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Laforin is required for the functional activation of malin in endoplasmic reticulum stress resistance in neuronal cells

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Mutations in either *EPM2A*, the gene encoding a dual-specificity phosphatase named laforin, or *NHLRC1*, the gene encoding an E3 ubiquitin ligase named malin, cause Lafora disease in humans. Lafora disease is a fatal neurological disorder characterized by progressive myoclonus epilepsy, severe neurological deterioration and accumulation of poorly branched glycogen inclusions, called Lafora bodies or polyglucosan bodies, within the cell cytoplasm. The molecular mechanism underlying the neuropathogenesis of Lafora disease remains unknown. Here, we present data demonstrating that in the cells expressing low levels of laforin protein, overexpressed malin and its Lafora disease-causing missense mutants are stably polyubiquitinated. Malin and malin mutants form ubiquitin-positive aggregates in or around the nuclei of the cells in which they are expressed. Neither wild-type malin nor its mutants elicit endoplasmic reticulum stress, although the mutants exaggerate the response to endoplasmic reticulum stress. Overexpressed laforin impairs the polyubiquitination of malin while it recruits malin to polyglucosan bodies. The recruitment and activities of laforin and malin are both required for the polyglucosan body disruption. Consistently, targeted deletion of laforin in brain cells from *Epm2a* knockout mice increases polyubiquitinated proteins. Knockdown of *Epm2a* or *Nhlrc1* in neuronal Neuro2a cells shows that they cooperate to allow cells to resist ER stress and apoptosis. These results reveal that a functional laforin–malin complex plays a critical role in disrupting Lafora bodies and relieving ER stress, implying that a causative pathogenic mechanism underlies their deficiency in Lafora disease.

Structured digital abstract
Malin physically interacts with **Laforin** and **GS1** by pull down (View interaction)
GS1 and **Laforin** colocalize by fluorescence microscopy (View interaction)
Laforin physically interacts with **Malin** by anti tag coimmunoprecipitation (View Interaction: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11)

Abbreviations
ATF6, activating transcriptional factor 6; CHOP, proapoptotic C/EBP homologous protein; EGFP, enhanced green fluorescent protein; EPM2A, epilepsy of progressive myoclonus type 2A; ER, endoplasmic reticulum; GS1, glycogen synthase 1; GSK3β, glycogen synthase kinase 3β; HEK293, human embryonic kidney 293; KO, knockout; LD, Lafora disease; PGB, polyglucosan body; PTG, protein targeting to glycogen; WT, wild-type.

Introduction

Laforin, encoded by the epilepsy of progressive myoclonus type 2A gene (*EPM2A*), is highly expressed in adult brain [1,2]. Loss-of-function mutations of *EPM2A* in humans cause Lafora disease (LD), an early-onset fatal epileptic neurodegenerative disorder marked by the accumulation of abnormally branched glycogen inclusions called Lafora bodies or polyglucosan bodies (PGBs), in brain neurons [3–6]. Although Lafora bodies are also distributed in glycogen metabolism-active tissues, such as liver and muscle, LD is not classified as a glycogen storage disease. Loss-of-function mutations of NHL repeat-containing 1 (*NHLRC1*), which encodes an E3 ubiquitin ligase named malin, can also cause LD [7]. Interestingly, although mutation of either gene can cause the development of LD, some LD patients do not harbor either mutation [8]. Mice with targeted deletion of either *Epm2a* or *Nhlrc1* do not recapitulate the early-onset lethal neurological features of LD [9–11], which indicates that the mutations themselves should be considered in the quest to elucidate the molecular mechanism(s) of LD development.

Laforin has two functional domains: a carbohydrate-binding domain and a dual-specificity phosphatase domain [3,12–14]. The carbohydrate-binding domain is critical for the *in vitro* binding of laforin to glycogen [13], the *in vivo* binding of laforin to polyglucosan [12,15] and the *in vivo* binding of laforin to itself [16]. Malin also has two functional domains: a RING finger E3 ubiquitin ligase domain and six repeats of NHL that are defined by (and named after) amino acid sequence homologies with NCL-1, HT2A and LIN41 proteins [17]. Three missense mutations of *NHLRC1* in the RING domain have been reported to impair malin's E3 ligase activity, whereas two missense mutations in the NHL repeats have been reported to impair the association of malin with laforin [18,19].

Interrelationship studies of laforin and malin have demonstrated that the combination of these proteins is responsible for reducing glycogen content in neuronal cells that ectopically express protein targeting to glycogen (PTG), a glycogenesis activator that induces protein phosphatase 1 to dephosphorylate glycogen synthase [20,21]. The combination of laforin and malin has also been shown to degrade PTG [22,23], misfolded proteins [24] and even laforin itself [18]; it can be stabilized and activated by AMP-activated protein kinase in hepatoma cells [25,26]. Laforin has been shown to be an *in vitro* phosphatase of glycogen; it removes phosphates from phosphate-labeled amylopectin, isolated muscle glycogen and muscle glycogen synthesized by muscle glycogen synthase (GS1) [15,27–29]. We and the Minassian laboratory have shown that laforin dephosphorylates and inactivates glycogen synthase kinase (GSK)3β at serine 9 in serum-starved, growth factor-stimulated cells [19,30]. However, under physiological conditions in *Epm2a* knockout (KO) mice, increased serine 9 phosphorylation in GSK3β was not observed in the soluble portion of the tissue lysate [28,31]. This suggests that laforin dephosphorylates GSK3β at serine 9 in a context-dependent manner.

We have also shown that laforin reduces and its mutants exaggerate the neuronal cell response to ER stress stimulation, and that laforin protects cells from apoptosis induced by energy-deprivation stress [32,33]. Consistent with this, increased ER stress in both *Epm2a* KO liver cells and an autopsy sample from an LD patient has been revealed [34]. Besides preventing these stresses, laforin with associated malin protect cells from thermal stress by activation of heat-shock factor 1, a transcriptional factor that activates heat-shock genes [35].

Here, we present data demonstrating that laforin recruits malin to PGBs, where it activates functional malin for PGB disruption. Both the PGB-binding ability and the phosphatase activity of laforin are required for the recruitment and activation of malin and the disruption of PGBs. Likewise, both the laforin-binding ability and the E3 ligase activity of malin are required for the disruption of PGBs by the laforin–malin complex. The functional assembly of the laforin–malin complex alleviates ER stress and prevents the apoptosis of neuronal cells exposed to stress stimuli.

Results

Malin and its mutants aggregate and are polyubiquitinated but do not contribute to ER stress

To determine the relationship between malin and laforin, we generated malin mutations at the same sites found in LD patients. These included three sites near the RING finger E3 ubiquitin ligase domain and nine sites in the first five of the six NHL repeats (Fig. 1A). Similar to some mutants previously reported in transformed monkey kidney fibroblast COS7 cells [36,37], we found that overexpressed wild-type (WT) malin and its mutants in mouse neuroblastoma Neuro2a (N2A) cells and human embryonic kidney (HEK)293 cells were expressed as monomers as well as polymerized

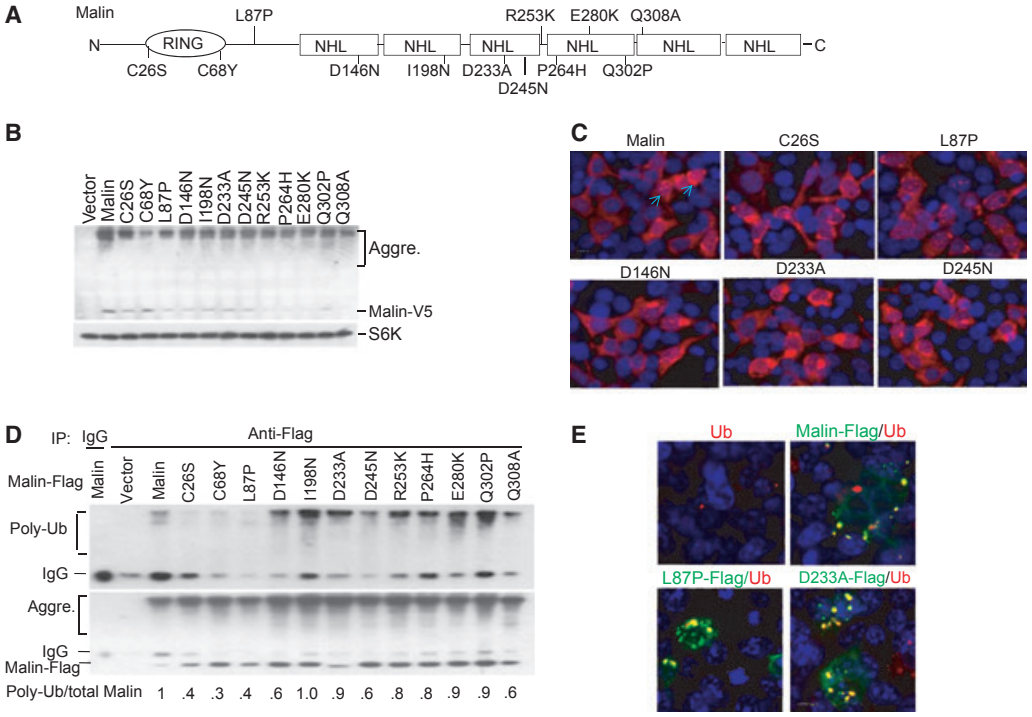

Fig. 1. Malin and its mutants aggregate and are polyubiquitinated. (A) The distribution of missense mutations identical to the sites in LD patients. (B) Aggregation of malin and its mutants. Malin and its mutants were transiently transfected into N2A cells for 24 h. The cell lysate was resolved by reducing heat-denaturing SDS/PAGE. (C) Aggregates of malin and its mutants are formed in or around the nuclei of transfected HEK293 cells. Twenty-four hours after transfection with malin or its mutants, HEK293 cells were fixed and stained with primary antibody to V5 followed by a secondary Texas Red-conjugated IgG. (D) Aggregates of malin and its mutants are polyubiquitinated. Immunoprecipitation (IP)–western blotting shows the polyubiquitinated aggregates of malin and its mutants in their Flag-tagged plasmid-transfected HEK293 cells 24 h before lysis for IP. The bottom digits on the gel show the densitometric quantitation of polyubiquitinated malin divided by the total malin as a relative ratio to WT malin that was arbitrarily set to 1. (E) Aggregates of malin and its representative mutants are polyubiquitin-positive. HEK293 cells transfected as in (D) were fixed and double-stained with antibodies to Flag and polyubiquitin. In all images nuclei were stained with 2,6-diaminopimelic acid and merged photos are shown. Scale bars, 10 μM.

aggregates (Fig. 1B). No statistically significant differences in protein stability were seen between WT malin and its mutants in these two cell types, although transfection efficiency varied in separate experiments. Immunocytochemistry revealed that in HEK293 cells, Flag-tagged aggregates of malin or its mutants mostly localized in the cytoplasm (Fig. 1C). Immunoprecipitation of the Flag-tagged malin or its mutants demonstrated that the aggregates were polyubiquitinated (Fig. 1D). RING domain mutants (C26S, C68Y and L87P) and NHL mutants (D146N, D245N and Q308A) were polyubiquitinated to a lesser extent than the WT protein, as quantified by densitometry (Fig. 1D). Some aggregates observed in the ligase-inactive RING mutant cells were polyubiquitin-negative (Fig. 1E), indicating that the polyubiquitination of aggregates is likely attributable to malin autoubiquitination. This is consistent with the characteristics of the RING finger E3 ubiquitin ligase family [38] and with studies showing that malin protein is autoubiquitinated *in vitro* [18]. In support of this, our results showed that the three E3 ligase-inactive RING finger mutants had much lower levels of ubiquitination than did WT or other malin mutants (Fig. 1D).

To test the effect of malin and its mutants on ER stress, we transfected them into N2A cells and treated the transfected cells with either thapsigargin or tunicamycin, two molecules that induce ER stress by reducing ER calcium pump activity and dysglycosylating ER proteins, respectively [39,40]. We then measured two common ER stress markers, ER chaperone 78-kDa glucose-regulated protein (GRP78) and the transcription factor proapoptotic C/EBP homologous protein (CHOP). Our results show that, in the absence of thapsigargin or tunicamycin, N2A cells expressing WT malin or its mutants expressed little or no CHOP. However, in the presence of either stressor, significant levels of CHOP were induced (Fig. 2A,B). Induced

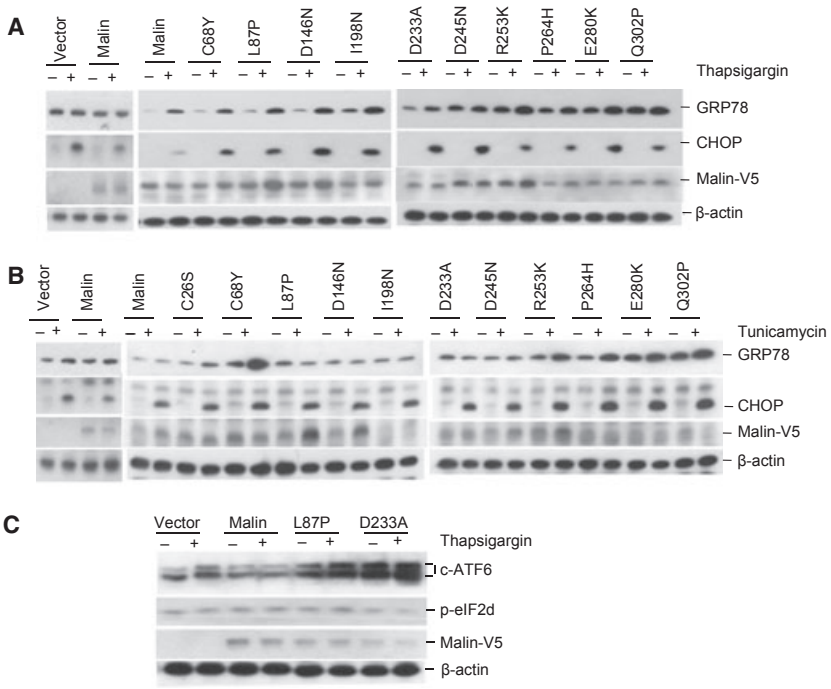

Fig. 2. WT malin prevents and malin mutants exacerbate ER stress in N2A cells. (A–C) Protein expression of ER stress markers. N2A cells were transiently transfected with V5-tagged malin or its mutants for 24 h and then treated with 2 μM thapsigargin (A,C) or 2 $\mu g \cdot mL^{-1}$ tunicamycin (B) or dimethylsulfoxide vehicle control. Eight hours after treatment, cells were directly lysed with 1% Triton X-100 lysis buffer. The ER stress marker proteins GRP78, CHOP, cleaved ATF6 (cATF6) and phosphor-eIF2α were detected by western blotting. All blots were performed on the same membrane after stripping. Representatives of two separate experiments are shown.

CHOP levels were higher in most of the cells transfected with malin mutants than in cells transfected by WT malin. The inconsistent induction of GRP78 and CHOP in some mutant transfected cells after exposure to stressors indicates that the mutants elicited signs of both early and late ER stress at different stages. Usually, GRP78 is an early-stage marker of ER stress, whereas CHOP is a late stage marker. Regardless of whether the cells were stressed, N2A cells transfected with malin mutants showed a marked increase in cleaved activating transcriptional factor 6 (ATF6), an active form of ATF6 and another ER stress marker. Neither WT nor mutant malin affected the levels of phosphor-eIF2α, an early-stage marker of ER stress, in transfected N2A cells exposed to stress stimulation (Fig. 2C). It is interesting to note that although overexpressed WT malin formed polyubiquitinated aggregates in the transfected N2A cells (Fig. 1C), cells transfected with malin showed a decreased ER stress response to stimuli in comparison with empty vector transfected cells (Fig. 2B), suggesting that WT malin plays a role in the prevention of ER stress. Taken together, these results showed that aggregated, polyubiquitinated WT malin alone does not induce ER stress. By contrast, the malin mutants themselves induce slight ER stress, but exacerbate this response upon the addition of exogenous ER stress inducers.

Malin mutants have impaired binding to laforin

We next characterized the ability of malin mutants to bind to laforin; although WT malin is known as a binding partner of laforin, the precise domains required for binding of malin to laforin remain undefined [18,19]. We constructed plasmids that expressed different truncated versions of laforin and malin (Fig. 3A,B). By analysis of immunoprecipitation data and cotransfection of these plasmids into HEK293 cells, we were able to define the reciprocal binding regions of laforin and malin. The region of laforin that binds malin was near the carbohydrate-binding domain end and included the entirety of exon 2; the region of malin that binds laforin began near the RING domain end and spanned the first five NHL repeats (Fig. 3A,B). The critical region of malin that binds laforin encompasses the end of the RING domain and extends to the first NHL repeat. Because the glycogen binding ability is maintained in exon-deleted mutants of laforin, cellular glycogen might cause nonspecific binding between the deleted forms of laforin and malin (Fig. 3A). Consistent with what is known about the region of malin that binds laforin, the RING domain mutants C26S, C68Y and L87P did not differ in their binding to laforin. By contrast, other NHL repeat mutants demonstrated an impaired ability to bind to laforin, with the exception of the two mutants D245N

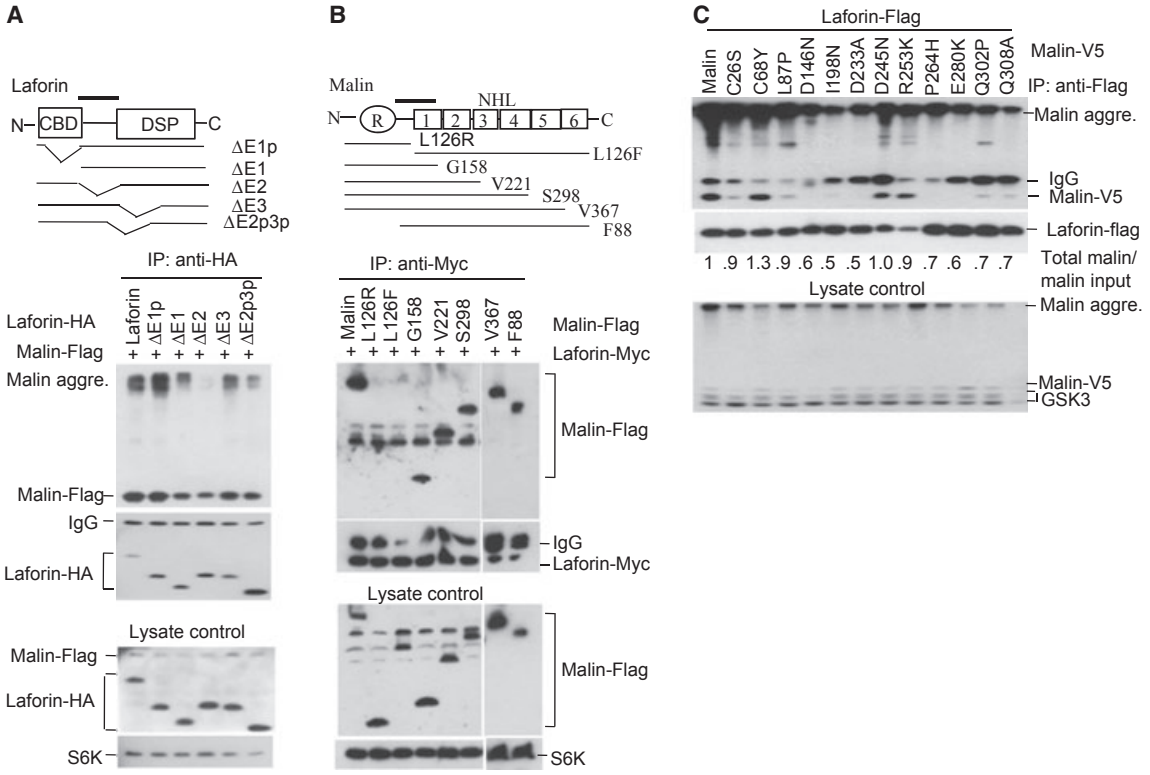

Fig. 3. Most NHL repeat mutants of malin impair their binding to laforin. (A,B) The reciprocal binding regions of malin and laforin are defined. HEK293 cells were transiently cotransfected with protein-expressing plasmids as indicated. Twenty-four hours after transfection, cells were directly lysed by 1% Triton X-100 plus 0.02% SDS lysis buffer and supernatants were immunoprecipitated and then blotted with the indicated antibodies. Areas critical for binding between malin and laforin are shown with bold lines. (C) Impaired binding ability of malin mutants to laforin. HEK293 cells were transiently cotransfected with V5-tagged malin or malin mutants together with Flag-tagged laforin, and then lysed directly with Triton X-100 plus 0.02% SDS buffer. The resultant supernatants were immunoprecipitated with Flag antibody and the immunoprecipitates were subjected to western blotting. Densitometric quantitation of total malin divided by total malin input of the lysate is represented as a relative ratio to WT malin that was arbitrarily set to 1.

in and R253K next to the third NHL repeat (Fig. 3C). These results demonstrated that most NHL repeat mutants of malin were defective in their ability to bind laforin.

Laforin is required for malin recruitment to polyglucosan

Subsequent experiments were undertaken to reveal the binding target that laforin–malin complex might work on. Based on the finding demonstrating that transgenic mice expressing GS1 under the control of skeletal muscle-specific promoter display elevated polyglucosan levels in the tissue [41], we constructed and transfected GS1 into HEK293 cells and found that it synthesized polyglucosan that was glycogen positive, periodic acid Schiff positive and resistant to α-amylase hydrolysis, as previously reported [41]. After cotransfecting or singly transfecting GS1 with laforin, laforin mutant C265S, malin or malin mutants L87P or D233A into HEK293 cells, we found that laforin and C265S, but not WT malin or its mutants, bound GS1-synthesized polyglucosan (Fig. 4A). In the triple combination indicated in Fig. 4A, only WT laforin and WT malin bound to and disrupted large PGBs into relatively small granules. In cells containing these small granules, GS1 was still distributed within the cytoplasm and nucleus. This indicates that if the laforin–malin complex degrades GS1 to limit polyglucosan formation, this process takes place in PGBs only (Fig. 4A). Neither phosphatase-dead mutant C265S with WT malin nor E3 ligase-inactive mutant L87P or laforin-binding-deficient mutant D233A in combination with WT laforin was able to disrupt the PGBs, demonstrating that the disruption of PGBs requires the activities of both laforin phosphatase and malin E3 ligase, as well as appropriate recruitment of the laforin–malin complex. To prove the requirement of laforin for malin in

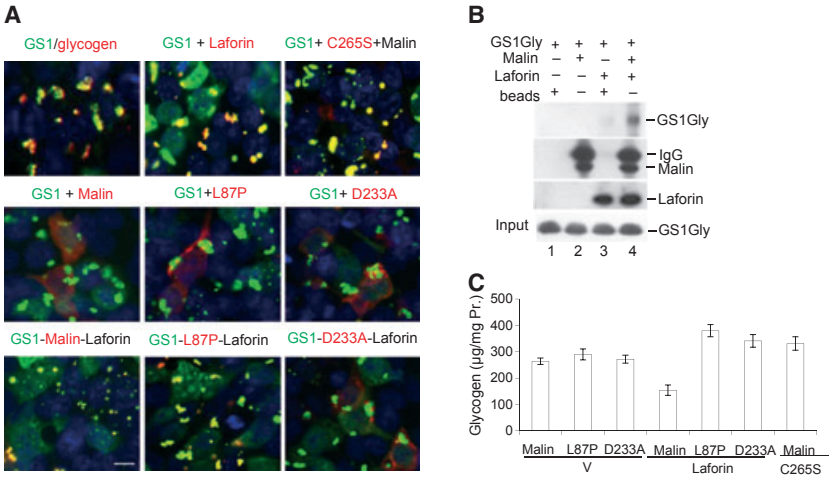

Fig. 4. Laforin recruits malin and both desrupt polyglucosan. (A) Laforin is required for malin recruitment to GS1-synthesized polyglucosan. HEK293 cells were transfected with a plasmid of GS1–Flag alone or in combination with the indicated plasmids of laforin–myc and malin–V5 or their mutants for 24 h. The transfected cells were fixed and double-stained with antibodies to glycogen or Flag, V5 or Myc tags. (B) Laforin is essential for malin binding to polyglucosan *in vitro*. Isolated GS1 polyglucosan was added to binding buffer containing malin–protein G beads or empty beads in the presence or absence of purified laforin protein. After 2 h of binding at 4 °C, the washed beads were lysed with 1× SDS loading buffer for western blotting to detect GS1 polyglucosan. (C) Laforin and malin together decrease glycogen content *in vivo*. HEK293 cells transfected with the indicated plasmids were directly lysed in NaAc buffer for glycogen determination. Results of glycogen determination are presented as the mean ± SEM of three separate experiments. Scale bars, 10 μM.

binding to PGBs, we combined purified laforin protein and malin immunoprecipitate with the isolated GS1 polyglucosan *in vitro* and found that the malin immunoprecipitated the GS1 polyglucosan only in the presence of laforin (Fig. 4B, lane 2 compared with lane 4). Also, by determining the glycogen content in HEK293 cells that possessed endogenous GS1 glycogen, we found that only cells transfected with both malin and laforin had a reduced glycogen content (Fig. 4C), which is consistent with results observed in neuronal cells that ectopically express PTG [20]. These results demonstrate that GS1 polyglucosan is a target of the laforin–malin complex, and that polyglucosan disruption requires the binding and activity of laforin and malin. This conclusion is supported by previous results showing that GS1, not PTG, accumulates in Lafora bodies [42].

Laforin and malin functionally depend on each other to prevent ER stress

Laforin recruits malin to PGBs, which both proteins disrupt in combination. Disruption of PGBs either provides glucose (energy) to cells to counteract ER stress stimulation (which results in a decrease in cellular energy), or releases laforin and malin for recycling. Based on this hypothesis, knockdown of either laforin or malin in N2A cells could enhance cell sensitivity to ER stress. As expected, knockdown of either laforin or malin in N2A cells increased thapsigargin-induced CHOP levels (Fig. 5A,B). Restoration of laforin did not diminish the increased sensitivity of malin-silenced N2A cells to CHOP induction by thapsigargin; likewise, restoration of malin did not diminish the increased sensitivity of laforin-silenced N2A cells to CHOP induction, even though they increased the resistance of cells transfected with scrambled small hairpin RNA to ER stress (Fig. 5B). To determine whether both phosphatase and E3 ligase activities were required to prevent ER stress-induced apoptosis, we transfected malin or its mutants into N2A cells expressing either laforin or C265S, and observed that only a combination of WT laforin and WT malin significantly prevented an ER stress-induced increase in annexin V-positive (apoptotic) cells (12.68% in WT laforin–WT malin versus 23.23% in WT laforin–mutant L87P). Other combinations could not prevent apoptosis (Fig. 5C). These results show that a functional laforin–malin complex is necessary for cellular resistance to ER stress.

Laforin and ER stressor impair malin autoubiquitination

The question of how autoubiquitinated malin becomes functionally activated once it has bound laforin

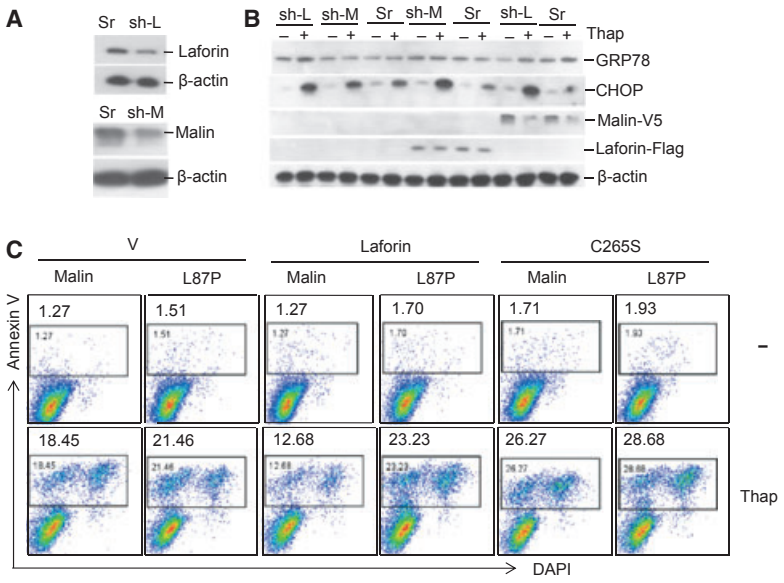

Fig. 5. Laforin and malin cooperate in ER stress relief and apoptosis prevention. (A) Knockdown of laforin or malin in N2A cells. Silencer of small hairpin RNA of laforin (sh-L) or malin (sh-M) was cotransfected with Flag-tagged laforin or malin into N2A cells for 24 h. Knockdown efficiency was determined by western blotting using anti-Flag IgG. (B) Codependence of laforin and malin in the prevention of ER stress. Scrambled sh (Sr), sh-L or sh-M cells of N2A were transiently transfected with vector, malin or laforin for 24 h and then treated with 2 μM thapsigargin or vehicle for an additional 8 h. After treatment the cells were lysed for western blotting and probed for ER stress proteins. (C) Activities of both laforin and malin are required for the inhibition of apoptosis induced by ER stressors. N2A cells expressing WT laforin or its mutant C265S were transiently transfected with malin–EGFP or malin mutant L87P–EGFP. Twenty-four hours after transfection, cells were divided into two test groups and treated with 1 μM thapsigargin or vehicle for 24 h in 2.5% fetal bovine serum Dulbecco's modified Eagle's medium. Treated cells were stained with annexin V (for apoptotic cells) and 2,6-diaminopimelic acid (for dead cells). The annexin V in the EGFP-positive population was analyzed by flow cytometry.

remained. To determine the mechanism by which this occurs, we cotransfected laforin along with malin or one of its mutants into HEK293 cells, and found that in malin immunoprecipitate containing laforin, malin became less ubiquitinated, whereas mutants of malin did not (despite the presence or absence of laforin in their immunoprecipitates) (Fig. 6A). To ascertain whether an ER stressor activates malin by impairing its autoubiquitination, we treated malin-transfected N2A cells with tunicamycin or thapsigargin. Ubiquitination of malin was significantly induced by both stressors (Fig. 6B). Detection of endogenous malin activation by laforin or ER stress stimulation cannot be performed because, at present, no convincing, commercially available malin antibodies exist. However, via immunoprecipitation using anti-polyubiquitin IgG [43], we were able to detect significantly more polyubiquitinated proteins in the brain cells of *Epm2a* KO mice than in age-matched WT mice (Fig. 6C,D). This indicates that laforin plays a role in preventing the accumulation of polyubiquitinated proteins, which is consistent previous work [44] showing increased polyubiquitinated proteins in the lysate of laforin-deficient human fibroblasts by western blotting. Taken together, these data demonstrated that laforin is required not only for the binding of malin to PGBs, but also for the activation of malin by preventing its autoubiquitination.

Discussion

Through a systematic analysis of malin, laforin and their missense mutants, and using KO approaches, we demonstrate for the first time the cooperation of laforin and malin that confers neuronal protection against ER stress. Although the exact mechanism underlying the prevention of ER stress by laforin–malin complexes remains to be defined, we hypothesize that polyglucosan disruption governed by laforin–malin complexes not only provides endogenous energy in the form of glucose, but also lowers the level of stress within the cytoplasm, easing the ER burden. We are the first to demonstrate that polyglucosan disruption absolutely requires both laforin and malin. However, these proteins alone are not sufficient. Thus, for disruption of PGBs, a consecutive process may be required. First, laforin recruits malin and other

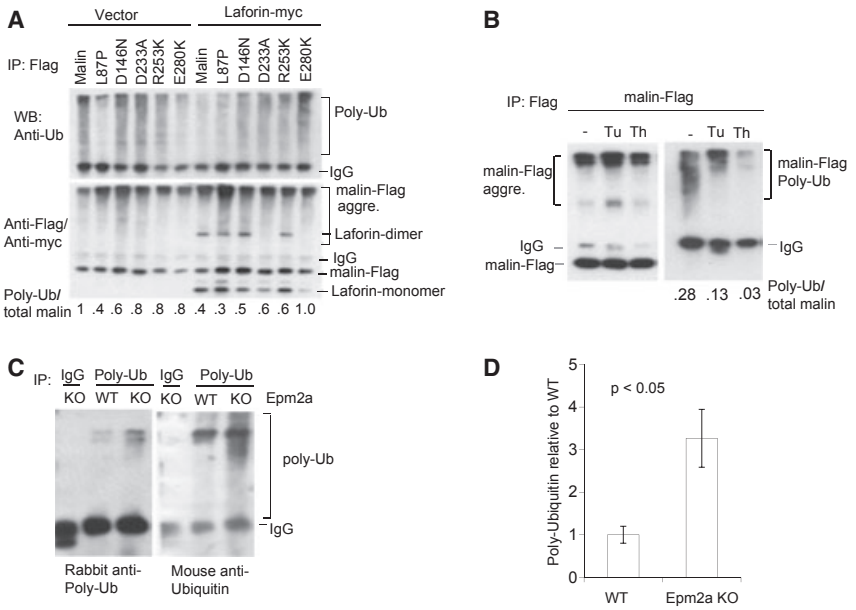

Fig. 6. Laforin and ER stressors promote malin deubiquitination. (A) Laforin favors the deubiquitination of malin. HEK293 cells cotransfected with malin or its mutants together with vector or laforin were lysed 24 h after transfection to detect polyubiquitination of malin by IP–western blotting using tag antibodies. (B) ER stress stimulation activates malin deubiquitination. N2A cells expressing malin–Flag were treated with 2 $\mu g \cdot mL^{-1}$ tunicamycin (Tu) or 1 $\mu g \cdot mL^{-1}$ thapsigargin (Th). 8 h after treatment, the cells were lysed for anti-Flag IP and anti-polyubiquitin blotting to detect malin ubiquitination. (C) Polyubiquitinated proteins accumulated in brain cells from *Epm2a* KO mice. Equal amounts of Triton X-100-soluble proteins in the brain lysate of 6-month-old *Epm2a* KO and WT mice were immunoprecipitated with polyubiquitin Lys48-linkage antibody. Ubiquitinated proteins were detected by mouse anti-ubiquitin IgG and rabbit anti-(Lys48-linkage polyubiquitin) IgG. (D) Graphical representation of polyubiquitinated protein levels in the two strains.

proteins or enzymes to PGBs, where the functional assembly of a laforin–malin complex disrupts PGBs into relatively small, 'normal' glycogen granules that can be degraded by conventional glycogen metabolic enzymes. GS1 is the key enzyme involved in PGB formation, and may be the first enzyme targeted by the functional complex of laforin and malin. The binding and recruitment of laforin and malin to GS1 polyglucosan suggests that the laforin–malin complex degrades GS1 and thus inhibits the ability of GS1 to synthesize polyglucosan, a process that takes place in an insoluble glycogen pool. Degradation of GS1 by the complex has been hinted at by a decrease in GS1 protein levels in the lysate of N2A cells expressing laforin, malin and PTG [20], and in the accumulation of GS1 protein in Lafora bodies from *Epm2a* or *Nhlrc1* KO mice [10,42,45,46]. Disruption of PGBs by the laforin–malin complex may subsequently prevent laforin and malin from becoming trapped in PGBs. The requirement for both laforin phosphatase activity and malin E3 ligase activity in PGB disruption suggests that the dephosphorylation and ubiquitination of key components in PGBs may take place simultaneously. These processes occurring in the insoluble glycogen pool may create a situation in which detecting alterations in the target protein becomes difficult [45].

Stresses that result in intracellular energy decline, such as energy deprivation and ER stress, may induce PGB formation; activation of laforin–malin complexes may subsequently disrupts PGBs, thereby supplying energy for cell recovery from stress. Furthermore, under homeostatic conditions, the laforin–malin complex plays a critical role in the surveillance and prevention of PGB formation; thus, deletion of either gene causes PGB accumulation [9,45–47]. The laforin–malin complex also prevents the formation of and disrupts PTG-activated GS1-synthesized abnormal, but not normal, glycogen [20,22,23], because laforin preferentially binds to polyglucosan over normal glycogen [15]. We also predict that polyubiquitination of malin under physiological conditions may be a form of self-inactivation to control its activity when it is not needed. Laforin is not a deubiquitinating enzyme, and therefore an unknown alternative may be recruited to PGBs or perhaps earlier in the malin–laforin pathway to counteract the cellular effects of ER stress. In addition, increased protein levels of ER stress markers were not observed in brain extracts from 9-month-old *Epm2a*

KO mice, probably because it is difficult to extract brain proteins from tissues with massive PGB accumulations [34].

Because PGBs are found in other neuronal disorders, including Alzheimer's disease [48] and temporal lobe epilepsy [49], decreased functional laforin and/or malin might also contribute to the progression of these diseases. Therefore, our study has clinical implications across a broad range of neurological disorders.

Experimental procedures

Mice and cells

The *Epm2a* KO mice (from a 129Sv strain) [9] used in this study have been backcrossed onto a C57BL/6 background for more than 10 generations. Experiments were performed using WT and *Epm2a* KO mice that were littermates born from homozygous breeding pairs. All mice were kept and used according to the procedures approved by the Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan.

HEK293 and Neuro2a cell lines were from Invitrogen (Grand Island, NY, USA) and ATCC (Manassas, VA, USA), respectively. The HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g glucose, 2 mM glutamine, 2% penicillin and 10% fetal bovine serum. The N2A cells were cultured in minimal essential medium supplemented with 2 mM glutamine, 2% penicillin and 10% fetal bovine serum.

Reagents and antibodies

Sources of rabbit polyclonal antibodies to specific proteins are as follows: Flag (Sigma, St Louis, MO, USA), phosphor–Ser52–eIF2α (Cell Signaling (Danvers, MA, USA)) and polyubiquitin Lys48 linkage for immunoprecipitation (clone Apu2; Millipore Billerica, MA, USA). The sources of mouse mAbs to specific proteins are as follows: ubiquitin for western blotting (P4D1; Santa Cruz, Santa Cruz, CA, USA), polyubiquitin for immunocytochemistry (Ubi-1; Thermo Scientific, Waltham, MA, USA), ATF6 (IMGENEX, San Diego, CA, USA), V5 and Myc tags (Invitrogen), Flag tag (M2, Sigma), Flag-Cy3 (Sigma), GRP78/Bip (BD Transduction Lab, Franklin, NJ, USA), S6K (H-9, Santa Cruz), β-actin (Sigma) and CHOP/ GADD153 (MA1–250, Thermo Scientific). The flow cytometry antibody to hycocyanin APC–annexin V used in the present study was from BD Pharmingen (San Diego, CA, USA). Monoclonal anti-glycogen IgM was from O. Baba (Tokyo Medical and Dental University, Tokyo, Japan). Thapsigargin and tunicamycin, enzymes and standard for amyloglucosidase (A7420), amylase (A6814) and glycogen (G0885), and glucose (GO) assay kit were all purchased from Sigma.

Plasmids and RT-PCR

The coding regions of cDNAs of human malin and mouse GS1 (Gys1) were amplified from reverse transcription mRNA of human cord blood cells and C57BL/6J bone marrow cells, respectively, and cloned into a pcDNA vector with Myc, V5 or Flag tag at the N-terminus (malin) or C-terminus (GS1). After sequencing confirmation of the WT malin, all point mutants were made by site-directed mutagenesis using WT malin as a template. Truncated forms of malin were generated by PCR using full-length malin as a template. Plasmids of laforin and its mutants have been described previously [16]. Gene-silenced sequences were: Epm2a, 5-TTCCAGACTGAATGGGAT A-3, and Nhlrc1, 5-ATTCTCTTCTTGTGCTGGA-3. These were cloned into small hairpin RNA lentiviral vectors with enhanced green fluorescent protein (EGFP) as a reporter. The lenti–sh vector was made by substituting the CMV prompter of plenti6–TOPO/V5 (Invitrogen) with the U6–siRNA–PGK–EGFP cassette. Malin and its mutants were cloned into a lenti–EGFP vector that was made by substituting the T7-blasticidin cassette of plenti6–TOPO/V5 with the PGK–EGFP cassette.

Transfection, western blotting and immunoprecipitation

In general, HEK293 and N2A cells were transiently transfected with 0.25 μg plasmid and 0.75 μL Lipofectamine 2000 (Invitrogen) per well on a 24-well plate for 24 h in 0.5 mL Opti-MEM containing 10% fetal bovine serum. To prevent cells from detaching when the culture medium was changed to 0.5 mL Opti-MEM, one-third of the original culture medium was not removed. Overnight-passaged cells grown to ~ 75% confluence were used for transfection. In double or triple transfections, the total DNA plasmids did not exceed 0.375 μg (double) or 0.5 μg (triple) per well in 24-well plates. Transfected cells were lysed with 1% Triton X-100 lysis buffer containing 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 40 mM NaF, 1 mM dithiothreitol and a protease and phosphatase inhibitor cocktail (Sigma). Supernatants of the lysate were used for western blots and resolved on a reducing and heat-denaturing 10% SDS/PAGE gel. Immunoprecipitation was carried out with protein G beads at 4 °C, overnight, with rotation. The supernatant was preincubated with protein G beads for 2 h, and cleared supernatant was used for immunoprecipitation. After washing three times with 1% Triton X-100 lysis buffer, the immunoprecipitates were dissolved in SDS loading buffer and resolved on a 10% SDS/PAGE gel.

PGB isolation and malin ubiquitination

To isolate PGBs from transfected cells, 0.55% NP-40 in Hepes buffer containing 10 mM Hepes, 1 mM EDTA and 1 mM EGTA was used for lysing cells. Nuclei were removed by centrifugation at 1000 ***g*** for 5 min and washed twice with Hepes buffer. The combined supernatants were centrifuged at 8000 ***g*** for 15 min to remove debris and were then centrifuged again at 18 000 ***g*** for 45–60 min to obtain pellets containing insoluble PGBs. To detect malin ubiquitination and the binding of malin to laforin, the pellets were digested with 1 $U \cdot mL^{-1}$ amyloglucosidase and 5 $U \cdot mL^{-1}$ amylase in NaCl/P_i, pH 7.4, at 37 °C for 2 h and then dissolved by 5× 1% Triton X-100 plus 0.02% SDS buffer. The pellet-digesting solution in combination with cytosolic supernatant was centrifuged and resultant supernatant was used for immunoprecipitation in a final solution containing 1% Triton X-100, 0.5% NP-40 and 0.02% SDS.

Immunofluorescence staining

Cells were fixed in cold methanol for 10 min and then permeabilized with 0.3% Triton X-100 in 10 mM Tris/HCl buffer for 30 min. Immunofluorescence staining with the primary antibody was performed overnight in 10 mM Tris/HCl buffer containing 2% BSA at 4 °C. Secondary antibody staining was carried out in 2% BSA Tris/HCl buffer at room temperature for 2 h.

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