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REVIEW ARTICLE

Lafora disease: from genotype to phenotype

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Abstract. The progressive myoclonic epilepsy of Lafora or Lafora disease (LD) is a neurodegenerative disorder characterized by recurrent seizures and cognitive deficits. With typical onset in the late childhood or early adolescence, the patients show progressive worsening of the disease symptoms, leading to death in about 10 years. It is an autosomal recessive disorder caused by the loss-of-function mutations in the *EPM2A* gene, coding for a protein phosphatase (laforin) or the *NHLRC1* gene coding for an E3 ubiquitin ligase (malin). LD is characterized by the presence of abnormally branched water insoluble glycogen inclusions known as Lafora bodies in the neurons and other tissues, suggesting a role for laforin and malin in glycogen metabolic pathways. Mouse models of LD, developed by targeted disruption of the *Epm2a* or *Nhlrc1* gene, recapitulated most of the symptoms and pathological features as seen in humans, and have offered insight into the pathomechanisms. Besides the formation of Lafora bodies in the neurons in the presymptomatic stage, the animal models have also demonstrated perturbations in the proteolytic pathways, such as ubiquitin-proteasome system and autophagy, and inflammatory response. This review attempts to provide a comprehensive coverage on the genetic defects leading to the LD in humans, on the functional properties of the laforin and malin proteins, and on how defects in any one of these two proteins result in a clinically similar phenotype. We also discuss the disease pathologies as revealed by the studies on the animal models and, finally, on the progress with therapeutic attempts albeit in the animal models.

Keywords. epilepsy; neurodegenerative disorder; locus heterogeneity; glycogen metabolism; stress response pathways.

Introduction

Much of our understanding on the neurodegenerative processes and the players involved in the neuronal survival pathways have come from the discoveries made in the pathobiology of rare forms of neurodegenerative disorders. For example, the very concept of mitophagy—a quality control mechanism that regulates the mitochondrial homeostasis (Corti and Brice 2013)—and its causal role in neurodegeneration have come from the study on parkin, a protein found to be defective in a small subset of Parkinson's disease patients (Kitada *et al.* 1998). Similarly, the discovery of the infectious agent of the rare disorder known as Creutzfeldt–Jakob disease (Field *et al.* 1969)—the agent later identified to be protein and hence named as prion (Collinge *et al.* 1996)—led to a revolutionary change in our understanding on a nonRNA/DNA mode transmission of disease which we believe could underlie the pathogenesis of several neurodegenerative disorders (Goedert 2015). Yet another example of a rare form of a disorder offering novel insights into the neuropathophysiology is the subset of amyotrophic lateral sclerosis (ALS), patients with mutations in the *FUS* or *TDP-43* gene—accounting to less than 3% of the ALS cases (Lattante *et al.* 2013). Functional studies on the mutant forms of *FUS* and *TDP*-43 lead to our current understanding on their regulatory role post-transcriptional regulation, and as to how defects in this process could underlie neurodegeneration (Lagier-Tourenne *et al.* 2010). These discoveries have led to our current understanding on the role of noncoding RNA in neurological disorders (Lourenco *et al.* 2015). Studies on yet another rare disorder known as Lafora disease (LD) and the topic of the current review, likewise offered novel insights into the role of glycogen metabolism in the neuronal survival. Dissecting the function of laforin and malin proteins (the two proteins defective in LD) in diverse cellular pathways, especially on the glycogen metabolism and autophagy, extended our understanding on the common pathways connecting diverse set of neurodegenerative disorders. This review aims to summarize the findings on

We dedicate this review article to our mentor and teacher Professor Rajiva Raman as a tribute to his constant encouragements and immense contributions to the field of genetics in the country.

LD and attempts to functionally link the disease genotype with the disease phenotype by providing a comprehensive overview on the LD biology.

Introduction to LD

LD is an adolescent onset neurodegenerative disorders, with the disease-defining symptoms of epileptic attacks, including myoclonus, tonic–clonic and absence seizures (Minassian 2001; Ganesh *et al.* 2006). The other symptoms include ataxia, dementia, hallucination, and dysarthria, and all those, including the seizures, show progressive worsening. Hence, LD is classified as one of the five forms of progressive myoclonus epilepsies (Delgado-Escueta *et al.* 2001). With typical age at onset around 12–15 years, the affected patients die around 25 years, often due to respiratory failure (Ganesh *et al.* 2006; Striano *et al.* 2008). Thus, LD is considered to be one of the most severe forms of epilepsies (Minassian 2001). LD shows an autosomal recessive inheritance with 100% penetrance and fatality. LD is named after Gonzalo Rodríguez Lafora (1886–1971), a Spanish neurologist who first described the presence of intracellular inclusions, which he referred to as 'amyloid bodies,' in the condition progressive myoclonic epilepsy (Nanduri *et al.* 2008). These inclusions were later referred to as 'Lafora bodies' in honor of the contributions of Gonzalo Lafora. LD, though a rare disorder, is relatively frequent in the Mediterranean region, the Middle East, eastern Europe and the South Asian populations (Singh and Ganesh 2009). There are a few reports of LD in the Japanese and Chinese population as well (Ganesh *et al.* 2001; Singh *et al.* 2005; Yildiz *et al.* 2017).

Genetic heterogeneity in LD

A genetic locus for LD was mapped on chromosome 6q24 (Serratosa *et al.* 1995; Sainz *et al.* 1997), and a causative gene, named *EPM2A*, was identified in the year 1998 (Minassian *et al.* 1998; Serratosa *et al.* 1999) (see figure 1). Subsequently, the full gene sequence was discovered and characterized (Ganesh *et al.* 2000). The coding sequence of the *EPM2A* gene spans 4 exons, and codes for a dual-specificity protein phosphatase named laforin (Ganesh *et al.* 2000). Besides the phosphatase domain at the carboxyl terminal, the laforin protein also harbors a carbohydrate-binding domain at the amino terminal (Wang *et al.* 2002; Ganesh *et al.* 2004) (see figure 1). Several loss-of-function mutations were identified in the gene, confirming that the *EPM2A* gene is indeed the causative gene for LD (see below). Some of the LD families did not show mutations in the *EPM2A* gene or mapped to the 6q24 locus, suggesting the presence of at least one more locus for LD (Minassian *et al.* 1999). Subsequent studies, specifically by using the non*EPM2A* LD families, identified a second locus for LD named as *EPM2B* and mapping at 6p23

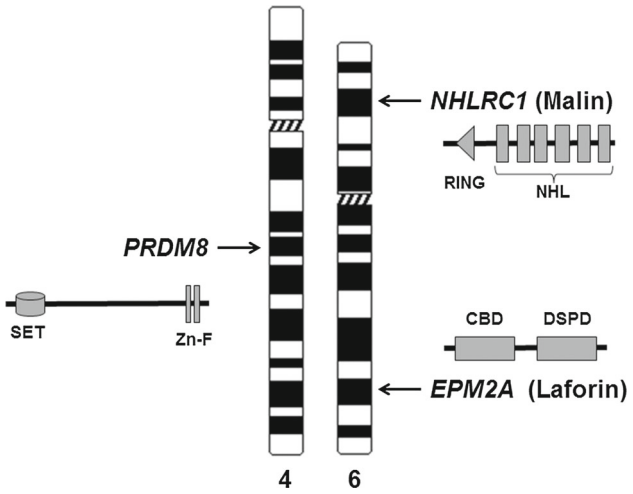

Figure 1. Schematic diagram showing the position of LD genes *EPM2A* and *NHLRC1* on chromosome 6 and *PRDM8* gene on the chromosome 4. The domain organization of the encoded proteins are also shown (drawn not to scale). SET, SET domain; Zn-F, Zinc-finger domain; RING, RING domain; NHL, NHL repeat domain; CBD, carbohydrate-binding domain; DSPD, dual-specificity phosphatase domain.

(Chan *et al.* 2003a,b). Sequence analyses of genes mapped in this region lead to the identification of *NHLRC1* gene as the second causative gene for LD (Chan *et al.* 2003a,b). The *NHLRC1* is a single exon gene (Chan *et al.* 2003a,b), coding for an E3 ubiquitin ligase protein named malin (Gentry *et al.* 2005) (see figure 1). A number of mutations have been identified in the *NHLRC1* gene and all of them appear to be loss-of-function mutations (see below), as expected for an autosomal recessive mode of inheritance of LD. It is of interest to note that the two genes showed population specific distributions of the mutations; e.g., mutations in the *EPM2A* gene is far more frequent in Spanish, French and the US population whereas the *NHLRC1* gene appears to be the major causative gene for LD in the Italian, Canadian, Arab, Indian and Brazilian populations (Singh and Ganesh 2009; Turnbull *et al.* 2016). Among the reported families, defects in the *EPM2A* accounts for nearly 50% of them, and the rest for the *NHLRC1* gene. There appears to be a third locus for LD since not all LD families show mutations in the *EPM2A* or the *NHLRC1* gene (Chan *et al.* 2004a,b). Indeed, a novel locus for LD was mapped on chromosome 4q21 in a family that showed early onset LD phenotype (Turnbull *et al.* 2012). Homozygosity mapping leads to the identification of a novel variant of the *PRDM8* gene in a family of Pakistani origin (Turnbull *et al.* 2012) (see figure 1). Although recent studies indicate a role for this gene in neuronal differentiation during development (Inoue *et al.* 2015; Jung *et al.* 2015; Iwai *et al.* 2018), the possible role for this gene in LD is yet to be established (see below). Nevertheless, the LD families that do not map to *EPM2A* and *NHLRC1* loci strongly indicate the presence of multiple genetic loci for LD.

Allelic heterogeneity in LD

Genetic screen for more than 250 LD families are available in the literature and more than 150 distinct mutations have been reported in the two genes (Singh and Ganesh 2009; Turnbull *et al.* 2016). These include large and small deletions, insertions, point mutations and mutations in the splice junctions (Singh and Ganesh 2009; Turnbull *et al.* 2016). This extreme allelic heterogeneity, with orphan mutations spreading across the gene region poses an obvious challenge for the genetic diagnostics. Haplotype analyses indicate that only a minor fraction of these mutations are due to the founder effect and a large number represents mutational hot spots (Singh and Ganesh 2009; Turnbull *et al.* 2016). For example, the p.R241X mutation in the *EPM2A* gene is often seen in the Spanish population and their decent seem to be due to the founder effect (Ganesh *et al.* 2002a). Similarly, the p.C26S mutation in the *NHLRC1* gene in the French Canadian appears to be due to a founder effect (Chan *et al.* 2003a,b; Singh *et al.* 2006). Similarly, quite a few discrete spots of high recurrent mutations have also been reported in both genes (Singh and Ganesh 2009; Turnbull *et al.* 2016). These include, but not limited to, the large deletions and p.G279S mutation in the *EPM2A* gene, and the p.P69A and p.G158fs16 mutations in the *NHLRC1* gene (Ganesh *et al.* 2002a). Heterozygous mutations were reported in a few families, suggesting the possible presence of undetected mutations in the other allele (Gómez-Garre *et al.* 2000; Ganesh *et al.* 2002a; Chan *et al.* 2003a,b; Singh *et al.* 2005). Some of these might carry deletions, since hemizygous deletions may go undetected in a conventional PCR. Indeed such instances of compound heterozygous mutations complicating the genetic diagnosis have been recorded and reported (Minassian *et al.* 2000).

Genotype–phenotype correlations in LD

LD is generally considered to be a clinically homogenous disorder. It is clinically difficult to differentiate LD patients who have mutations in *EPM2A* gene from those who have in *NHLRC1*. Few reports did suggest that the *NHLRC1* defective patients may show a slower disease progression (Singh *et al.* 2006). However, such slow progressive forms have also been identified for a few patients with *EPM2A* defects (Jara-Prado *et al.* 2014), suggesting that the observed difference could be mutation specific (Ferlazzo *et al.* 2014). Similarly, subsyndromes of LD have also been reported; an atypical form of LD, with childhood onset learning disabilities was shown to be associated with the mutations in the first exon of the *EPM2A* gene (Ganesh *et al.* 2002a; Annesi *et al.* 2004). Since the first exon of the *EPM2A* gene codes for the carbohydrate binding domain (CBD), a positive correlation for the CBD and atypical form was also proposed (Ganesh *et al.* 2002a). Later, the possible differential effect of mutations on the alternatively spliced transcript of the *EPM2A* gene was suggested (Dubey *et al.* 2012). However, such correlations could not be extended to other patients with exon 1 mutations (Ganesh *et al.* 2002a), and more importantly, a founder effect mutation in an Arab family showed variable expression, suggesting the possible presence of 'modifier' genes for LD (Lesca *et al.* 2010; Singh and Ganesh 2012b). A strong support to this notion comes from a study where the same mutation on the *EPM2A* gene showed variable pathologies, and a rare sequence variant in the *PPP1R3C* gene coding for the protein targeting to glycogen (PTG)—a protein that interacts with laforin—caused slower progression of the disease (Guerrero *et al.* 2011). Given that laforin and malin are shown to interact with a number of proteins, a possible disease modifying role for these interacting proteins are suspected (Singh and Ganesh 2012b). Recently, several variants of LD clinical course are reported. For example, LD with obsessive compulsive symptoms (Nasri *et al.* 2017) and a late onset LD with Parkinsonism (Lynch *et al.* 2016) are observed. Another example is the early onset of LD associating with the *PRDM8* gene mutation, thus highlighting the primary defect—in one or more genes—associating with subsyndromes of LD. Clearly further work is needed to correlate genetic defects in other genes with the variation in clinical symptoms.

Functional properties of laforin phosphatase and malin ubiquitin ligase

The cellular functions of the two LD associated gene products—laforin and malin—are reasonably well characterized (table 1). The suggested functions for the LD proteins include a critical role in the glycogen metabolism (Roach 2015), ubiquitin-proteasome pathway (Mittal *et al.* 2007; Garyali *et al.* 2009; Vernia *et al.* 2009; Rao *et al.* 2010a), autophagy (Knecht *et al.* 2010; Puri and Ganesh 2010, 2012), heat shock response (Sengupta *et al.* 2011; Jain *et al.* 2017), ER stress response (Vernia *et al.* 2009; Sharma *et al.* 2013), oxidative stress response (Romá-Mateo *et al.* 2014, 2015; Sánchez-Elexpuru *et al.* 2017a,b), translational regulation (Ganesh *et al.* 2000), RNA metabolism (Singh *et al.* 2012a), cell death pathway (Upadhyay *et al.* 2015), and mitochondrial homeostasis (Upadhyay *et al.* 2017) (see table 1) (also discussed below). The *EPM2A* gene product laforin is a dual-specificity protein phosphatase (Ganesh *et al.* 2000; Minassian *et al.* 2000; Girard *et al.* 2006). Although the phosphatase activity of this protein is well established, and a number of interacting proteins are identified for laforin, it is yet not clear as how many of them are substrates of laforin (table 2). *In vitro* studies have identified Tau (Puri *et al.* 2009), HIRIP5 (Ganesh *et al.* 2003), malin (Gentry *et al.* 2005; Lohi *et al.* 2005a), AMPK (Moreno *et al.* 2010) to be a few of the

Table 1. Table listing the known cellular pathways in which laforin and/or malin were shown to play a regulatory role.

Cellular pathways	Specific functions	References
Glycogen metabolic pathways	Laforin is a glycan phosphatase — binds to and removes phosphate group from glycogen	Roach (2015)
	Laforin regulates glycogen chain length	Nitschke *et al.* (2017)
	Malin ubiquitinate and degrade PTG/R5 in laforin-dependent manner	Fernández-Sánchez *et al.* (2003); Worby *et al.* (2008)
	Laforin-malin complex degrades muscle isoform of glycogen synthase	Vilchez *et al.* (2007)
	Laforin-malin complex ubiquitinate and regulate cellular levels of AMPK, an energy sensor of the cell	Moreno *et al.* (2010)
	Laforin-malin complex regulate levels of glycogen debranching enzyme and brain isoform of glycolgen phospholyrase	Liu *et al.* (2013)
	Laforin-malin negatively regulate glucose uptake in the cell, by modulating the surface expression of the glucose transporters	Singh *et al.* (2012a)
	Malin regulates the levels of neuronatin - a known regulator of glycogen synthesis	Sharma *et al.* (2011)
Proteolytic pathways	Laforin-malin complex promotes the clearance of misfolded proteins via ubiquitin-proteasome pathway	Garyali *et al.* (2009)
	Loss of laforin or malin results in compromised proteasome function	Garyali *et al.* (2014)
	Laforin and malin regulate autophagy flux	Aguado *et al.* (2010); Knecht *et al.* (2010); Puri *et al.* (2012); Criado *et al.* (2012); Knecht *et al.* (2012); Jain *et al.* (2017)
	Laforin negatively regulates mTOR activity via SGK1	Singh *et al.* (2013)
	Laforin-malin regulate the cellular functions of p62 and ubiquitin conjugating enzyme E2-N – two key players in autophagy	Sánchez-Martín *et al.* (2015)
	Laforin regulate the expression levels of Fox3a – a key transcription factor in autophagy	Jain *et al.* (2016)
Cellular stress response	Laforin and malin are recruited to the aggresome in response to proteotoxic stress	Mittal *et al.* (2007)
	Laforin and malin are involved in endoplasmic reticulum stress response	Vernia *et al.* (2009); Zeng *et al.* (2012)
	Laforin and malin are required for the activation of heat shock transcription factor HSF1 and to protect cells under heat shock	Sengupta *et al.* (2011)
	Loss of laforin or malin results in increased oxidative stress in neurons; laforin-malin involved in anti-oxidant pathway	Romá-Mateo *et al.* (2014); Romá-Mateo *et al.* (2015)
	Laforin and malin negatively regulate Hipk2, a pro-apoptotic factor activated during cellular stress	Upadhyay *et al.* (2015)
	Malin is required for activating the autophagy and proteasomal function during a recovery from heat shock	Jain *et al.* (2017)
Mitochondrial homeostasis	Laforin and malin regulate the cellular levels of the mitochondrial fission GTPase Drp1	Upadhyay *et al.* (2017)
	Cells lacking functional laforin or malin show compromised mitochondrial function	Romá-Mateo *et al.* (2015)

Table 1. (*contd*)

Cellular pathways	Specific functions	References
Post-transcriptional gene regulation	Laforin associates with ribosomes and polysomes	Ganesh *et al.* (2000), Minassian *et al.* (2001)
	Malin is recruited to processing bodies and modulate the stability of mRNAs via the decapping enzyme Dcp1a	Singh *et al.* (2012a)

potential substrates of laforin. Similarly, for malin, the potential substrates are laforin (Gentry *et al.* 2005; Lohi *et al.* 2005a; Solaz-Fuster et al. 2008), PTG (Worby *et al.* 2008), MGS (Valles-Ortega *et al.* 2011), Dishevelled (Sharma *et al.* 2012), Neuronatin (Sharma *et al.* 2011), and abnormally misfolded proteins (Garyali *et al.* 2009; Rao *et al.* 2010a; Jain *et al.* 2017). It may be noted here that a majority of these studies relied upon cell models, and overexpressed laforin or malin since no reliable antibodies were available for both the proteins for further characterization of the interactions.

Studies using overexpression constructs have shown exclusively cytoplasmic localization for laforin (Ganesh *et al.* 2000; Ganesh *et al.* 2002b; Wang *et al.* 2002), while malin appears to localize both in cytoplasm and nucleus (Chan *et al.* 2003b; Mittal *et al.* 2007). Moreover, their localization pattern appears to be dynamic and aligned to the physiological state of the cell. For example, a heat shock or glucose starvation induces the nuclear localization of laforin (Sengupta *et al.* 2011; Singh *et al.* 2012a) (see figure 2) (also see below). Studies have also documented the subcellular compartment in which laforin and malin might localize (see figure 2). Both laforin and malin appear to localize in the endoplasmic reticulum and in the polysomal fraction (Ganesh *et al.* 2000; Minassian *et al.* 2001; Chan *et al.* 2003b). *In situ* localization revealed that laforin colocalizing with glycogen particles in the cell (Wang *et al.* 2002; Tiberia *et al.* 2012) and has been shown to dephosphorylate glycogen (Ganesh *et al.* 2004; Worby *et al.* 2006). The transgenically overexpressed laforin was also found to localize with the Lafora inclusion bodies in the LD mice (Chan *et al.* 2004a,b). Malin was shown to localize with processing bodies in the cells (Singh *et al.* 2012a; Singh and Ganesh 2012b).

Laforin harbours two functional domains—the amino terminal CBD which enables laforin to bind to glycogen, and the carboxyl terminal dual-specificity phosphatase domain (DSPD) which confers the phosphatase activity to laforin (figure 1). Intriguingly, laforin functions as a phosphatase in dimeric form (Liu *et al.* 2006; Dubey and Ganesh 2008; Dubey *et al.* 2012; Sankhala *et al.* 2015) while it binds to glycogen as monomer (Dubey and Ganesh 2008). At least in humans, the *EPM2A* gene codes for multiple isoforms, each with varying localization and possible function (Ganesh *et al.* 2002b; Ianzano *et al.* 2004; Dubey and Ganesh 2008; Dubey *et al.* 2012). For example, one of the minor isoforms harbor both CBD and DSPD, yet inactive as a phosphatase due to a sequence variation at the carboxyl terminal, and it localizes to the nuclear compartment as well (Dubey *et al.* 2012). In addition, this isoform can potentially interact with the major form, to form a heterodimer and block its phosphatase activity (Dubey *et al.* 2012). The other isoforms, resulting from the alternative mRNA splicing harbor one of the

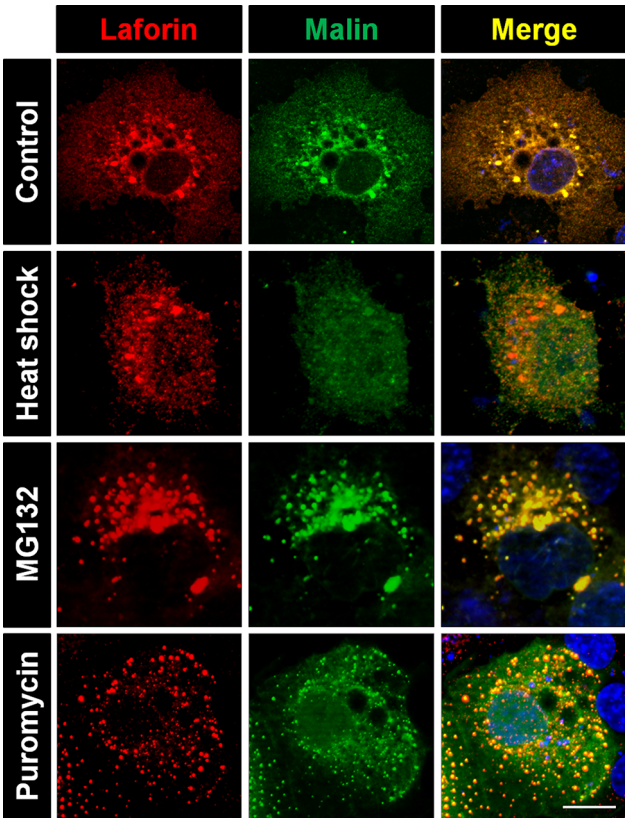

Figure 2. Representative images showing the exclusively cytoplasmic localization pattern of transiently expressed laforin (red) and malin (green) in normal condition (top row), their import to nucleus on exposure of COS7 cells on heat shock (second row), their perinuclear aggresomal localization upon proteasomal blockade (third row) or their recruitment to processing bodies upon puromycin treatment, as indicated (scale 10 μm).

functional domains or a novel protein sequence with unknown function (Dubey *et al.* 2012). The biological significance of these potential isoforms of laforin is not known although a majority of them interact among each other, and with malin. Malin, however, do not appear to function as a dimer although malin–malin interaction has been established (Mittal *et al.* 2015). This self-interaction perhaps promotes the autoubiquitination of malin when the cellular levels of its substrates are low (Mittal *et al.* 2015). Laforin could possibly minimize the autoubiquitination of malin by presenting itself as a substrate (Mittal *et al.* 2015). Intriguingly, malin appears to have a higher affinity for the monomeric form of laforin, suggesting a regulatory role for malin in laforin's phosphatase activity (Mittal *et al.* 2015). Conversely, laforin seems to phosphorylate malin although its biological significance is not clear (Gentry *et al.* 2005).

Animal models of LD

The mouse lines deficient for laforin or malin, created by targeted disruption of the *Epm2a* or the *Nhlrc1* gene, offer attractive models for the LD (Ganesh *et al.* 2002c; DePaoli-Roach *et al.* 2010; Criado *et al.* 2012). Each of the models replicated most of the symptoms and pathological features of the LD. These include the formation of polyglucosan bodies (figure 3), epileptic attacks, abnormal motor coordination, behavioural and cognitive deficits (Ganesh *et al.* 2002c; DePaoli-Roach *et al.* 2010; Criado *et al.* 2012; Berthier *et al.* 2015; Sánchez-Elexpuru *et al.* 2017a). Unlike the LD in humans, the animal models however do not die early possibly due to the species specific difference in the disease progression. Detailed studies on these models has led us to discover the following: (i) mutation in both the genes and thus both the animal models develop similar pathological and clinical course (García-Cabrero *et al.* 2012); (ii) formation of Lafora bodies predates the epileptic symptoms (Ganesh *et al.* 2002c; Sánchez-Elexpuru *et al.* 2017a); (iii) Lafora bodies develop first in the neurons (as early as in one-month-old animals) followed by other tissues such as liver and muscle (from 3–4 months onwards) (Ganesh *et al.* 2002c; (iv) the Lafora bodies increase in size and dimension with age of the mice (Ganesh *et al.* 2002c); (v) the Lafora bodies are lesser branched than normal glycogen, rich in phospho content and are insoluble in water (Tagliabracci *et al.* 2008; Roach 2015); (vi) an unique form of cell death—called dark cell death—predate the formation of Lafora bodies in the neurons (Ganesh *et al.* 2002c; Machado-Salas *et al.* 2012); (vii) several of such degenerating neurons do not show visible Lafora bodies (Ganesh *et al.* 2002c; Machado-Salas *et al.* 2012), suggesting multiple causes for the neuronal death; (viii) the Lafora bodies are positive for ubiquitin, p62—an adaptor protein for autophagy targets, and advanced glycation end-products, hereby suggesting

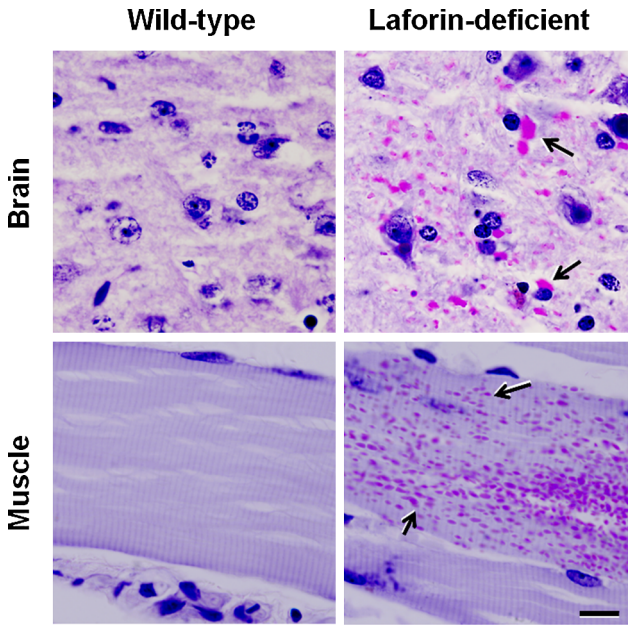

Figure 3. Representative images showing the periodic acid-schiff (PAS)-positive Lafora inclusion bodies (identified by arrows) in the brain and the skeletal muscle tissues of the laforin-deficient mouse and their absence in the wild-type littermate, as indicated (scale 10 μm).

a higher amount of protein content in the glycogen-rich inclusions (Ganesh *et al.* 2002c; Puri *et al.* 2012; Criado *et al.* 2012; Duran *et al.* 2014); (ix) the affected neurons show hyperphosphorylated neurofibrillary tangles, intraneuronal abeta deposits, senile plaques (Puri *et al.* 2009; Machado-Salas *et al.* 2012) and abnormally large lysosomes (Puri and Ganesh 2012); (x) the neurites showed shunted projections, abnormally structured endoplasmic reticulum and the Gogli networks (Ganesh *et al.* 2002c; Puri and Ganesh 2012) and fragmented mitochondria (Ganesh *et al.* 2002c; Upadhyay *et al.* 2017); (xi) the brain showed increased gliosis indicating persistent insult to the neurons (Puri *et al.* 2012; Pederson *et al.* 2013; Turnbull *et al.* 2014; Sánchez-Elexpuru *et al.* 2017a; Rai *et al.* 2017); (xii) brain tissue showed increased levels of long-lived proteins (Aguado *et al.* 2010; Criado *et al.* 2012), and insoluble ubiquitinated proteins (Puri *et al.* 2012); (xiii) autophagic defects in the brain and other tissues occurring prior to the formation of Lafora bodies (Aguado *et al.* 2010; Puri and Ganesh 2012; Criado *et al.* 2012); (xiv) increased levels of neuroinflammatory markers revealing increased innate inflammatory responses in the LD model (López-González *et al.* 2017); (xv) increased susceptibility to drug-induced seizures (Sánchez-Elexpuru *et al.* 2017a,b); (xvi) cardiomyopathy characterized by hypertrophy and systolic dysfunction, suggesting pathologies beyond the neurological defects (Villalba-Orero *et al.* 2017). In addition to the transgenic mouse models for LD, a selected breed of dogs displayed myoclonic seizures and Lafora

bodies in the brain, showed an expanded repeat mutation in the *Nhlrc1* gene confirming the natural occurrence of LD in the canine species (Lohi *et al.* 2005b).

Pathomechanisms in LD

The locus heterogeneity and clinical homogeneity in LD suggest that the products of the two LD genes are nonredundant partners in physiological pathways that are defective in the LD. Thus, defect in any one of them will likely to affect the same set of pathways, leading to clinically identical symptoms and pathologies. Consistent with this model, a number of studies showed that both laforin and malin as a functional complex and are involved in multiple pathways, and loss of anyone of them would affect the cellular physiology (see tables 1 and 2). Notwithstanding the substantial progress made in deciphering the functions of the laforin and malin in cellular context, whether or not they contribute to the disease defining symptoms—epilepsy and other neurological defects as seen in LD patients—is yet to be unequivocally established. Grossly, the possible functions of the LD proteins (laforin/malin) can be grouped in to the following three generic cellular processes: (i) cellular stress response mechanisms, (ii) proteolytic pathways and (iii) glycogen metabolic pathways. And each one of them can contribute to the defect in the other. For example, the polyglucosan bodies can trap ubiquitin, proteasome, chaperones and other players of the protein quality control pathways, thus leading to a compromise in their function (Rao *et al.* 2010a,b; Criado *et al.* 2012; Puri *et al.* 2012). Similarly, the increased glycogen level in the LD tissues can block autophagy through the AMPK-mTOR axis (Singh *et al.* 2013). Conversely, the oxidative stress, for example, can lead to increased glycogen in the neuronal cells (Wang *et al.* 2013; Saez *et al.* 2014; Rai *et al.* 2018), possibly, as a transient protective response to the cellular stress (Rai *et al.* 2018). Thus, each of these three axes can potentially feed-in to the other, and it would therefore be difficult to dissect the cause-consequence relationship. The narrative below provides a brief summary of the each of these pathways in the context of LD.

Lafora bodies—the disease defining pathology of LD represent phospho-rich, lesser branched form of glycogen. These conspicuous inclusions in the neurons led to the suggestion that LD could be possibly a metabolic disorder, and these inclusions are causative for the symptoms seen in LD (Criado *et al.* 2012; Singh *et al.* 2013; Duran *et al.* 2014). Consistent with this hypothesis, the laforin phosphatase was found to bind to the glycogen and dephosphorylate the same (Irimia *et al.* 2015; Raththagala *et al.* 2015). The findings that the Lafora bodies are rich in phosphate moieties, and that they tend to form water insoluble, 'sticky' aggregates support this notion (Tagliabracci *et al.* 2008). Thus, the addition of phosphate group was thought to be an error in the glycogen synthetic process, and that laforin is an error fixing enzyme (Roach 2011). Thus, the 'hyperphosphorylated' abnormal glycogen, resulting from the loss of laforin function was considered to be the primary cause for LD (Tagliabracci *et al.* 2008). However, the recent reports that the abnormal chain length pattern and not the hyperphosphorylation could be the cause for the genesis of Lafora bodies (Nitschke *et al.* 2017). More recently, a transgenic mouse expressing a phosphatase inactive mutant of laforin was shown to correct the LD pathology in the laforin-deficient mouse (Gayarre *et al.* 2014), suggesting defects in functions other than the phosphatase activity of laforin is critical for pathology. An alternate model on the role for laforin/malin in glycogen metabolism was that these proteins regulate the glucose uptake in the cells (Singh *et al.* 2012a; Singh and Ganesh 2012b; Singh *et al.* 2013). It is long known that in addition to the Lafora bodies the LD tissues including the brain showed abnormally higher levels of glycogen. Studies have shown that laforin, and possibly malin, functions as energy sensors in the cell, and that they negatively regulate the cellular glucose uptake by regulating cell surface localization of glucose transporters by modulating the activity of the serum/glucocorticoid-induced kinase 1 (SGK1) (Singh *et al.* 2012a; Singh and Ganesh 2012b; Singh *et al.* 2012c, 2014). Thus, loss of either malin or laforin is expected to promote excessive glucose uptake and its conversion into glycogen. These observations explain as to why the LD tissues show higher levels of intracellular glycogen, and that the increased glycogen may play a role in the aetiology. Consistent with this notion, a recent report demonstrates an increased level of glycogen in the astrocytes in a LD mouse model, and that astrocyte glycogen could contribute to the LD pathology (Rubio-Villena *et al.* 2018).

Studies have shown that the Lafora bodies are positive for ubiquitin, proteasome, p62 and other markers of proteolytic pathways (Ganesh *et al.* 2002c; Criado *et al.* 2012; Duran *et al.* 2014), suggesting that Lafora bodies could contribute defects in the proteolytic pathways. Subsequently, a few reports suggested a direct involvement of laforin and malin in the ubiquitin-proteasome pathway (Mittal *et al.* 2007; Garyali *et al.* 2009), and autophagy–lysosome pathway (Aguado *et al.* 2010; Puri *et al.* 2012; Criado *et al.* 2012; Knecht *et al.* 2012). Intriguingly, studies have shown that the defects in autophagy predate the formation of visible Lafora bodies in the LD mouse models (Criado *et al.* 2012), suggesting that the proteolytic defects could be one of the primary triggers in LD. However, the defects in glycogen metabolism contributing to the compromised autophagy process cannot be ruled out. For example, a link between intracellular glucose level and the mTOR activation was shown in cellular LD models (Singh *et al.* 2013), and reducing the glycogen build-up in the LD animals restored the autophagy defects (Duran *et al.* 2014). Thus, metabolic changes in the LD tissues can

Table 2. Table listing reported interacting partners for laforin and/or malin, and their cellular functions.

Interacting partners	Interacts with*		Proposed/established cellular functions of the partner	Reference
	Laforin	Malin		
AMP-activated protein kinase (AMPK)	Yes	Yes	Malin interacts with and ubiqutinate AMPK subunit beta; AMPK interacts with and phosphorylate laforin; the AMPK interaction might facilitate malin-mediated degradation of laforin	Solaz-Fuster et al. (2008); Moreno *et al.* (2010); Singh *et al.* (2012a)
Carboxyl terminus of Hsp70-interacting protein (CHIP)	Yes	Yes	The HSP70 co-chaperone CHIP requires malin and laforin for its protective function during heat stress	Rao *et al.* (2010b); Sengupta *et al.* (2011)
DCP1a	No	Yes	Malin promotes the degradation of Dcp1a via the ubiquitin-proteasome pathway; microRNA pathway may be altered	Singh and Ganesh (2012b)
Dishevelled2	Not reported	Yes	A key player in Wnt signalling pathway; shown to be regulated by malin via its degradation	Sharma *et al.* (2012)
EPM2AIP1	Yes	Not reported	Interacts with glycogen synthase (GS) and allosterically activates GS	Ianzano *et al.* (2003); Turnbull *et al.* (2013)
Glycogen synthase kinase 3 (GSK3)	Yes	Yes	Laforin and malin bind to GSK3, and laforin dephosphorylates GSK3; GSK3 is involved in a variety of pathways including neuronal development, body pattern formation and cellular homoeostasis	Lohi *et al.* (2005a); Wang *et al.* (2006)
Glycogen-debranching enzyme (AGL)	Not reported	Yes	Malin interacts with and promotes the ubiquitination of AGL	Cheng *et al.* (2007)
Heat shock factor-1 (HSF1)	Yes	Yes	Laforin and malin contribute to the activation of heat shock transcription factor, HSF1	Sengupta *et al.* (2011)
Heat shock protein 70 (HSP70)	Yes	Yes	HSP70 requires presence of laforin and malin for its chaperone activity	Garyali *et al.* (2009)

Table 2. *(Contd)*

Interacting partners	Interacts with*		Proposed/established cellular functions of the partner	Reference
	Laforin	Malin		
Muscular isoforms of pyruvate kinase (PKM1 and PKM2)	Yes	Yes	Both PKM1 and PKM2 are polyubiquitinated by the laforin-malin complex	Viana *et al.* (2015)
Neuronatin	Not reported	Yes	A proteolipid that regulates ion channels and contribute to the brain development; shown to stimulate glycogen synthesis in a malin-dependent way.	Sharma *et al.* (2011)
NFU1 (HIRIP5)	Yes	Not reported	NFU1 localizes on mitochondria plays critical role in iron-sulfur cluster biogenesis. Alternatively spliced variants are known; laforin was shown to interact with a cytoplasmic variant.	Ganesh *et al.* (2003); Navarro-Sastre *et al.* (2011)
Parkin	Yes	Yes	Laforin and malin positively regulate the ubiquitin ligase activity of parkin	Upadhyay *et al.* (2017)
PPP1R3D (R6)	Yes	Yes	This interaction leads to the malin-dependent ubiquitination of R6, thus regulating the R6 glycogeneic activity	Rubio-Villena *et al.* (2013)
Protein targeting to glycogen (PTG/R5)	Yes	Yes	PTG is a scaffold protein that binds to glycogen and many other enzymes; PTG promote the activity of glycogen synthase; the stability of PTG is regulated by the malin–laforin complex	Fernández-Sánchez *et al.* (2003); Solaz-Fuster et al. (2008); Worby *et al.* 2008; Guerrero *et al.* (2011)
Sequestosome-1 (p62)	Yes	Yes	p62 is an adapter protein involved in autophagy; Laforin and malin were shown to interact with p62 to promote autophagy flux	Sánchez-Martín *et al.* (2015)
Starch-binding domain-containing protein 1 (STBD1)	Yes	Not reported	A protein potentially involved in glycogen meatabolism	Zhu *et al.* (2014); Sun *et al.* (2016)
Tau protein	Yes	Not reported	Laforin interacts with and dephosphorylates the microtubule-binding protein tau	Puri *et al.* (2009)
Ubiquitin-conjugating enzyme E2 N (UBE2N)	Yes	Yes	E2-N interacts with laforin and malin and regulates malin's E3 ubiquitin ligase activity	Sánchez-Martín *et al.* (2015)

*Interaction refers to direct physical interaction established by yeast two-hybrid screes and/or immunoprecipitation/pull-down assays. 'Not reported' indicates the absence of any published report testing the interaction and proving it in one way or the other

modulate the other processes, including the proteolytic, stress response and inflammatory pathways. The findings that the LD tissues show elevated ROS level (Romá-Mateo *et al.* 2014), ER stress (Vernia *et al.* 2009; Zeng *et al.* 2012), compromised chaperone activity (Sengupta *et al.* 2011; Rao *et al.* 2010a,b) and the elevated inflammatory response (López-González *et al.* 2017) point to the possibility of defective cellular processes. Thus restoring the glycogen metabolic flux might fix most of the other pathologies (also see below).

Therapy for LD

Despite the advances made in identifying the genes involved in LD and elucidating their cellular functions, the treatment for LD remains at best symptomatic (Goldsmith and Minassian 2016; Michelucci *et al.* 2016). The current treatments for LD include the use of channel blockers such as valproic acid and recently the AMPA antagonist perampanel was shown to be more efficient in controlling the seizures (Goldsmith and Minassian 2016). Given the glycogen load in the LD tissues, a few studies have also attempted ketogenic diet for LD but did not show an appreciable effect (Cardinali *et al.* 2006; Kossoff *et al.* 2014). Therefore, the LD animal models are being used to identify and test 'druggable' targets for effective therapy. Obviously, the best way would be to block the glycogen synthesis since genetic inactivation of the glycogen synthase or its positive regulators led to the suppression of Lafora bodies and seizure susceptibility in the mouse models (Pederson *et al.* 2013; Turnbull *et al.* 2014; Sánchez-Elexpuru *et al.* 2017a,b; Rai *et al.* 2017). Thus attempts are being made to identify small molecules that can block glycogen synthase activity (Solmesky *et al.* 2017). Similarly, gene therapy is also being tested in the animal models (Cornford *et al.* 2016) to restore the function of laforin or malin. As a pharmacological approach, treatment with metformin, an activator of AMP-activated protein kinase (AMPK), was shown to reduce the Lafora body load, neurodegeneration and seizure susceptibility in LD animals (Berthier *et al.* 2015; Sánchez-Elexpuru *et al.* 2017a,b), although its effect on epilepsy or seizure susceptibility was not documented. Similarly, sodium selenite administration was shown to decrease the seizure susceptibility possibly by protecting neurons from oxidative stress (Sánchez-Elexpuru *et al.* 2017a,b). Recently, a study from our own group has demonstrated that blocking the leptin signalling in the brain could reduce the glycogen level, Lafora body load and seizure susceptibility possibly by lowering the neuronal glucose uptake (Rai *et al.* 2017). Nonetheless how the loss of laforin or malin results in the epileptic episodes, and whether epilepsy is primary symptom or secondary to the Lafora bodies or neuroinflammation are not studied well. Similarly, the one-to-one correlation on the specific pathologies and the disease symptoms (such as epilepsy, dementia, psychosis and ataxia) have not been established.

Concluding statement

The first 20 years since the discovery of the first LD gene, *EPM2A*, has seen a remarkable progress in DNA diagnostics, understanding the gene function, developing models and deciphering the pathomechanisms. However not much has changed with regard to the prognosis or the treatment; LD continues to be a debilitating condition affecting hundreds of families worldwide. Often termed as a 'rare orphan disorder', work on LD genes and LD models continue to shape our understanding on the biology of glycogen metabolism, and its relevance to neuronal function. With the availability of LD models, the next two decades is likely to witness a remarkable progress in our understanding behind the epilepsy and other symptoms of LD and contribute to the management and treatment of LD in humans.

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