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Novel glycogen synthase kinase 3 and ubiquitination pathways in progressive myoclonus epilepsy

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Lafora progressive myoclonus epilepsy, caused by defective laforin or malin, insidiously present in normal teenagers with cognitive decline, followed by rapidly intractable epilepsy, dementia and death. Pathology reveals neurodegeneration with neurofibrillary tangle formation and Lafora bodies (LBs). LBs are deposits of starch-like polyglucosans, insufficiently branched and hence insoluble glycogen molecules resulting from glycogen synthase (GS) overactivity relative to glycogen branching enzyme activity. We previously made the unexpected observation that laforin, in the absence of which polyglucosans accumulate, specifically binds polyglucosans. This suggested that laforin's role is to detect polyglucosan appearances during glycogen synthesis and to initiate mechanisms to downregulate GS. Glycogen synthase kinase 3 (GSK3) is the principal inhibitor of GS. Dephosphorylation of GSK3 at Ser 9 activates GSK3 to inhibit GS through phosphorylation at multiple sites. Glucose-6-phosphate is a potent allosteric activator of GS. Glucose-6-phosphate levels are high when the amount of glucose increases and its activation of GS overrides any phospho-inhibition. Here, we show that laforin is a GSK3 Ser 9 phosphatase, and therefore capable of inactivating GS through GSK3. We also show that laforin interacts with malin and that malin is an E3 ubiquitin ligase that binds GS. We propose that laforin, in response to appearance of polyglucosans, directs two negative feedback pathways: polyglucosan–laforin–GSK3–GS to inhibit GS activity and polyglucosan–laforin–malin–GS to remove GS through proteasomal degradation.

INTRODUCTION

Lafora disease (LD) is an autosomal recessive epilepsy that attacks mainly normal teenagers with progressively worsening seizures and dementia, culminating in continuous seizures, a protracted vegetative state, and death within 10 years (1,2). In retrospect, most families report an insidious cognitive decline over some months prior to epilepsy onset. At autopsy, neuronal degeneration is seen (3–5), and in addition, neurons, myocytes and hepatocytes are found occupied by conspicuous polyglucosan masses known as Lafora bodies (LBs). Polyglucosans are malformed glycogen molecules lacking the symmetric branching that allows correctly constructed glycogen to suspend in the cytoplasm. They are thus akin to starch, and like starch, they precipitate in the cell (1,2). LD is caused by mutations in the *EPM2A* gene encoding laforin, a protein comprised a carbohydrate-binding domain (CBD) and a dual-specificity phosphatase (DSP) domain (6–10), or in the *EPM2B* gene encoding malin, a putative E3 ubiquitin ligase (11). Also there is at least one additional gene yet to be identified (12). Here, we explore the roles of laforin and malin in LB formation.

In LD, LBs occur in brain in four conditions, with differences in the locations of the LB. In LD, LBs are exclusively found in neuronal perikarya and dendrites (2). In glycogen branching enzyme deficiency disease (GBED), they are found in axons (GBED patients have dementia but not epilepsy) (13,14). During normal aging, numerous LBs called corpora amylacea are found in astrocytes 15), with greater numbers in patients with Alzheimer's disease (16). Finally,

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florid astrocytic corpora amylacea are also present in occasional temporal lobes resected at epilepsy surgery (17,18).

Normal glycogen is fashioned through coordinated action of glycogen synthase (GS) and glycogen branching enzyme (BE). The process initiates with GS adding glucose units to a glycogenin-bound oligosaccharide. As GS elongates the saccharide, BE removes every six- to seven-residue oligomer formed and re-attaches it inside the chain, creating a new end for GS to extend. The process resumes and continues at each end, expanding the molecule in a globular fashion (19). In GBED, LBs form because the GS/BE balance is disturbed in favor of GS (14). This mechanism also explains the LB formation in skeletal muscle in muscle phosphofructokinase deficiency, where glucose-6-phosphate (G6P), the allosteric activator of GS (19,20), accumulates and overdrives GS, and in the transgenic mice, it overexpresses GS in muscle (21). It is also the leading theory for the generation of LB in LD, as discussed subsequently.

Laforin preferentially binds starch over glycogen (10), suggesting that its function begins after the appearance of excessively long, improperly branched strands. It likely serves a quality control role detecting such strands and counteracting their further formation. GS is activated by dephosphorylation by protein phosphatase 1, which is brought into contact with it by the R5 protein. Laforin binds the same region of R5 as GS and can therefore displace GS from R5 and prevent its activation (22). Could laforin also induce GS inhibition? The main GS inhibitor is glycogen synthase kinase 3 (GSK3) (19). This is a component of a wide variety of cellular pathways including insulin signaling and is dysregulated in several neurodegenerative diseases (23–26). In insulin signaling, Akt kinase inactivates GSK3 by phosphorylation at Ser 9 (numbering according to β isoform) (19,27). In this study, we show that laforin is a GSK3 Ser 9 phosphatase, and thus it is able to activate GSK3 to inhibit GS. We also show that laforin interacts with malin and that malin is an E3 ubiquitin ligase that binds GS. We propose a double-pronged negative feedback pathway whereby laforin, following detection of polyglucosans, inhibits GS through GSK and also removes GS through malin-mediated proteasomal degradation.

RESULTS

Laforin and malin interaction

We first overexpressed full-length laforin and malin in yeast under selective growth media and noted that they interact (Fig. 1A). To further verify the specificity of the interaction under conditions that allow for post-translational changes to the hybrid proteins, we used a mammalian two-hybrid system, in which the interaction is detected by the secretion of alkaline phosphate into the media. Co-expression of malin and laforin recombinants in Chinese hamster ovary (CHO) cells activated the co-transfected secreted alkaline phosphatase (SEAP) reporter gene, demonstrating binding of the two proteins (Fig. 1B). Finally, we performed *in vivo* co-immunoprecipitation binding assays. We expressed hemagglutinin A (HA)-tagged malin and myc-tagged laforin in HEK293 cells. Immunoprecipitation with anti-myc antibody co-precipitated malin and with anti-HA antibody co-precipitated laforin (Fig. 1C) confirming the interaction.

To further map the critical regions involved in the interaction, we constructed several plasmids with different laforin and malin domains (Fig. 1D) and expressed them in yeast. Expression of truncated laforin comprised of exons 1 and 2 or exons 2, 3 and 4 with full-length malin maintained the interaction, but expression of laforin exon 1 or exons 3 and 4 did not. These results indicate that the region encoded by exon 2, between the laforin CBD and the phosphatase domain, is important for the interaction. We also tested a laforin mutant where the catalytic Cys residue in the phosphatase domain is inactivated to Ser (10) and an alternative nuclear isoform of laforin (28), both of which interacted with malin (data not shown).

We then analyzed the binding properties of individual malin domains (Fig. 1D). Malin is comprised a RING finger and an NHL domain including six NHL repeats (11). Expression of the RING finger or the NHL domain alone with full-length laforin disrupted interaction, suggesting that full-length malin is required for proper binding. Finally, we tested and found no interaction between the malin and the laforin interacting protein EPM2AIP1 (29) (data not shown).

Laforin is a GSK3 phosphatase and GSK3 associates with LB

To test whether laforin interacts with GSK3, we used the mammalian two-hybrid system. Co-expression of GSK3 and laforin recombinants in CHO cells led to the activation of the SEAP reporter gene, demonstrating binding of the two proteins (Fig. 2A). Interaction was further confirmed by expressing the proteins in COS7 cells. Immunoprecipitation with anti-GSK3 antibodies co-precipitated laforin (Fig. 2B). To determine whether laforin dephosphorylates GSK3 Ser 9, we transfected GSK3 into NIH3T3 cells and induced Ser 9 phosphorylation by platelet-derived growth factor (PDGF). Phosphorylated GSK3 was then immunoprecipitated, incubated with purified glutathione *S*-transferase (GST)-laforin and tested for phosphorylation at Ser 9 using a Ser 9 specific anti-phospho-GSK3 antibody. As shown in Figure 2C, laforin greatly reduces GSK3β Ser 9 phosphorylation. Similar results were obtained, *in vivo*, by co-expressing GSK3 with laforin in COS7 cells (Fig. 2D).

We previously showed that LBs of malin-deficient LD patients stain strongly positive for laforin (10). If GSK3 is involved in mediating laforin's function, then it might also be present on these LBs. Using immunohistochemistry, we show that the GSK3 antibody intensely stains LB in a malin-deficient patient (Fig. 3A). If this GSK3 presence on LB depends on laforin, then GSK3 should be absent from LB of laforin-deficient LD patients, which we show to be the case (Fig. 3B). We then asked whether GSK3's presence on LB requires laforin molecules with functional phosphatase domains. We addressed this question in our $Epm2a^{cys-ser}$ transgenic mice. These mice overexpress Cys–Ser phosphatase-inactive laforin 100-fold, which competes with wild-type laforin and results in LB, densely populated by the mutant laforin (10). Figure 4 and Table 1 show that GSK3 is greatly depleted from these LBs, indicating that GSK3's LB localization requires phosphatase-active laforin.

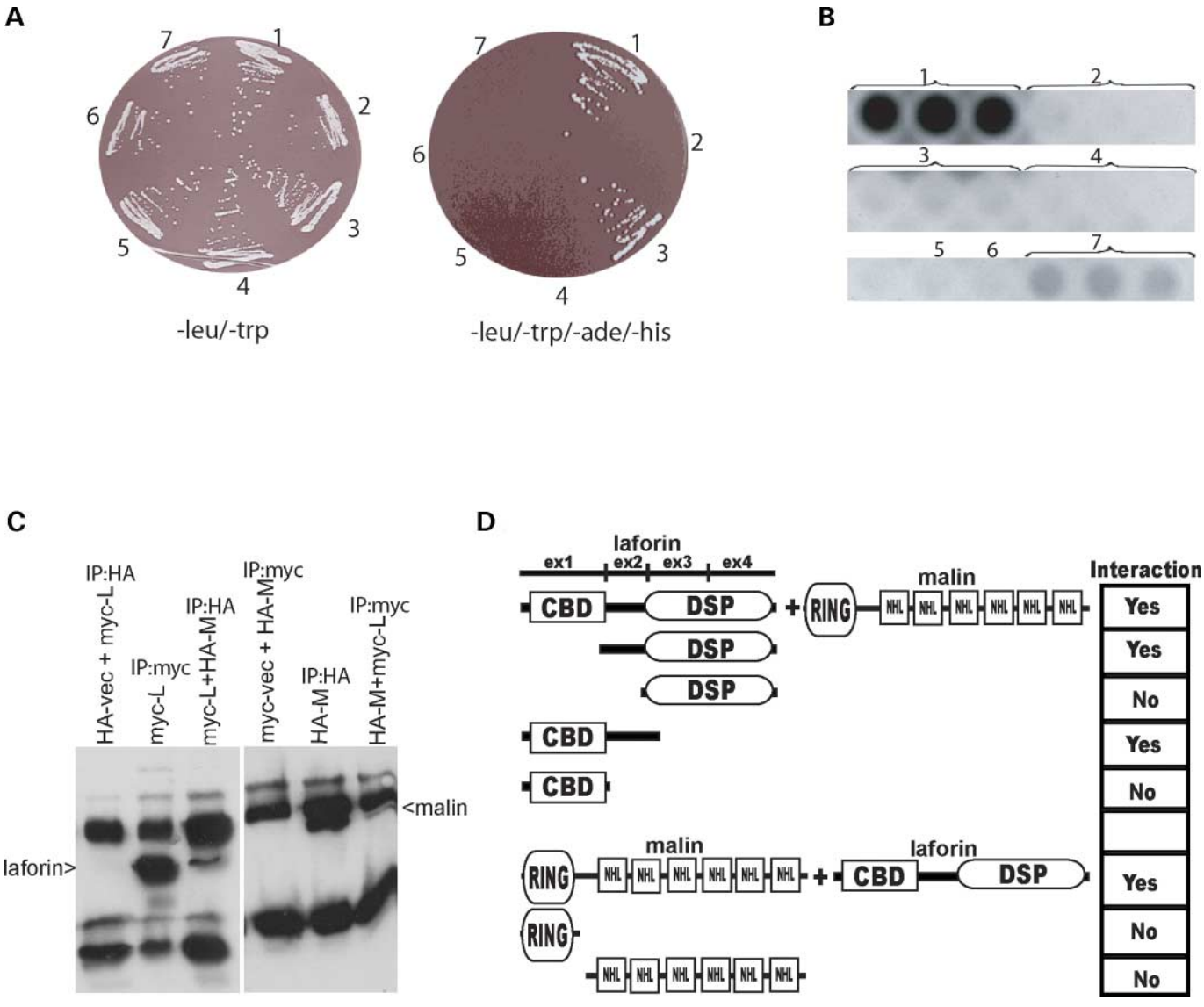

Figure 1. Laforin and malin interact. (**A**) Yeast two-hybrid interaction experiment between full-length laforin and malin (and malin deletion constructs): 1, positive control; 2, negative control; 3, pGBKT7-laforin and PACT2-malin; 4, pGBKT7-laforin and PACT2-malin RING domain; 5, pGBKT7-laforin and PACT2-malin NHL domain; 6, pGBKT7-laforin and PACT2 vector and 7, pGBKT7 vector and PACT2-malin. (**B**) Mammalian two-hybrid experiment with full-length laforin and malin: 1, positive control (pM3-VP16 combined plasmid contains both binding and activator domains); 2, negative control (pM53 and pVP16-CP); 3, pM-laforin and pVP16; 4, pVP16-malin and pM3; 5, untransfected cells; 6, basal control (pM and pVP16) and 7, pM-laforin and pVP16-malin. (**C**) Co-immunoprecipitation of laforin and malin: IP, immunoprecipitation using indicated anti-tag (HA or myc) antibody; L, laforin; M, malin; left, immunoblot stained with myc antibody; right, with HA; vec, vector with indicated tag but no insert. (**D**) Depiction of constructs used in mapping interaction regions in yeast two-hybrid experiments.

Malin is an E3 ubiquitin ligase and binds GS

NHL domains are regions at which protein–protein interaction occurs, but do not shed further light on function. However, presence of the RING finger in malin suggests that it might be an E3 ubiquitin ligase (11). To test this, we expressed full-length malin in COS7 cells, which resulted in a thick diffuse band on western blot, typical of a polyubiquitinated protein, with lower edge at ~50 kDa, the size of malin. Expression of a malin protein containing a mutation in the RING domain, Cys26Ser, known to cause LD (11) produced a single discrete 50 kDa band (Fig. 5A). These results suggested that malin self-ubiquitinates. To further confirm this result, we transfected a truncation construct consisting of malin's RING domain alone. Western analysis revealed a distinctive ladder of fragments separated by ~8 kDa, the size of ubiquitin. Introduction of a disease causing mutation, Phe33Ser (11), abolished the ladder producing a single ~15 kDa band, the size of the RING (Fig. 5B). Finally, co-expression of malin with ubiquitin reproduced the thick diffuse band on immunoblots, which was again absent in the presence of the RING mutant (Fig. 5C and D). Collectively,

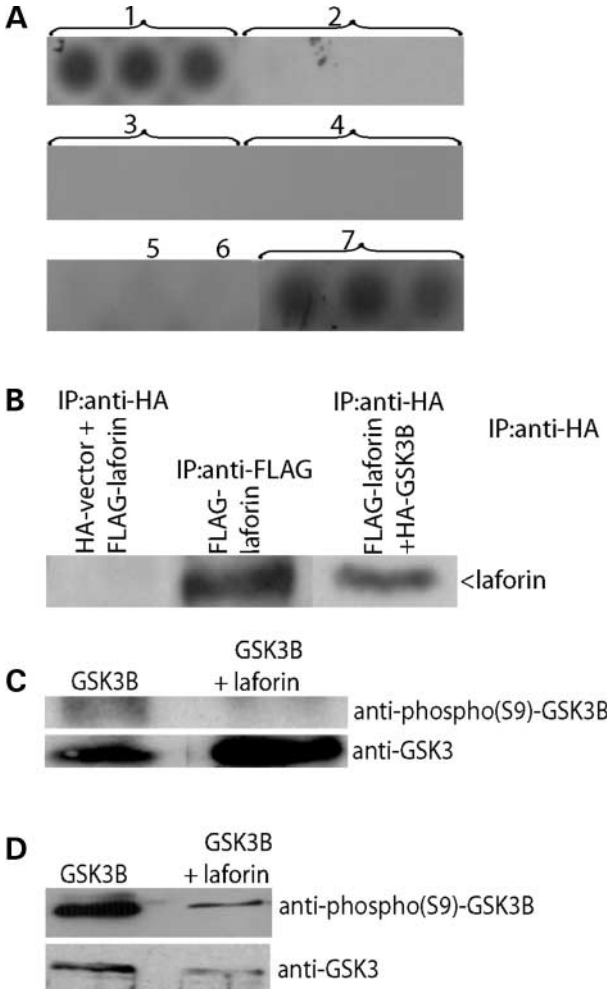

Figure 2. Laforin is a GSK3 phosphatase. (**A**) Mammalian two-hybrid experiment with full-length laforin and GSK3β: 1, positive control (pM3-VP16 combined plasmid contains both binding and activator domains); 2, negative control (pM53 and pVP16-CP); 3, pM-laforin and pVP16; 4, pVP16-GSK3β and pM3; 5, untransfected cells; 6, basal control (pM and pVP16) and 7, pM-laforin and pVP16-GSK3β. (**B**) Co-immunoprecipitation of laforin and GSK3β: IP, immunoprecipitation using indicated anti-tag (HA or FLAG) antibody; immunoblots then stained with FLAG antibody. Laforin dephosphorylates GSK3β Ser 9 *in vitro* (**C**) and *in vivo* (**D**): in the presence of laforin (right column), the Ser 9 phosphorylated GSK3β portion (top row) of total GSK3β (bottom row) is reduced.

these results demonstrate that malin's RING domain is an E3 ubiquitin ligase. They also show that at least two disease mutations inactivate malin's ubiquitination function.

Most E3 ubiquitin ligases self-ubiquitinate under experimental conditions, as described earlier (30–32). Physiologically, however, their role commonly is to ubiquitinate specific interacting partners. Ubiquitination serves one of the two functions, direction of the ubiquitinated protein to the proteasome for degradation (31) or activity regulation of the targeted protein (32). Malin's function, as an LD gene product, is to prevent polyglucosan formation. If it is to accomplish this through removal or regulation of a specific protein, a candidate target would be GS, the polyglucosan-synthesizing enzyme.

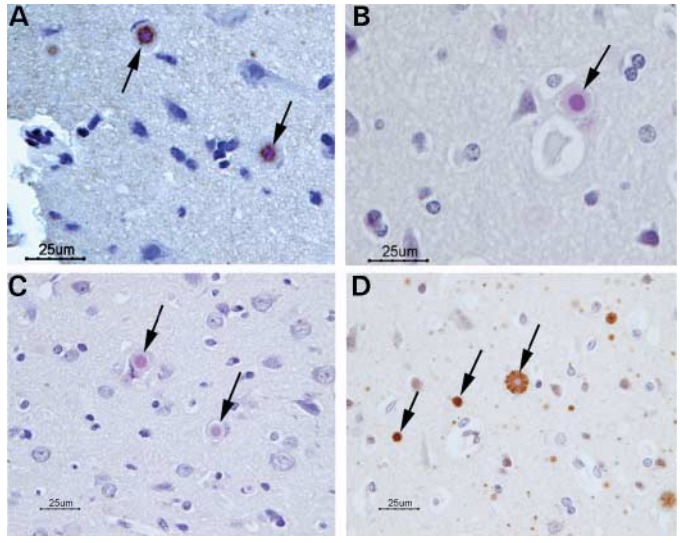

Figure 3. GSK3 localizes on malin-deficient LB and ubiquitin is present on laforin-deficient LB. (**A**) Anti-GSK3 stained section from brain of a malin-deficient LD patient (homozygous *EPM2B* F33S[11]); note intense labeling of LB (arrows). (**B**) Same stain in a laforin-deficient LD patient (homozygous *EPM2A* 800insA[7]); note absence of LB staining. (**C**) Anti-ubiquitin stained section from patient in (A); her LBs are not ubiquitinated. (**D**) Anti-ubiquitin from patient in (B); her LBs are strongly ubiquitinated.

To determine whether malin binds GS, we incubated lysates of HA-malin-expressing cells with purified GS and separately immunoprecipitated each protein. Immunoprecipitation of HA-malin co-precipitated GS and of GS co-precipitated malin, demonstrating that the two proteins interact (Fig. 5E). Whether malin ubiquitinates GS and whether the outcome of this ubiquitination is destruction or regulation of GS are currently under investigation.

Malin is required for ubiquitin positivity of LB

Corpora amylacea strongly react to ubiquitin antibodies (15). LBs in the laforin-deficient mouse are likewise ubiquitin positive (5). It is thought that ubiquitin positivity of CA and LB is due to non-specific ubiquitinated but undegraded proteins trapped within them (5,15). We tested an alternative hypothesis, namely, LB ubiquitin positivity is specific and dependent on the presence of malin. We show that LBs in human patients and animal models with LD because of malin deficiency are devoid of ubiquitin, whereas LBs in human patients and animals models with LD because of laforin deficiency are ubiquitinated (Figs 3C, D and 6; Table 2). Which protein(s) is ubiquitinated on LB in the presence of malin is unknown, but may be GS.

DISCUSSION

We present experimental evidence demonstrating that the two known LD-associated proteins, laforin and malin, interact and function in the same biological pathway. The subneuronal localization of malin is yet to be determined, but laforin localizes in neuronal perikarya and dendrites (10). In the absence

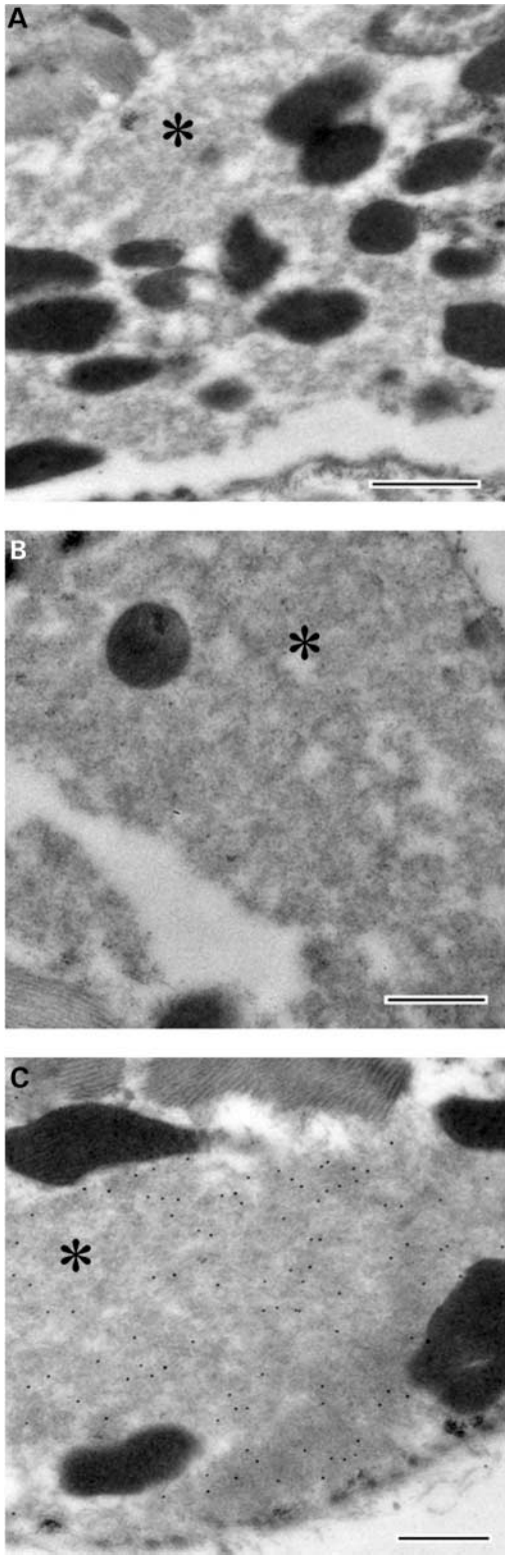

Figure 4. GSK3 localization on LB requires presence of phosphatase-active laforin (immunogold electron microscopy on skeletal muscle sections; primary antibody is anti-GSK3; asterisks indicate LB; all bars equal 0.5 μm). (**A**) Epm2a$^{cys-ser}$ transgenic mouse model of LD (10); note absence of labeling. (**B**) Laforin-deficient LD patient (homozygous *EPM2A* 800insA[7]); note absence of labeling. (**C**) Malin-deficient canine model of LD (48); note intense labeling.

Table 1. GSK3 numbers (gold attached to secondary antibody) per LB surface area

Genotype	Particles/μm^2
Functional laforin-deficient Epm2a$^{cys-ser}$ mouse (10)	2.6 ± 1.2
Laforin-deficient human (*EPM2A* 800insA[7])	1.2 ± 0.9
Malin-deficient dog (48)	36 ± 6.8

Tissue is skeletal muscle; counts are expressed as a mean in 50 LBs ± standard error; $P > 0.001$.

of either laforin or malin, large spherical LBs form in neuronal perikarya, as shown in Figure 4, but smaller LBs form in countless dendrites, including at synapses where they often completely occupy the post-synaptic space (2,10,15). Their sheer numbers and extent of synaptic invasion alone suggest that LBs almost certainly contribute to neurologic symptoms in LD. The fact that the full N-terminal third of laforin is a polysaccharide-binding domain (7,10,33–36) was an important first step in understanding the genesis of LB. This domain belongs to the CBM20 family of evolutionarily conserved starch-binding proteins (http://afmb.cnrs-mrs.fr/cazy/) (7,34), and its binding preference has been confirmed to be for starch (polyglucosan) rather than for glycogen (10,36). This suggests that polyglucosans do form normally in cells. Their accumulation being clearly harmful, laforin's role likely involves their detection and initiation of measures to counter their further formation. The only known process by which polysaccharides of any kind are made in mammalian cells is glycogen synthesis, and polyglucosans most likely are byproducts of glycogen synthesis. Therefore, to understand the role of laforin in regulating polyglucosan formation, we sought links between the laforin and the glycogen synthesis machinery.

We show that laforin is a GSK3 phosphatase. GSK3 is an intermediary of the insulin pathway (19,23). In the absence of insulin, it is active, directly inhibiting GS, but also driving, through second messengers, transcriptional upregulation of glucose-6-phosphatase (G6Pase) (37,38), the enzyme that converts G6P to glucose for secretion [neurons do possess G6Pase, compartmentalized, like laforin and LB, in the soma and dendrites (39,40)]. With insulin, GSK3 is inhibited, activating GS and decreasing G6Pase, the latter leading to increased intracellular G6P and strong further allosteric activation of GS (19,20). Laforin's phosphatase action on GSK3 Ser 9 would activate GSK3 to inhibit GS and to increase G6Pase and decrease G6P and its allosteric effect on GS. BE would not be affected, as it is not subjected to GSK3 phosphoregulation or G6P activation (19). The laforin effect would, therefore, move the GS/BE balance away from polyglucosan synthesis.

In the absence of laforin, polyglucosans form and precipitate into LB. Both laforin and GSK3 are absent from these LBs. However, when LBs form because of malin deficiency, laforin densely populates them (10), and so does GSK3. These observations confirm the involvement of GSK3 in LB biology and the dependence of this involvement on the presence of laforin.

GSK3 is employed in a variety of cellular functions unrelated to glycogen metabolism (23), and therefore, laforin's

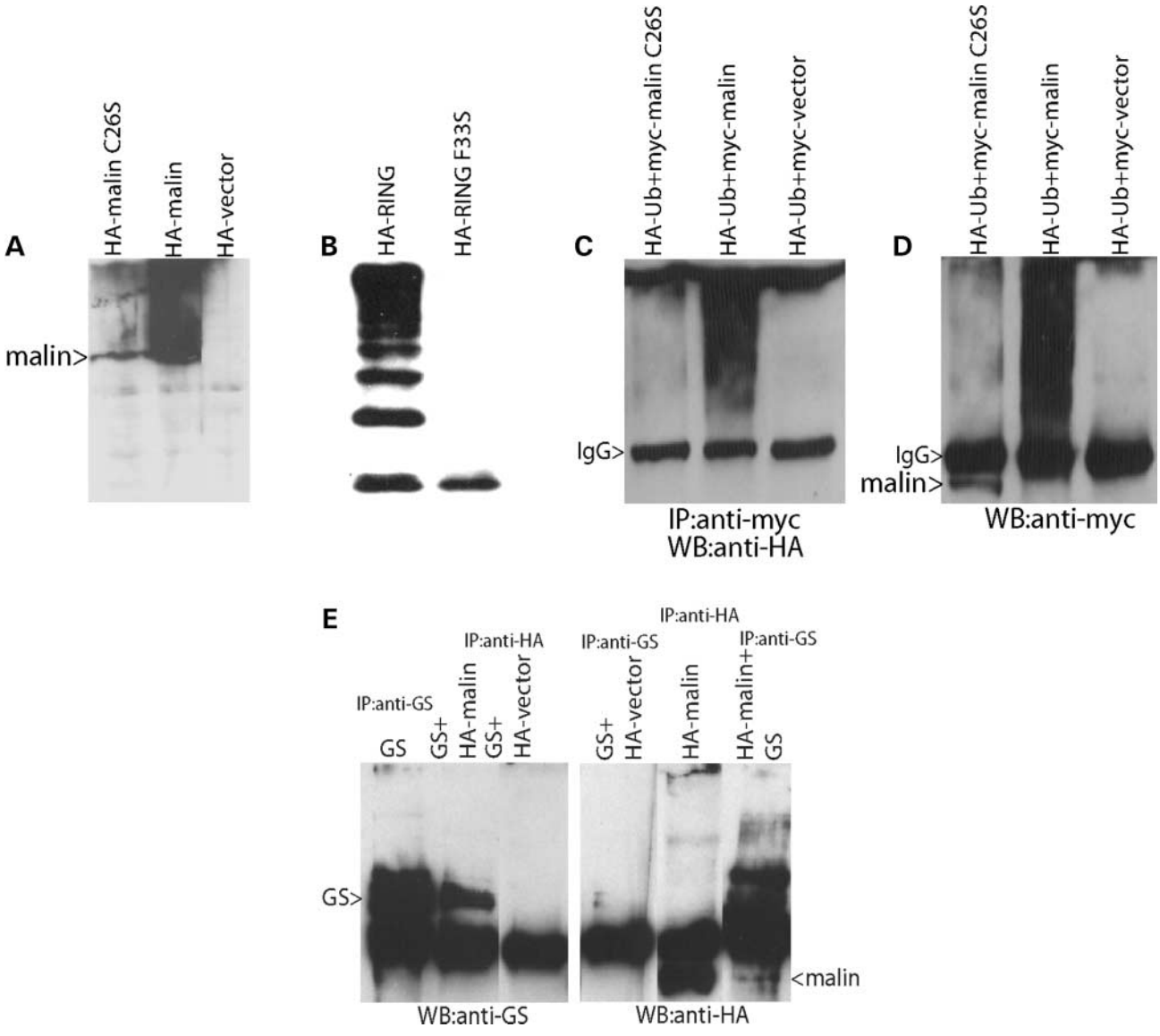

Figure 5. Malin is an E3 ubiquitin ligase and interacts with GS. (**A**) Expression of HA-tagged malin results in a smear of sizes, typical of self-polyubiquitinating proteins, which are eliminated by the C26S LD causing mutation. (**B**) Expression of HA-tagged malin RING domain (amino acids 1–91) produces bands separated by ~8 kDa, the size of ubiquitin, which are eliminated by the F33S LD mutation. (**C**) Co-expression of myc-tagged malin with HA-tagged ubiquitin again results in a smear of sizes eliminated by the C26S mutant; IP, immunoprecipitation; WB, western blot. (**D**) Same membrane stained with anti-myc shows the presence of the malin band (lane 1), confirming that the absence of ubiquitination with the C26S mutant is not due to the lack of protein expression; the malin band is not seen in lane 2, because all of malin is ubiquitinated (note strong smear). (**E**) Co-immunoprecipitation of HA-malin and GS; IP, immunoprecipitation using indicated antibody; left, immunoblot stained with GS antibody; right, immunoblot with HA antibody; note: anti-HA detects additional higher molecular weight bands, likely representing ubiquitinated malin.

utilization and regulation of GSK3 are likely normally restricted to sites of glycogen metabolism so as not to affect other GSK3 activities. However, spillover effects of laforin deficiency on GSK3 activities in other systems cannot be ruled out. This possibility is important in the light of observations in the *Epm2a* (laforin gene) knockout mouse. This animal allowed pre-symptomatic pathological analyses and revealed neurodegenerative changes (5), including neurofibrillary tangle-like formations (41), prior to the appearance of LB. This suggested that LD, in addition to being a glycogen metabolic disorder, is also a primary neurodegenerative disease. GSK3 is tau kinase, the principal phosphoregulator of microtubular tau (42) and kinesin light chains (KLCs) (43). GSK3 dysregulation would disturb tau and KLC phosphorylation and disrupt microtubular stability and cellular transport, both early deficits in Alzheimer's disease (AD) (44) and precursors of neurofibrillary tangle formation in AD, tauopathies and other neurodegenerative diseases (45).

Allosteric activation of GS by G6P is extremely potent and overrides any phospho-inactivation of GS, including GSK3 (19). When the amount of glucose increases, laforin-mediated GS downregulation may therefore require greater inhibition than that provided by phosphorylation or may necessitate removal of GS. We show in this article that laforin interacts

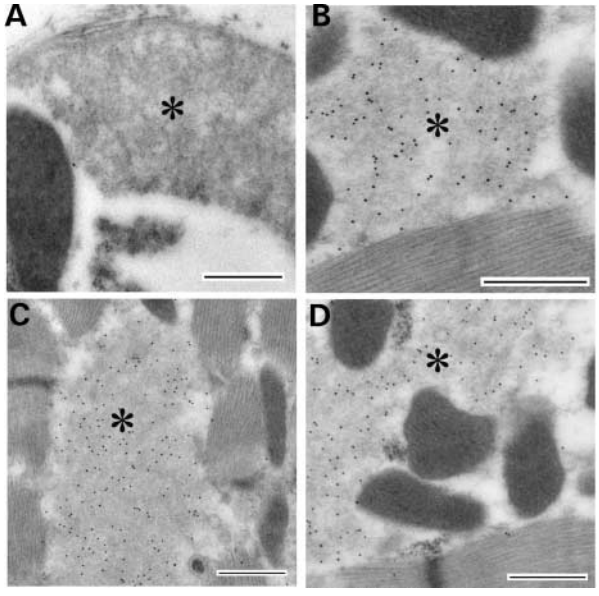

Figure 6. Malin is required for LB ubiquitination (immunogold electron microscopy on skeletal muscle sections; primary antibody is anti-ubiquitin; asterisks indicate LB; bars equal 0.5 μm). (**A**) Malin-deficient dog (48); note absence of labeling. (**B**) Unpublished canine model of LD with genetic linkage to neither *Epm2a* nor *Epm2b*; likely represents canine equivalent of LD with mutations in the third yet unidentified gene (12); note intense labeling. (**C**) Laforin-deficient LD patient (homozygous *EPM2A* 800insA[7]). (**D**) Epm2a$^{cys-ser}$ transgenic mouse (7).

with malin and that malin is an E3 ubiquitin ligase that binds GS. We theorize that malin strongly inhibits GS or directs GS to proteasomal degradation. This hypothesis remains to be tested. In the absence of laforin, malin still appears to be active, as evidenced by the LB in laforin-deficient patients being ubiquitinated. However, without laforin, malin's action would no longer be coordinated with polyglucosan appearance and its function in regulating GS may be futile once polyglucosans have already formed and precipitated into LB.

Our results reveal the following molecular connections: polyglucosan–laforin–GSK3–GS and polyglucosan–laforin–malin–GS. They suggest a double-pronged negative feedback pathway, connecting polyglucosans to polyglucosan downregulation. Disruption of the arm including malin appears to be of greater clinical consequence (malin mutations are sufficient to cause LD). This evokes a potential therapeutic possibility. If this arm of the feedback pathway is indeed needed only when the amount of glucose increases, then it might be obviated with glucose deficiency, as can be induced with the ketogenic diet (46).

While our article was in review, Gentry *et al.* (47) showed, using very similar yeast two-hybrid experiments, that laforin and malin interact. Interestingly, they concluded that malin's NHL domain is sufficient for the interaction. Their malin NHL construct starts at Cys-78, whereas our construct starts at His-118, which suggests that the intervening 40 amino acids are important for the interaction. In contrast, they also concluded that full-length laforin is required for the two proteins to interact (47). However, their laforin deletion constructs do not include the 40 amino acids between Gly-120 and Leu-161, 38 of which are encoded by exon 2, which we mentioned earlier to be important in the interaction (Fig. 1D). In conclusion, laforin and malin appear to interact through their middle portions. In malin, this is the region between the RING finger and the NHL domains, and in laforin, the stretch between the CBD and the DSP domain.

Table 2. Ubiquitin numbers (gold attached to secondary antibody) per LB surface area

Genotype	Particles/μm^2
Malin-deficient dog (48)	1.7 ± 0.6
Non-malin nor laforin-deficient dog (unpublished data)	44 ± 9.4
Laforin-deficient human (*EPM2A* 800insA[7])	37 ± 9.3
Functional laforin-deficient Epm2a$^{cys-ser}$ mouse (10)	46 ± 5.3

Tissue is skeletal muscle; counts are expressed as a mean in 50 LBs ± standard error; $P > 0.001$.

Gentry *et al.* (47) also showed that malin polyubiquitinates and degrades laforin. Noting the apparent conflict of this result with the genetics of LD, they discussed two interesting theoretical scenarios that might resolve the conflict, both involving existence of an unknown protein 'X' onto which malin ubiquitination and/or laforin phosphorylation need to act. If GS is the protein 'X' onto which malin and laforin act (the latter indirectly through GSK3), then it would appear to make sense that having destroyed GS, malin would also destroy laforin (and perhaps GSK3; interestingly, in this respect, LBs in malin-deficient LD are highly positive in both laforin and GSK3). Otherwise, GSK3 would remain activated with possible negative consequences on the multiple cellular pathways it subserves. In any case, this all remains theoretical, until malin's effect on GS is experimentally verified.

MATERIALS AND METHODS

Yeast two-hybrid analysis

The GAL4-based system was used according to manufacturer's instructions (Clontech). Full-length laforin (and de letion or mutation construct) and malin (and deletion construct) cDNAs were cloned into pGBKT7 and pACT2 plasmids, respectively, co-transfected into yeast strain YH109 and grown under selection in media lacking leucine, tryptophan, adenine and histidine amino acids. Plates were incubated at 30°C for 5–7 days before colony counting. Possible interaction of malin with EPM2AIP1 (NM_014805) was studied in the same way. Protein expressions were verified in yeast lysates by western blotting to avoid misinterpretations due to possible protein instabilities especially with truncated constructs.

Mammalian two-hybrid analysis

The BD Matchmaker Mammalian Assay Kit 2 was used according to manufacturer's instructions (BD Biosciences). Full-length laforin was cloned into the pM vector as a fusion with the GAL4 DNA-binding protein, and full-length malin or GSK3β was inserted into the pVP16 plasmid as a fusion with the VP16 activation domain. The SEAP reporter gene was in pG5SEAP. All three were co-transfected into

CHO cells. After 48 h, interaction was assayed by measuring SEAP expression using the Great EscAPe SEAP Chemiluminescence Detection Kit (BD Biosciences). The specificity of the system was verified using several positive, negative and control plasmids included in the kit. Fusion protein expressions were verified using western blots of transfected cell lysates.

Immunoprecipitation assays

HA-tagged malin inserted into the pHM6 plasmid and myc-tagged laforin in pcDNA3.1 were co-expressed in HEK293 cells for 48 h. Cells were then treated with 1 mM sodium vanadate before lysing in lysis buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1% TritonX-100). Overnight immunoprecipitations at 4°C were performed using mouse monoclonal anti-HA or anti-myc antibodies (Santa Cruz). Co-precipitated malin or laforin was then detected by immunoblotting using corresponding tag antibodies. Interaction between laforin and GSK3β was studied by co-transfecting human HA-tagged GSK3β (pcDNA3.1HA-GSK3B) and FLAG-tagged laforin (pcDNA3.1FLAG-laforin) into COS7 cells. After 48 h, cells were lysed and HA antibody was used to pull-down the GSK3β–laforin complex. Co-precipitated laforin was detected by immunoblotting using anti-FLAG antibody (Sigma). Interaction between malin and GS was studied by expressing HA-tagged malin (pHM6HA-malin) in COS7 cells and incubating the cell lysate with purified GS from rabbit skeletal muscle (Sigma) for 1 h at room temperature. The lysate was then subjected to immunoprecipitation using mouse monoclonal anti-GS antibody (MAB3106, Santa Cruz) and anti-HA antibodies as described earlier. Co-precipitated GS and malin were then detected using corresponding antibodies.

Dephosphorylation assay

In vitro: NIH3T3 cells were transiently transfected with mouse GSK3β cloned into the pcDNA3.1 plasmid. Two days after transfection, cells were treated with 50 ng/ml of PDGF (Sigma) for 30 min to enhance phosphorylation of the Ser 9 of GSK3β. Cells were then lysed in lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP-40, 5 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate], and GSK3β was immunoprecipitated using rabbit anti-GSK3 antibody [Santa Cruz, GSK3β(H-76): sc-9166]. GSK3β was then bound to protein G beads and washed. Dephosphorylation was performed by adding 500 ng of purified recombinant GST–laforin in dephosphorylation buffer (50 mM imidazole, 0.1% β-mercaptoethanol) to the beads and incubating at 30°C for 15 min. GST–laforin fusion protein was produced previously (10). Dephosphorylation activity of laforin was analyzed by immunoblotting and using mouse monoclonal anti-phospho-GSK3β antibody against Ser 9 (cell signaling). Dephosphorylation results were normalized against total GSK3β protein using the rabbit anti-GSK3 antibody in the same membranes. *In vivo* dephosphorylation of GSK3β Ser 9 was studied by expressing pVP16-GSK3β alone or with pM-laforin in COS7 cells and inducing phosphorylation with PDGF. Phosphorylation status was analyzed from immunoblots as described earlier.

Immunohistochemistry and immunogold electron microscopy

Immunohistochemistry was performed on microwave antigen-retrieved paraffin sections, which were incubated with the aforementioned GSK3 antibody or an anti-ubiquitin antibody [Santa Cruz, Ub(P4D1): sc-8017] for 1 h and immunostained using an ABC staining kit (Dako) and counterstained with hemotoxylin prior to light microscopic examination.

For immunogold electron microscopy, tissues were fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, or 2.5% glutaraldehyde in phosphate buffer and minced into millimeter cube pieces for >4 h. Tissues fixed in glutaraldehyde were rinsed in buffer, post-fixed in 2% aqueous OsO_4, dehydrated in an ascending series of ethanols and infiltrated, embedded and polymerized in epon araldite. Thick sections were cut and stained with toluidine blue to identify LB containing blocks. Ultrathin sections were then cut, mounted on grids and stained with ethanolic uranyl acetate and lead citrate prior to examination in the transmission electron microscope (TEM). Tissues fixed in paraformaldehyde were infused with 2.3 M sucrose overnight, frozen in liquid nitrogen, substituted at −85°C for 48 h in absolute methanol containing 2% uranyl acetate, rinsed in cooled methanol, infiltrated in lowicryl HM20 resin overnight at −20°C, embedded and cold polymerized using ultraviolet light. Ultrathin sections of LB containing blocks were cut and mounted on formvar-coated nickel grids, which were immunogold labeled with either the GSK3 or ubiquitin antibodies (Santa Cruz). Briefly, sections were blocked with phosphate-buffered saline containing 0.5% bovine serum albumin prior to incubation with antibody for 1 h. Following a thorough rinse, sections were incubated in 10 nm gold complexes attached to either goat anti-rabbit IgG or goat anti-mouse IgG. Grids were thoroughly washed prior to staining in uranyl acetate and lead citrate, examined in the TEM and images are captured using a CCD digital camera. Labeling density was determined using an image analysis program (SCION Image analysis) to determine the projected areas of each LB. Gold particles within the LB were manually counted and particle density for each LB was determined. A minimum of 50 LBs were analyzed from each group. Data were expressed as mean ± standard error, and a Student's *t*-test was carried out to determine the significance.

Ubiquitination assays

Constructs described in Figure 5 were cloned into the pHM6 plasmid and expressed in COS7 cells for 2 days. Cells were then lysed and pellet fractions were analyzed by western blot using anti-HA antibody. *In vivo* self-ubiquitination of malin was studied by co-expressing myc-tagged wild-type or mutant (Phe33Ser) malin with HA-tagged ubiquitin in COS7 cells for 48 h. Cells were lysed and malin was immunoprecipitated using anti-myc antibodies. Ubiquitination of malin was detected by staining the immunoblots with anti-HA. Myc-vector (pcDNA3)-transfected cells were used as a control. Protein expression was verified by staining the same immunoblot with anti-myc.

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Conflict of Interest statement. The authors declare that they have no conflicts of interests.

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