a mouse lacking laforin [58]. In addition, many earlier studies of patient tissue came to similar conclusions [11,36,37].

A second hypothesis was that laforin was involved in 'destroying' LBs, possibly by targeting them to lysosomes [12,34,35,58–60]. This model was proposed by Ganesh *et al.* and Minassian and co-workers and was based on the observation that laforin binds LBs in preference to glycogen. Later, this hypothesis was bolstered by the work described above, which demonstrated no change in glycogen metabolizing enzymes in the LD mouse [23,58,59]. These groups suggested that laforin's function begins after the appearance of 'nascent LBs' and that laforin is involved in monitoring and preventing the accumulation of LBs [23,59]. Additionally, they speculated that laforin might promote the transport or destruction of 'nascent LBs' before they become detrimental. This hypothesis is supported by recent work demonstrating that deletion or mutation of laforin exacerbates the unfolded protein response due to endoplasmic reticulum stress [61,62].

We proposed a third hypothesis when we discovered that laforin possesses the unique ability to dephosphorylate phospho-glucans [63], dephosphorylating glycogen molecules as they are synthesized. We postulated that in the absence of laforin glycogen becomes hyperphosphorylated, phosphate molecules disrupt and decrease normal branching, and a LB forms (Figure 3). Surprisingly, two groups reported almost 40 years ago that LBs from human patients contain more phosphate and less branching compared to glycogen [46,47]. Although LBs contain increased phosphate, no data exist that would place laforin on the anabolism or catabolism side of glycogen metabolism (Figure 1c). Thus, a theme similar to that described above, but placing laforin on the catabolism side of glycogen metabolism, is equally as likely. In this scenario, glycogen metabolism enzymes would release glucose from glycogen and laforin would remove phosphate from glucose as it was exposed. During glycogen metabolism, the outer glucose tiers (Figure 2a) are released and the inner tiers serve as the foundation for subsequent rounds of glycogen anabolism. One could envision that phosphate groups

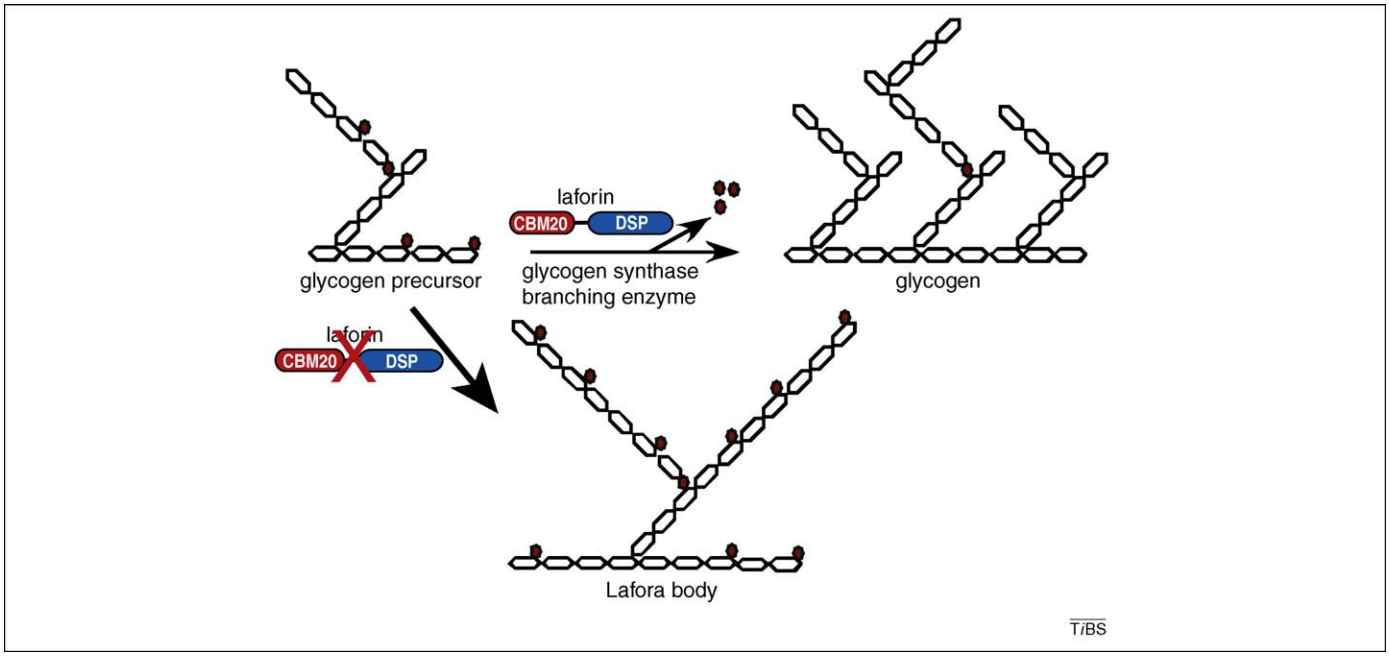

Figure 3. Model of Lafora body formation caused by loss of laforin. Glucose moieties are depicted as hexagons. Glucose is linked by α-1,4-glycosidic linkages with branches via α-1,6-glycosidic linkages. Glycogen contains small amounts of covalently linked phosphate (0.25% w/w), present as both phosphomonoesters and phosphodiesters [41–43,65]. Phosphate is represented by red filled circles, with phosphomonesters adjacent to glucose hexagons and phosphodiesters between two glucose hexagons. As nascent glycogen molecules are being synthesized by glycogen synthase and branching enzyme, phosphomonoesters and phosphodiesters accumulate by an unknown mechanism. Laforin removes phosphomonoesters so that glycogen production proceeds normally. In the absence of laforin, phosphomonoesters accumulate and negatively impact glycogen branching and lead to formation of the Lafora body (LB). LBs contain more phosphate and less branching compared to glycogen, and these two characteristics make LBs insoluble in water.

could block the action of glycogen catabolism enzymes, similar to that described recently in plants and discussed below (Figure 6a) [64]. In the absence of laforin, each round of glycogen metabolism would result in a slightly more phosphorylated glucan and would eventually result in a LD mouse model. Roach and colleagues have suggested a similar model in which laforin acts as part of a "repair or corrective mechanism" and in the absence of laforin, glycogen gradually accumulates 'structural defects' that eventually develop into LBs [65].

In support of the above glucan phosphatase models, Roach and colleagues confirmed the earlier reports of hyperphosphorylated LBs by demonstrating that mice lacking laforin have increased glucan phosphate and

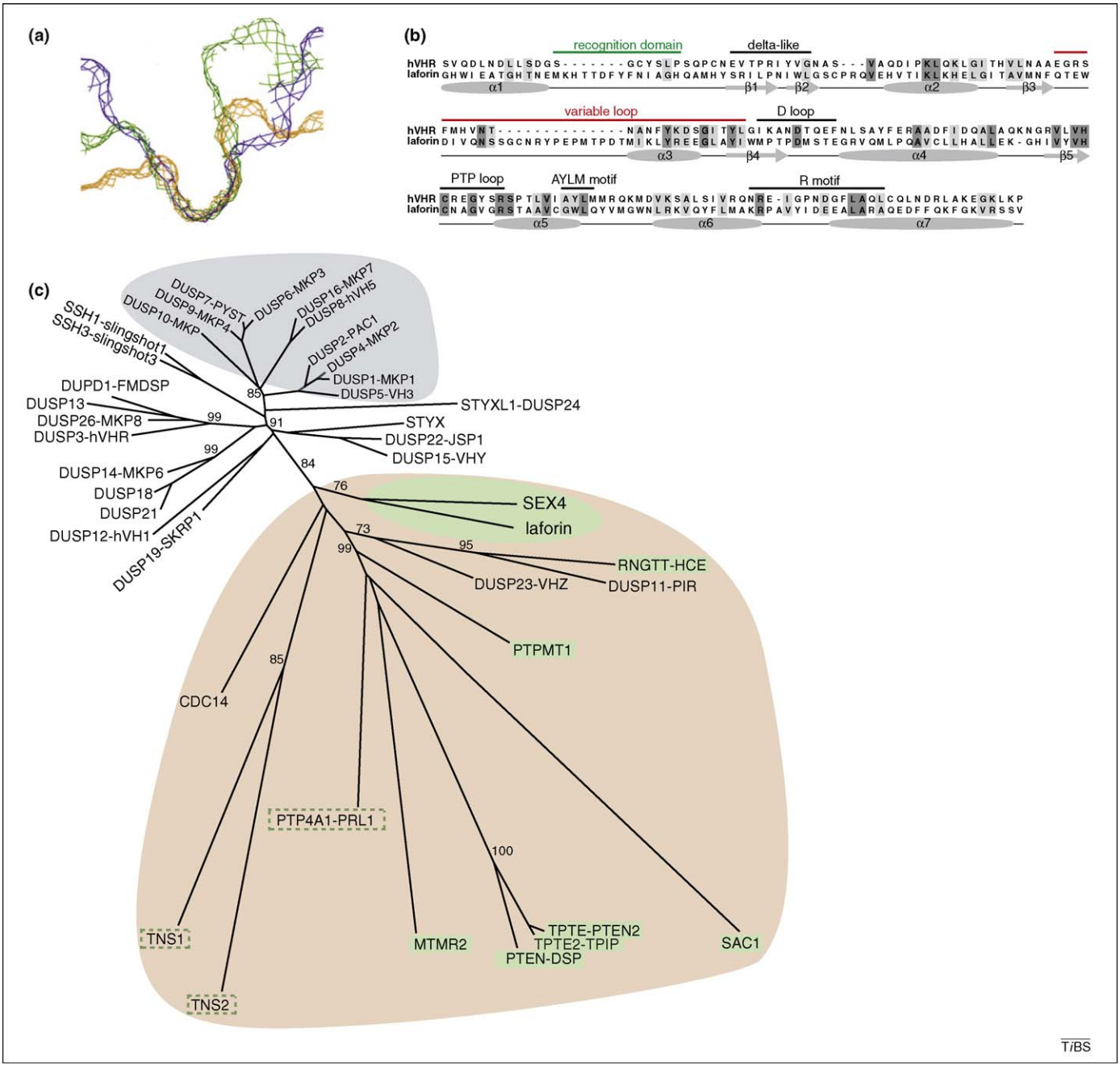

Figure 4. Structural and bioinformatic properties of laforin. (**a**) Slices of the active site surface of three classes of PTPs: (1) the deep and wide active site of the phosphoinositol phosphatase MTMR2 in blue; (2) the deep and narrow active site of the pTyr-specific phosphatase PTP1B in green; and (3) the shallow and narrow active site of the dual-specificity phosphatase VHR in orange. Reproduced with permission from [101]. (**b**) An alignment and secondary structure prediction of human laforin and VHR (hVHR) were generated using PROMALS [102]. The accepted phosphatase motifs are indicated above each segment and the secondary structure is indicated below. Similar amino acids are boxed in light grey and identical amino acids are boxed in dark grey. The recognition domain and variable loop are highlighted in green and red, respectively. (**c**) A phylogeny built using the catalytic domain of the dual-specificity phosphatases. The more recently evolved MAPK phosphatases or 'classical' DSPs are highlighted with a grey background. The more ancient and divergent 'atypical' DSPs fall into two groups. One group is relatively tightly clustered and utilizes proteinaceous substrates and this group has no highlighted background. The second group is more divergent and has a tan background, and includes laforin and SEX4. Most of the DSPs within this clade dephosphorylate non-proteinaceous substrates (e.g. phosphoinositols, RNA, and glucans), highlighted in green boxes. Some of the DSPs in this clade have undefined *in vivo* substrates, but they have activity against non-proteinaceous substrates *in vitro*, highlighted with a green broken line. The phylogenetic tree was generated from a PROMALS multiple sequence alignment using PROTDIST and FITCH from the PHYLIP 3.65 software package and displayed using HYPERTREE 1.0.0 [102,103].

decreased branching compared to wild-type controls [65,66]. In addition, they demonstrated that wild-type glycogen contains measurable amounts of phosphate and that laforin releases phosphate from glycogen. Therefore, the above two models are supported by both biochemical and patient data and have been recapitulated in an LD mouse model.

This hypothesis is bolstered by bioinformatic data and structural properties of laforin. The catalytic cleft of most dual-specificity phosphatases (DSPs) is shallow and narrow, to accommodate both pSer/pThr and pTyr (Figure 4a). This architecture is typical of the DSPs that dephosphorylate proteinaceous substrates. Alternatively, this region is deep and narrow in protein tyrosine phosphatases so that they only accommodate pTyr and are not capable of dephosphorylating pSer/pThr (Figure 4a). Phosphoinositol phosphatases exhibit a deep and wide catalytic cleft that can accommodate the large phosphoinositol head groups (Figure 4a) [71,67–69]. The architecture of this cleft is largely due to the length of the recognition region and variable loop of the phosphatase, with DSPs possessing fewer amino acids in these regions [71,70]. The recognition domain and variable loop of laforin are both twice as long as that of human VHR, a prototypical proteinaceous DSP (Figure 4b). Thus, laforin is predicted to have a deeper and wider cleft, more similar to that of the phosphoinositol phosphatases (e.g. PTEN and the myotubularins) than to proteinaceous DSPs. This deeper and wider cleft could accommodate phosphorylated glucans more easily. Furthermore, when one generates a phylogeny of all DSPs using only the phosphatase domain there are three distinct clusters (Figure 4c). The more evolutionarily recent 'classical' DSPs cluster together tightly, away from the more ancient and divergent 'atypical' DSPs [71]. Within the atypical DSPs is a very divergent group that includes laforin. Many of the DSPs within this clade dephosphorylate non-proteinaceous substrates (e.g. phosphoinositols, RNA, and glucans), whereas others have non-defined substrates and/or have activity against non-proteinaceous substrates *in vitro*. Collectively, these structural qualities suggest that laforin does not dephosphorylate a proteinaceous substrate and support our finding that laforin is indeed a glucan phosphatase. However, the definitive structural data will come from a crystal structure of laforin.

Although the above model resolves many questions about laforin and LD, a fourth hypothesis was proposed recently. A mouse expressing simian virus 40 large tumor antigen was engineered with a transgenic rearranged T-cell receptor (TCR) [72]. These mice are immunocompromised and develop a high rate of lymphoma [73]. Zhang and colleagues later showed that the *TCR* transgene is serendipitously inserted into the laforin gene locus [73]. They presented convincing data that laforin suppresses tumor growth in these immunocompromised mice. In addition, they presented data and stated that laforin dephosphorylates glycogen synthase kinase 3-beta (GSK3β), but did not recapitulate this finding *in vitro* using recombinant laforin. Instead, they over-expressed laforin in HEK293 cells, immunoprecipitated it, and showed that this mixture of proteins dephosphorylated a 20-mer peptide containing pSer9 of GSK3β. Subsequently, both we and Roach and colleagues demonstrated that laforin does not dephosphorylate GSK3β at Ser9 *in vitro*, nor is there an increase in phosphorylation of GSK3β at Ser9 in multiple tissues from laforin-deficient mice [63,66]. In contrast to these findings, using wild-type and laforin-deficient MEFs, Zheng and Minassian subsequently reported that laforin dephosphorylates Ser9 of GSK3β [74]. Surprisingly, they did not examine the state of GSK3β Ser9 in tissue from wild-type versus laforin-deficient mice.

Although there is no consensus concerning the endogenous substrate(s) of laforin, it is striking that no one has reported an increase of tumors in either LD patients or laforin-deficient mice. In addition, Roach and colleagues reported a convincing correlative study where they took multiple lines of transgenic mice that accumulated more glycogen than normal and found that laforin protein levels increase with glycogen levels, suggesting that more laforin is 'needed' as glycogen levels increase to presumably dephosphorylate the excess glycogen [75]. The exact molecular etiology of Lafora disease is still unclear and each of the above models could contribute to the pathology of the disease. However, we feel the data presented above strongly support laforin as a glucan phosphatase and that this role explains, at least partially, how laforin inhibits LD.

Lessons from plants and protists

It is rare and very informative when fields as diverse as neuroscience and plant starch metabolism intersect. Niittyla *et al.* discovered a gene in plants they called *starch excess 4* (*SEX4*), which contains a DSP followed by a CBM, the same domains as laforin but in the opposite orientation (Figure 5a) [76]. Strikingly, *SEX4* mutations result in a cellular phenotype similar to that seen in LD patients; namely, an increase in insoluble glucans. We went on to demonstrate that SEX4 has the same biochemical properties as laforin (i.e. it binds glucans, possesses phosphatase activity, and releases phosphate from glucans) and showed that laforin is a functional equivalent of SEX4 by rescuing the plant phenotype with human laforin [54].

The *Arabidopsis* experiments were initiated when we discovered that the gene encoding laforin is not confined to vertebrates, as previously thought, but that it is present also in a small set of protists (Figure 5b) [54]. These protists all produce floridean starch, which is very similar to LBs and plant amylopectin (Table 1) [48,77]. We found that each protist that contains laforin produces floridean starch. Conversely, protists that do not produce floridean starch or a similar glucan lack laforin. Thus, laforin is absent from the majority of protists, as well as yeast. Although laforin is conserved in vertebrates and a small subset of non-vertebrate organisms, SEX4 is found in all organisms of green algal descent (Figure 5c) [78]. The fact that SEX4 is conserved in all members of kingdom Archaeplastida/Plantae argues for a conserved necessary function from unicellular alga to multicellular plants.

Our understanding of the role of glycogen phosphorylation is still in its infancy, but plant researchers have elucidated a mechanistic cause and effect for starch phosphorylation. *Arabidopsis* has two kinases that phosphorylate starch directly. Glucan water dikinase (GWD)

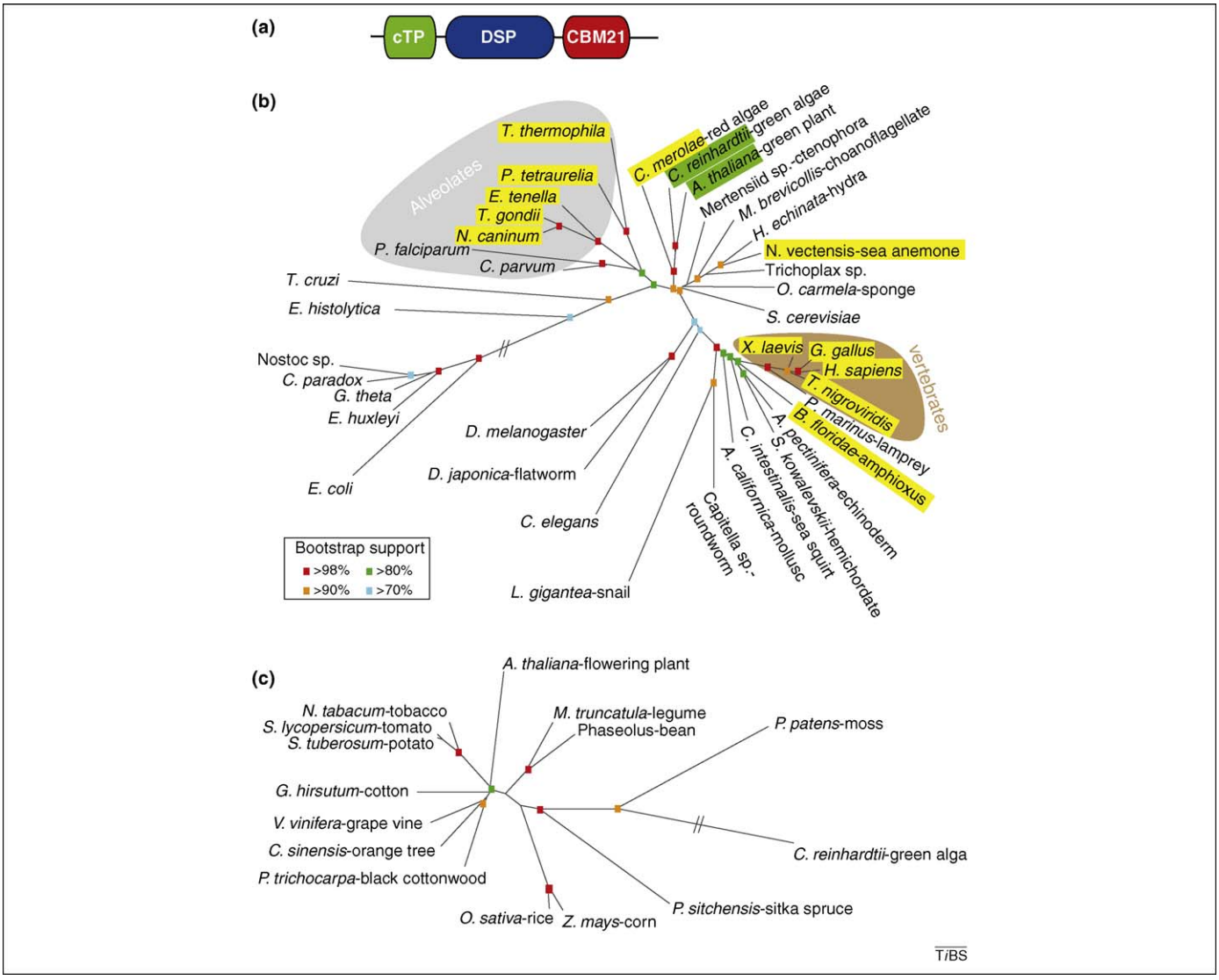

Figure 5. Evolutionary conservation of laforin and SEX4. (**a**) Schematic of SEX4, which is composed of a chloroplast-targeting peptide (cTP), dual-specificity phosphatase domain (DSP), and carbohydrate-binding module family 21 (CBM21). (**b**) Unrooted phylogeny of the small subunit ribosomal RNA (SSU rRNA) from organisms representing many evolutionary niches (modified [78]). Organisms containing laforin are boxed in yellow and those containing SEX4 are boxed in green. Alveolates are shaded with a grey background and vertebrates are shaded with a brown background. Bootstrap values are indicated by colour coding in the inset. (**c**) Unrooted phylogeny of all SEX4 orthologs. Bootstrap values are as in (**b**). The phylogenetic trees were generated as in Figure 4. Double hash marks indicate a place where the intervening segment was removed due to space limitations.

transfers the β-phosphate of ATP onto the C6 position of glucose in starch. Similarly, phosphoglucan water dikinase (PWD) phosphorylates the C3 position after GWD has phosphorylated the C6 position [79–84]. Mutations in either *GWD* or *PWD* result in a starch excess accumulation similar to that seen in plants with mutations in *SEX4*. The emerging theme of starch phosphorylation is described in detail in Figure 6a but, simply stated, it appears that glucan phosphorylation solubilizes the outer surface and allows access to the degradation machinery, then phosphate is removed at the C3 and/or C6 position by SEX4 so that another round of degradation can begin [64].

Although much progress has been made regarding glucan phosphorylation in plants, the very existence of phosphate in glycogen remained unproven until the 1980s when two groups showed definitively that phosphate is present in both a mono- and diester form [41,43,85]. As glycogen, like starch, contains both phosphate and a phosphatase to remove phosphate, one could envision a theme for glycogen similar to that described in Figure 6A for starch. Towards this end, we and others have performed bioinformatics searches to identify vertebrate homologues of GWD and/or PWD, but none have been identified to date. However, one group identified an activity in rabbit skeletal muscle that positions glucose 1-phosphate on the C6 position of glucose residues in glycogen and is likely to account for the phosphodiester in glycogen [43]. They named this enzme UDPglucose:glycogen glucose 1-phosphotransferase, but it has not been purified. They proposed that phosphomonoester groups in glycogen could arise by removal of glucose moieties originally transferred as glucose 1-phosphate. The phosphatase activity of laforin could be necessary to counter-balance these events.

Collectively, it seems that laforin and SEX4 are involved in degrading insoluble glucans. We propose that in protists and plants, laforin and SEX4 dephosphorylate glucans

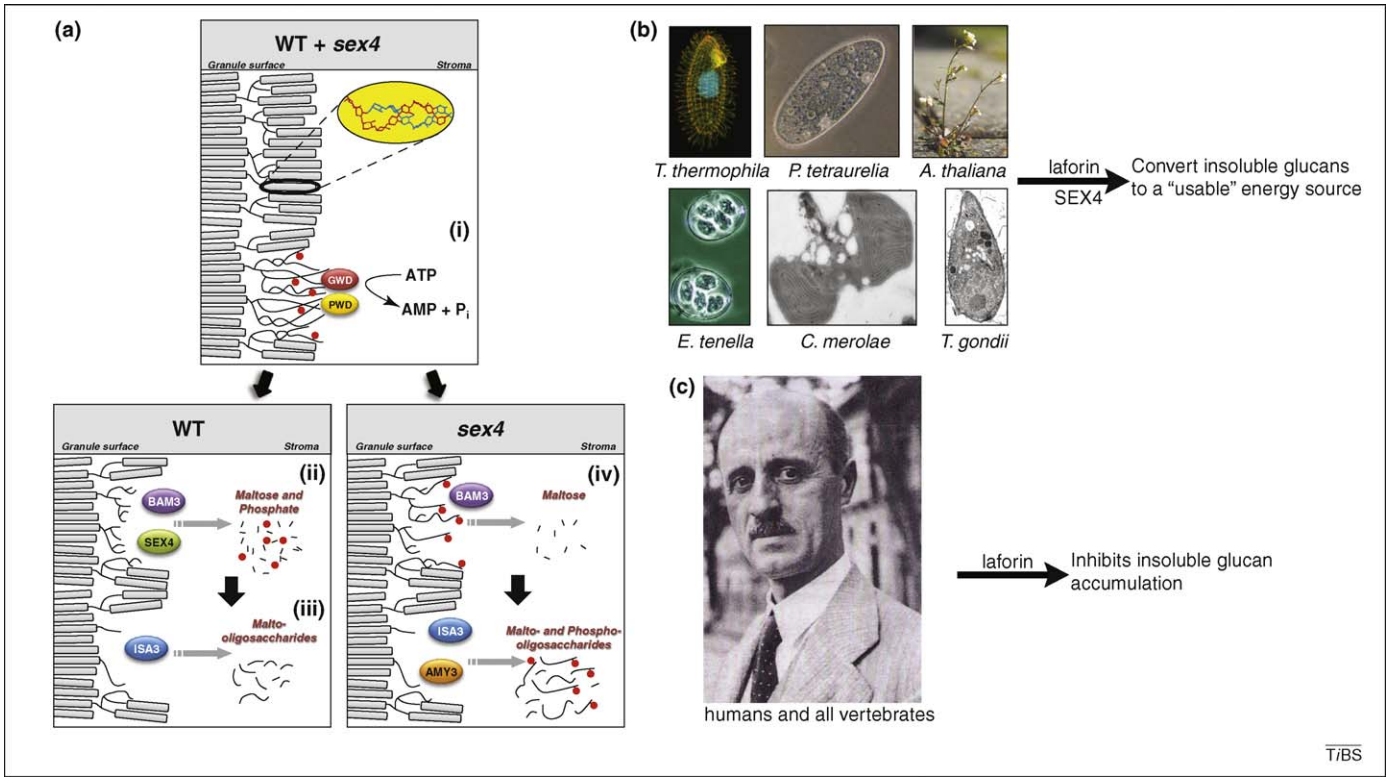

Figure 6. Models depicting the role of laforin and SEX4 in glucan metabolism. (**a**) Proposed model of starch breakdown (Copyright American Society of Plant Biologists; www.plantcell.org) [64]. Starch is phosphorylated (red circles) at night by GWD and PWD (i), leading to unwinding of amylopectin double helices. In WT plants, β-amylase isozyme 3 (BAM3) and SEX4 release maltose and phosphate, respectively (ii), and isoamylase 3 (ISA3) hydrolyses branch points and releases malto-oligosaccharides (iii). In *sex4* mutants, phosphate is not hydrolysed by SEX4, leading to reduced maltose release by BAM3 (iv). Subsequently, α-amylase (AMY3) and ISA3 release both malto- and phospho-oligosaccharides. Following degradation of the outer layer, a new round of degradationcan begins with the phosphorylation of the granule surface by GWD and PWD (i). (**b**) We propose that in plants and protists, laforin and SEX4 are involved in converting insoluble glucans into usable energy. (**c**) In humans, all other vertebrates, and at least two invertebrates (*Nematostella* and *Branchiostoma*), laforin inhibits insoluble glucan accumulation by dephosphorylating nascent glycogen molecules as proposed in Figure 3. The photograph of Dr Gonzalo Rodriguez Lafora and the image of a T. thermophila was reproduced with permission [104]. All other images were generated by the authors or obtained from non-restricted copyright sources.

during catabolism, that this event is downstream of the action of GWD and PWD, and that dephosphorylation is necessary for energy production through release of the stored glucans (Figure 6b). Similarly, in vertebrates laforin inhibits or degrades insoluble glucans before detrimental Lafora bodies form (Figure 6c). We feel it is probable that laforin dephosphorylates nascent glycogen molecules, as presented in Figure 3, but the details of this model have not been fully elucidated and the exact mechanism in plants and humans are likely to differ to some degree.

The destructive side of laforin

Although glucan dephosphorylation is likely to be part of the molecular mechanism driving LD, it is not the entire story. We found that malin binds laforin directly, ubiquitinates it, and triggers the laforin degradation [28]. Based purely on genetics, this finding is unexpected, as both malin and laforin inhibit LB formation. Why would malin trigger the destruction of laforin?

We and others have noted that laforin binds glucans very efficiently *in vitro* and once bound, it is not readily released [17,60]. If laforin functions to dephosphorylate glycogen as it forms, ubiquitination could be a method to release laforin from glycogen. Once released, laforin could be de-ubiquitinated and recycled, or it could be targeted for degradation.

In addition to ubiquitinating laforin, we and others found that malin has other targets, although this finding is disputed [65]. The targets of malin are all involved in glycogen metabolism; they include protein targeting to glycogen (PTG), glycogen synthase (GS), and glycogen-debranching enzyme (AGL/GDE) [29–32]. Surprisingly, laforin is needed for malin to ubiquitinate and trigger degradation of PTG and GS [29,30,32]. Therefore, laforin acts as a scaffold to bring malin to additional substrates and malin might ubiquitinate multiple proteins concentrated in this area. Thus, the laforin–malin complex might act as a controlled 'garbage disposal' to ubiquitinate and degrade many proteins involved in glycogen metabolism. This is a mechanism that might be shared with other E3 ubiquitin ligases, given their propensity to ubiquitinate multiple substrates.

One signal that regulates these events was described recently by Sanz and colleagues when they found that AMP-activated protein kinase (AMPK) phosphorylates PTG [33,86]. AMPK is a heterotrimeric protein that senses and responds to perturbations in both the cellular AMP:ATP ratio as well as glycogen stores, and thus is considered a key regulator of energy metabolism [87,88]. Sanz also demonstrated that phosphorylation of PTG by AMPK increases the malin–laforin-dependent degradation of PTG [33]. They provided biochemical data from tissue

culture models, and they confirmed these findings by using LD patient data, significantly strengthening this previously disputed result. The signals that regulate these degradation events and the timing of the events are currently being elucidated.

Why do only neurons die?

Whereas glycogen is generated in virtually all liver and skeletal muscle cells, glycogen is generated only in astrocytes and not in neurons in the mature brain [89]. Paradoxically, whereas neurons do not produce or store glycogen, neurons of LD patients accumulate LBs and are the only cells in LD patients reported to exhibit a cellular phenotype [34]. Recent work by Guinovart and coworkers solved the perplexing problem of how neurons were capable of generating LBs without generating glycogen. They demonstrated that neurons express low levels of muscle glycogen synthase (MGS) and that MGS in neurons is kept in an inactive, hyperphosphorylated state [30]. In addition, they demonstrated that a malin–laforin complex utilizes ubiquitination to ensure low levels of MGS in neurons and they elegantly showed that when MGS is dephosphorylated by protein phosphatase 1 (PP1) an aberrant poorly branched polyglucan forms, eventually leading to LBs in LD patients. This work has greatly expanded our knowledge of neuronal metabolism, but it does not define why only neurons undergo cell death in LD patients. The molecular mechanism that triggers neuronal apoptosis in LD patients is unknown, but we present four hypotheses that are not mutually exclusive. Given that LD symptoms take 15 years to manifest, it may be that multiple mechanisms contribute to the pathogenesis.

Glial cells outnumber neurons approximately 10:1 and provide them with most energy needs. Neurons store minimal to no glycogen and have increased energy needs due to numerous ion channels, and inherently live in an "energy crisis." Thus, glial glycogen is thought of as a safety net that ensures neurons maintain an energy source during periods of intense activation (reviewed in Refs [89,90]). The degree of branching and the length of glucose chains in glycogen are optimized for maximal glucose storage in the smallest volume and maximal energy release [39,91]. As neurons have very limited energy stores and are both dependent on glial glycogen and utilize large amounts of energy, they are hypersensitive to energy perturbations. LBs are likely to accumulate a significant amount of 'trapped' and 'unusable' energy. If this trapped energy causes a temporary disruption in energy release, then cells that lack their own energy stores, e.g. neurons, would be the first to undergo apoptosis. In this scenario, neurons are the 'canary in the coal mine' and are responding to decreased energy availability. Neuronal apoptosis leads to an early death of the patient, so no other cells have a chance to undergo apoptosis or cell death.

A second possibility is that LBs present a major trafficking problem. Whereas a neuron is 4–100 μm in diameter, neuronal LBs range from 3 to 40 μm [13]. Therefore, LBs might form a blockade in the cytoplasm. In addition, the neurons transport intracellular cargos over considerably further distances than other cells. Thus, a trafficking defect could first present in them and later result in neuronal apoptosis.

Third, neuronal death in LD patients could be the only cellular phenotype because of the age of neurons. Neurons abolish mitotic division and have a markedly increased lifespan compared to most other cell types. Neurons in LD patients might be the only cells undergoing apoptosis because of their advanced 'age', and other cell types might not live long enough to experience the detrimental affects. This hypothesis would explain why neurons in murine models that exhibit LBs do not undergo widespread apoptosis and why LD mouse and dog models do not die at a young age [92–94]. The lifespan of murine and canine neurons is not as long as that of human neurons and would not be long enough for LBs to cause massive apoptosis, which takes 15+ years in humans.

Lastly, it is possible that LBs are not the cause of LD, but rather a cellular defense mechanism to sequester and dispose of aberrantly folded glucans. This hypothesis has gained support among researchers studying the multiple neurodegenerative diseases involving proteinaceous accumulations. Corroborating this hypothesis in LD is the fact that not all neurons that undergo cell death in the laforin-deficient mouse model have visible LBs. This result could mean that LBs are the end result of multiple aberrant steps of glycogen synthesis, and that the earlier, non-visible products are the pathogenic cause of neuronal apoptosis. Alternatively, LBs might not be the pathogenic cause of LD. Ganesh and colleagues have suggested that a defect in autophagy or the ubiquitin proteasome system is a driving force in LD and LD pathology [95,96]. However, their studies examining autophagy in cell models utilize over-expressed proteins and treatment with proteasomal inhibitors; thus, the data needed to support this hypothesis are not yet entirely convincing. Nonetheless, it is striking that, like the proteinopathies, mutations in an E3 ubiquitin ligase, malin, result in LD.

Concluding remarks and future perspectives

Collectively, biochemistry, mouse models, cell biology, and LD patient data suggest two essential roles for laforin: (1) dephosphorylation of glycogen, or nascent glucans, to inhibit excess glycogen phosphorylation and LB formation; and (2) recruitment of malin to the site of glycogen synthesis so that malin can ubiquitinate PTG, GS, AGL, laforin, and possibly other proteins to inhibit LB formation. Thus, laforin performs two essential functions, and malin one, in maintaining proper glycogen metabolism. This idea of laforin having two roles also is supported by LD patient data. LD patients with malin mutations live 25% longer than patients with laforin mutations [97]. Thus, these data suggest that the function of laforin could be downstream of malin, or that laforin has a disproportionate role in glycogen metabolism.

As many pathways are regulated by ubiquitination, it is not overly surprising that ubiquitylation also regulates glycogen metabolism. However, we now must identify and define both the auxiliary proteins mediating these events and the signals that regulate these proteins at the cellular, tissue and organismal levels. As discussed above, an emerging regulator of these events is AMPK;

however, the extent to which AMPK orchestrates this regulation is still being determined.

A surprising discovery from the Lafora disease field is the identification of a glucan phosphatase activity that is conserved from plants to humans. Although the picture is becoming increasingly clear as to how plants utilize glucan phosphorylation and dephosphorylation to store and release energy, respectively, it is not clear how or why vertebrate glucans become phosphorylated. Is there a glucan kinase in vertebrates, similar to GWD and PWD in plants? Many groups have performed bioinformatic searches but have yet to identify a similar kinase in vertebrates. Alternatively, the actions of a phospho-glucotransferase could result in glycogen phosphorylation, but this enzyme has not been identified. Is glycogen phosphorylation the result of an evolutionary remnant, i.e. a mistake, or does it have an undefined purpose? Lastly, as yeast, flies, and worms all lack laforin and malin, how do they deal with insoluble glucan accumulations and can we identify an alternative pathway in these model organisms? The answers to these questions will further define Lafora disease at a molecular level, are likely to uncover potential therapies, will further identify similarities and differences between glycogen and starch metabolism, and may provide mechanisms to modulate glucan (i.e. energy) production in a variety of organisms.

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