

Laforin, the most common protein mutated in Lafora disease, regulates autophagy

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Lafora disease (LD) is an autosomal recessive, progressive myoclonus epilepsy, which is characterized by the accumulation of polyglucosan inclusion bodies, called Lafora bodies, in the cytoplasm of cells in the central nervous system and in many other organs. However, it is unclear at the moment whether Lafora bodies are the cause of the disease, or whether they are secondary consequences of a primary metabolic alteration. Here we describe that the major genetic lesion that causes LD, loss-of-function of the protein laforin, impairs autophagy. This phenomenon is confirmed in cell lines from human patients, mouse embryonic fibroblasts from laforin knockout mice and in tissues from such mice. Conversely, laforin expression stimulates autophagy. Laforin regulates autophagy via the mammalian target of rapamycin kinase-dependent pathway. The changes in autophagy mediated by laforin regulate the accumulation of diverse autophagy substrates and would be predicted to impact on the Lafora body accumulation and the cell stress seen in this disease that may eventually contribute to cell death.

INTRODUCTION

Lafora disease (LD) is an autosomal recessive, progressive myoclonus epilepsy that manifests during adolescence with generalized tonic–clonic seizures, myoclonus, absences, drop attacks and visual hallucinations. The disease results in progressive neurodegeneration, and death follows about 10 years after onset (1,2). The pathological hallmark of LD is the accumulation of polyglucosan inclusion bodies, called Lafora bodies, in the cytoplasm of cells in many organs. Lafora bodies contain around 90% of a poorly branched form of glycogen resembling amylopectin and 6% protein (3) and are also decorated by anti-ubiquitin antibodies (4,5).

The extent of Lafora body deposition correlates with neuronal cell death and seizure frequency. Thus, it has been suggested that Lafora bodies may cause the pathology (6), but this has not yet been firmly established.

The great majority (90%) of mutations causing LD have been identified in two genes: *EPM2A*, which encodes laforin, a member of the dual-specificity protein phosphatase family, and *EPM2B*, which codes for malin, a protein with an NH₂-terminal RING finger domain characteristic of an important group of E3-ubiquitin ligases (7–9). There is also evidence for a third minor locus (10). Patients carrying homozygous loss-of-function mutations in laforin or malin are indistinguishable suggesting that both proteins work together in the same

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physiological pathway. However, it is unclear how laforin and malin loss-of-function are related with the disease. Laforin interacts physically with malin and the complex causes specific ubiquitination and proteasome-dependent degradation of proteins involved in the regulation of glycogen biosynthesis (11–15). In addition, laforin acts as a glycogen phosphatase, and it appears that it can suppress excessive glycogen phosphorylation, thereby preventing the formation of the phosphorylated, poorly branched and aggregate-prone glycogen polymers that comprise Lafora bodies (16–19). While LD could be an error of carbohydrate metabolism, it has also been proposed to be a disorder of protein clearance (20).

Here we have considered the possibility that a deficiency in autophagy may be a feature of LD, and that it may contribute to the accumulation of Lafora bodies. Macroautophagy (which we will henceforth call autophagy) is a process that cells use to degrade intracytoplasmic proteins, protein complexes/oligomers, organelles and certain intracellular pathogens (21–24). It is initiated when cells form a double-layered vesicle, an autophagosome, around a portion of cytoplasm. Autophagosomes are trafficked to lysosomes where they ultimately fuse, allowing their contents to be degraded by the lysosomal hydrolases. We tested if autophagy was affected by the laforin mutation for three main reasons. First, autophagy is a crucial route for the removal of disease-associated, intracytoplasmic, aggregate-prone proteins, since it can engulf oligomeric and larger structures that cannot enter the narrow channel of the proteasome barrel (21–24). When autophagy is inhibited, then the clearance of such proteins is retarded, leading to increased aggregate formation. Second, when autophagy is inhibited in otherwise normal mice, then proteins accumulate in inclusions that are decorated by anti-ubiquitin antibodies (25). Third, autophagy is an important route for intracellular glycogen degradation (26,27). Our data in patient cells, laforin knockout mice and in cell culture systems show that laforin is a positive regulator of autophagy, via mammalian target of rapamycin kinase (mTOR), and therefore suggest that autophagy compromise may contribute to the pathology seen in LD.

RESULTS

Lack of laforin inhibits autophagosome formation

To assess macroautophagic activity, we first used the classical autophagosome marker LC3. During the formation of autophagic vacuoles, the cytoplasmic LC3-I isoform is converted by lipidation into LC3-II that has increased mobility in SDS–PAGE. LC3-II is the only known protein that associates specifically with autophagosomes and not with other organelles and is degraded in an autophagy-dependent fashion in lysosomes. Therefore, the levels of LC3-II (relative to actin or tubulin) correlate with the numbers of autophagosomes in the cells. The number of autophagosomes can be altered due to either changes in synthesis or in degradation. If one clamps LC3-II/autophagosome degradation by treating cells with lysosomal inhibitors (28), then changes in the levels of LC3-II will reflect alterations in the rate of LC3-II/autophagosome formation.

To further assess autophagic responses in our experiments, we have cultured cells in serum- and amino acid-free medium (Krebs–Henseleit medium) to stimulate autophagy via the

well-described starvation response (H, for high proteolysis), or in full medium to assess basal autophagy (L, for low proteolysis). Human fibroblasts from LD patients lacking laforin (Supplementary Material, Fig. S1) had lower levels of LC3-II in the presence and absence of the lysosomal inhibitor bafilomycin A₁, compared with control cells (Fig. 1A–C, and see also Supplementary Material, Fig. S2 for comparison with other control fibroblasts). Likewise, mouse embryonic fibroblasts (MEFs) from laforin null mice (*Epm2a*^{-/-}, Supplementary Material, Fig. S1) had lower levels of LC3-II in the presence and absence of the lysosomal inhibitor bafilomycin A₁, compared with control cells, in both full medium and starvation conditions (Fig. 1D–F). Thus, a lack of laforin impairs autophagosome synthesis both in human patient fibroblasts and in MEFs. This is consistent with a reduction in the numbers of autophagic vesicles in the LD patient cell lines (Laf-), as assessed by GFP-LC3 vesicle numbers (Supplementary Material, Fig. S3). Note that LC3-I levels vary between different cell types, as does LC3-II induction in response to starvation. There may be some clonal variability in different cell lines, and that is why we have tested different control and patient human cells lines as well as MEFs. In all cases, we observed a reduction in autophagy in the laforin-defective cells.

Long-lived protein degradation is impaired in LD cells

Under basal conditions, autophagy only degrades a very small proportion of long-lived proteins. However, when cells are starved and autophagy is induced, then this process contributes to a substantial proportion of long-lived protein degradation. Consistent with our LC3 data above, long-lived protein degradation was impaired in LD cell lines, compared with normal fibroblasts, and also in MEFs from laforin null mice, compared with control wild-type MEFs (Fig. 2A and B). This was associated with a defect in autophagic protein degradation, which one can assess by measuring the amount of long-lived protein degradation that is sensitive to the autophagy inhibitor 3-methyladenine. When autophagy is impaired, there is an increase in ubiquitinated proteins (25). Conversely, the levels of ubiquitinated proteins are decreased in normal cells under starvation/high autophagy conditions. Levels of ubiquitinated proteins were elevated in LD fibroblasts, compared with control cells (Fig. 2C and D and Supplementary Material, Fig. S4A and B), and in laforin null MEFs, compared with control MEFs (Supplementary Material, Fig. S5), in both starvation and full media. These differences in the levels of ubiquitinated proteins were still present in cells treated with the proteasome inhibitor MG132 (Fig. 2D and Supplementary Material, Figs S4B and S5B and D), compatible with the observed differences among laforin and wild-type cells being proteasome-independent and likely due to autophagy.

Autophagy is impaired in laforin knock-out mice

In order to test whether laforin regulates autophagy *in vivo*, we have studied laforin knockout mice (*Epm2a*^{-/-}, Supplementary Material, Fig. S1). In these mice, we can assay autophagosome number in the liver and relate this to synthesis by measuring LC3-II in the presence or absence of the lysosomal inhibitor leupeptin. Leupeptin is a cysteine protease

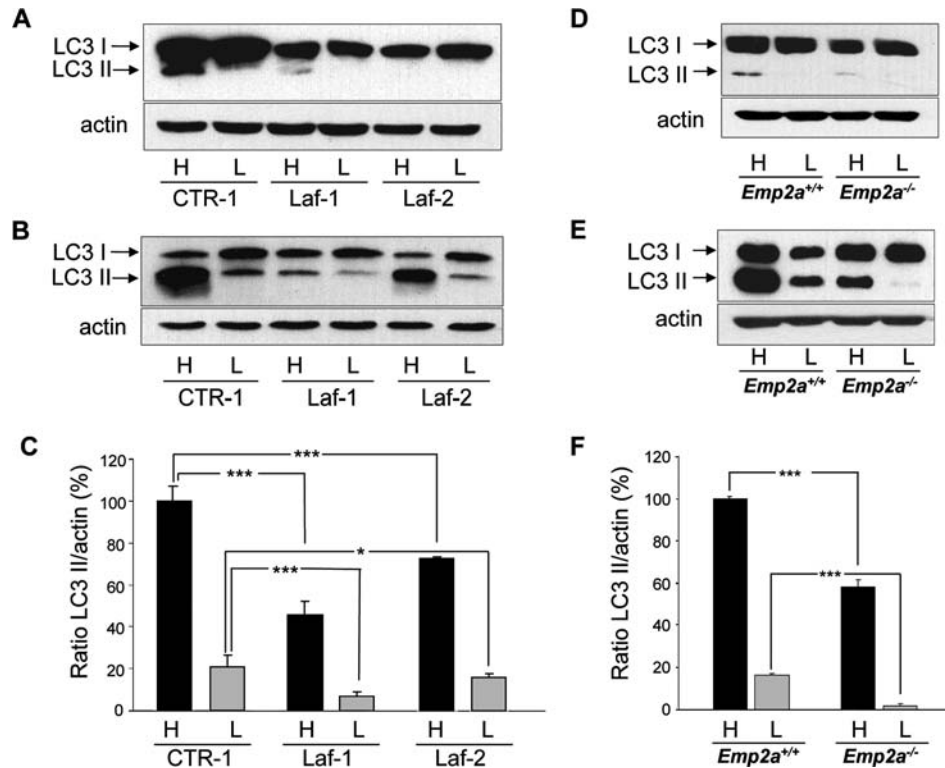


Figure 1. Loss of laforin decreases formation of autophagosomes. (A, B, D, E) Representative immunoblots, using antibodies which recognize LC3 or, as a loading control, actin, with extracts (75 μ g protein) of human fibroblasts from a control individual (CTR-1) and from two different LD patients (Laf-1 and Laf-2) (A and B), or of MEFs from control (*Emp2a*^{+/+}) and laforin-deficient (*Emp2a*^{-/-}) mice (D and E), incubated for 2 h without (A and D) and with (B and E) bafilomycin A1 (400 nM) under conditions of high (H, Krebs-Henseleit medium) and low (L, full medium) proteolysis. The positions of LC3-I and LC3-II bands are indicated on the left. (C and F) The LC3-II bands from three independent experiments similar to those shown in (B) and (E), respectively, were quantified by densitometry and normalized to the corresponding actin bands. Data are expressed in percent relative to control cells.

inhibitor which is probably incorporated into the cells by endocytosis and inhibits important lysosomal cathepsins. Therefore, it can be used as an equivalent to bafilomycin A1 in this context, but we have used leupeptin for the *in vivo* studies as it may have less organismal toxicity. Consistent with our data in the patient cell lines, liver extracts from laforin knockout mice have decreased numbers of autophagosomes that can be attributed to impaired autophagosome synthesis. This is apparent both in mice of 3 and 12 months of age (Fig. 3A and B).

To confirm the observed autophagy impairment, we also assayed the levels of the endogenous autophagy substrate p62, which was increased in laforin-null mice (Fig. 3C and D). Likewise, the levels of ubiquitinated proteins were increased in both fed and starved laforin null mice compared with wild-type controls (Fig. 3E and F and Supplementary Material, Fig. S4C and D). While small differences were seen at 1 month of age and in 12 h starved mice at 3 months of age, these differences became very clear in 3-month-old mice starved for 24 h.

The mTOR pathway is upregulated by laforin deficiency

The mTOR is a major negative regulator of autophagy (29). mTOR can be inhibited, leading to autophagy induction, by starvation (30). Indeed, starvation of both control fibroblasts

and wild-type mice leads to decreased phosphorylation (at the mTOR site) of the mTOR substrate p70S6 kinase (a conventional readout for mTOR kinase activity) (Fig. 4). Laforin deficiency in human fibroblasts (Fig. 4A) and *in vivo* (Fig. 4B) correlated with a marker of increased mTOR activity. This suggested that the autophagy defect in laforin null cells may be mTOR-dependent.

Laforin overexpression induces autophagy

We also assessed whether laforin overexpression could induce autophagosome formation. Consistent with the laforin null data above, overexpression of laforin increased the levels of LC3-II in the presence of bafilomycin A1, suggesting an increase in autophagosome synthesis in both COS-7 and SK-N-SH (neuroblastoma) cell lines (Fig. 5A). Furthermore, laforin overexpression increased EGFP-LC3 vesicle numbers (Fig. 5B).

One way of assessing autophagic substrate clearance is to examine if a perturbation changes the percentage of cells with aggregates after expression of exogenous EGFP-HDQ74, an enhanced green fluorescent protein-tagged version of mutant huntingtin exon 1 with 74 polyglutamine (polyQ) repeats. Mutant huntingtin exon 1, which is associated with Huntington's disease, is an excellent autophagy substrate, and its levels and the percentage of cells with aggregates

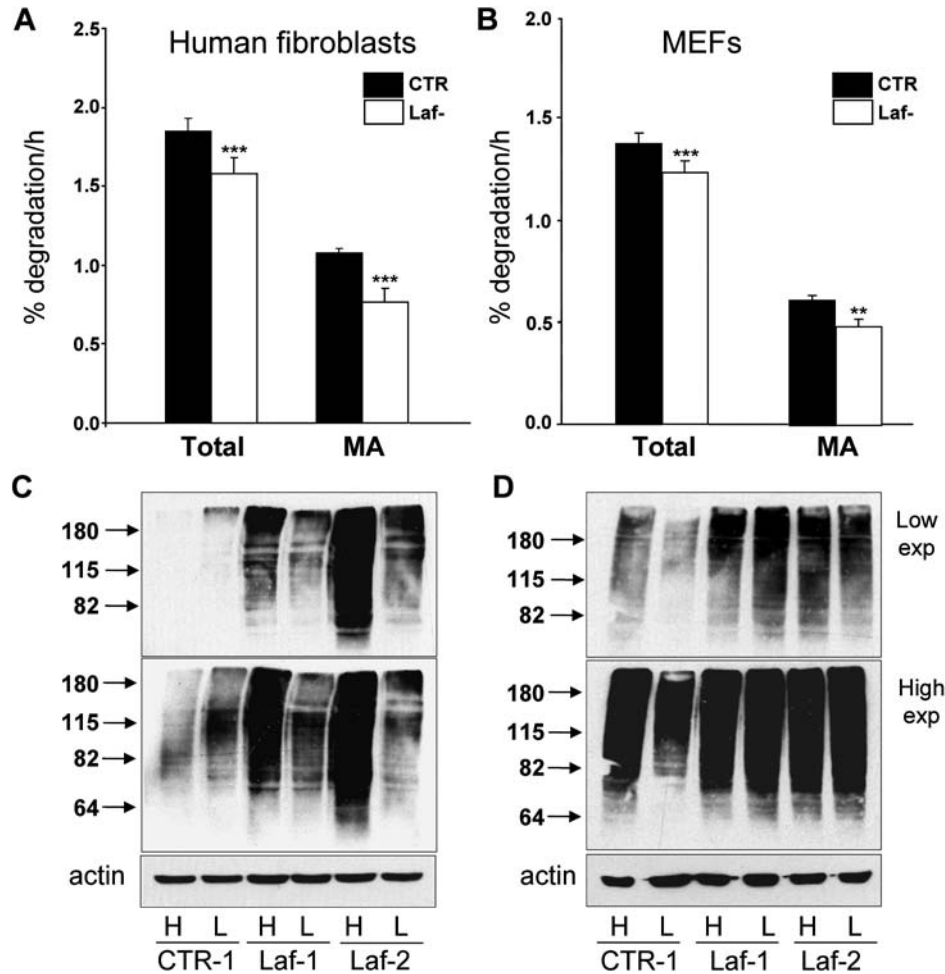


Figure 2. Loss of laforin decreases the degradation of long-lived proteins by macroautophagy and increases accumulation of polyubiquitinated proteins. (**A** and **B**) Intracellular protein degradation (total) and the amount of protein degraded by macroautophagy (MA) was calculated as described in Materials and Methods in control (CTR, black histograms) and laforin-deficient (Laf-, white histograms) human fibroblasts (**A**) and in MEFs from control (*Epm2a*^{+/+}, black histograms) and laforin-deficient (*Epm2a*^{-/-}, white histograms) mice (**B**). Each value represents the mean from three different experiments with duplicated samples, using in each case two different control cell lines and two different laforin-deficient cells. (**C** and **D**) Extracts (75 μ g protein) of fibroblasts from a representative control individual (CTR-1) and from two different LD patients (Laf-1 and Laf-2), incubated for 2 h, without (**C**) or with (**D**) the proteasomal inhibitor MG-132 (20 μ M) under conditions which produce high (H, Krebs–Henseleit medium) and low (L, full medium) proteolysis, were analysed on western blots using antibodies that recognize ubiquitinated proteins and actin, which serves as a loading control. The positions of molecular-mass markers and their size in kDa are indicated on the left. Upper gels: low exposure (exp.), lower gels: high exposure.

increases when autophagy is impaired. Likewise, the percentage of cells with aggregates is reduced when autophagy is induced (31). Laforin overexpression reduced the percentage of cells with mutant huntingtin aggregates, which was also associated with lower cell death (Fig. 5C). This was autophagy-dependent, as laforin only had these effects in autophagy-competent MEFs (*atg5*^{+/+}), but not in *Atg5* null, autophagy-incompetent cells (*atg5*^{-/-}) (Fig. 5D).

Laforin induces autophagy via the mTOR pathway

Consistent with the laforin knockout data above, laforin overexpression decreased mTOR activity, as assessed by phosphorylation of p70S6K (and the levels of phosphorylation of its substrate S6P) (Fig. 6A). To test whether laforin-mediated autophagy was mTOR-dependent, we tested its effects in cells

where mTOR was constitutively activated due to loss of its upstream inhibitor TSC2 (32). While laforin overexpression together with mutant huntingtin exon 1 increased autophagosome numbers (Fig. 6B) and decreased the percentages of cells with polyQ aggregates (Fig. 6C) in wild-type MEFs (*tsc2*^{+/+}), the mitigating effects of laforin overexpression were not seen in TSC2 null MEFs (*tsc2*^{-/-}), which, as expected, had fewer autophagosomes and more aggregates due to impaired autophagy. These data suggest that laforin modulates autophagy either at the level of TSC2 or above this protein.

DISCUSSION

Our data show that a lack of laforin decreases autophagy. This was assessed using a range of assays. In patient cells, MEFs and mice, we observed decreased autophagosome formation

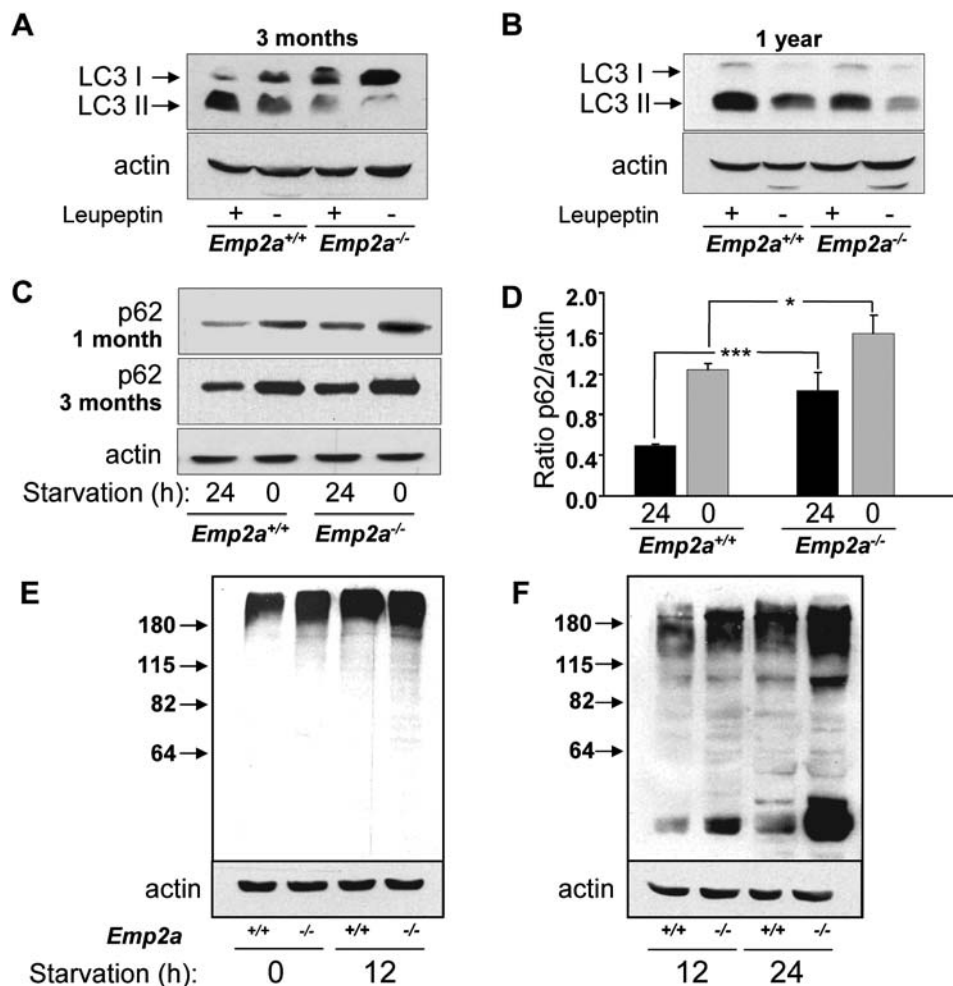


Figure 3. Loss of laforin slows macroautophagy *in vivo*. (A and B) Representative immunoblots using anti-LC3 or anti-actin of mouse liver lysates (100 μ g protein) from control (*Epm2a^{+/+}*) and laforin-deficient (*Epm2a^{-/-}*) mice, 3 months (A) and 1 year (B) old, starved for 24 h and injected (+) or not (-) with 2 mg/100 g weight leupeptin. The positions of LC3-I and LC3-II bands are indicated. (C) Mouse liver lysates (50 μ g protein) of 1-month and 3-month-old mice, starved for 24 h (high proteolysis) or fed (0, low proteolysis), were immunoblotted with p62 and actin antibodies. (D) The bands from four independent experiments (two from each 1-month and 3-month-old mice) similar to those shown in (C) were assessed by densitometry and normalized to the corresponding actin bands. (E and F) Control (*Epm2a^{+/+}*) and laforin-deficient (*Epm2a^{-/-}*) mice, 1 (E) and 3 (F) months old, were fed *ad libitum* (0) or fasted (starvation) for 12 or 24 h. Mouse liver lysates (100 μ g protein) were analysed on western blots using antibodies that recognize ubiquitinated proteins and actin, which serves as a loading control. The positions of molecular-mass markers and their size in kDa are indicated on the left.

when laforin activity was lost, as assessed by LC3-II blotting. This was associated with decreased clearance of long-lived proteins via autophagy and the accumulation of ubiquitinated proteins and the endogenous autophagy substrate, p62. The impaired autophagy associated with the lack of laforin correlated with increased activity of the autophagy inhibitor mTOR. In agreement with these observations, it has been recently shown that laforin-deficient mice have higher levels of the autophagy substrate tau (33) in their brains (34). Consistent with these data, we found that laforin overexpression induced autophagy and enhanced autophagy substrate clearance only in autophagy-competent cells. This was assessed by measuring the formation of autophagosomes by LC3-II western blotting, by immunofluorescence quantification of EGFP-LC3 vesicles (autophagosomes) and by assessing the accumulation of mutant huntingtin exon 1, an autophagy substrate, in wild-type and autophagy-incompetent (*atg5^{-/-}*)

cells. Thus, the ability of laforin to stimulate autophagy is not saturated in cells.

The accumulation of ubiquitinated proteins is a well-described phenomenon that occurs in autophagy-compromised tissues (25). This may occur if some proteins are specifically targeted to autophagy via ubiquitination and subsequent binding to adaptor proteins that link to the autophagic machinery (35,36). However, another reason for the accumulation of ubiquitinated proteins may be because autophagy inhibition results in a secondary reduction in flux through the ubiquitin-proteasome system. This phenomenon is driven by the accumulation of p62, which appears to impair the delivery of ubiquitinated proteins to the proteasome (37). Thus, it is possible that the reduced rates of autophagy in LD may have effects on the other major intracellular proteolytic system, the ubiquitin proteasome pathway.

The effects of laforin on autophagy are mTOR-dependent, as no effects are seen in *tsc2^{-/-}* cells, and it is well known

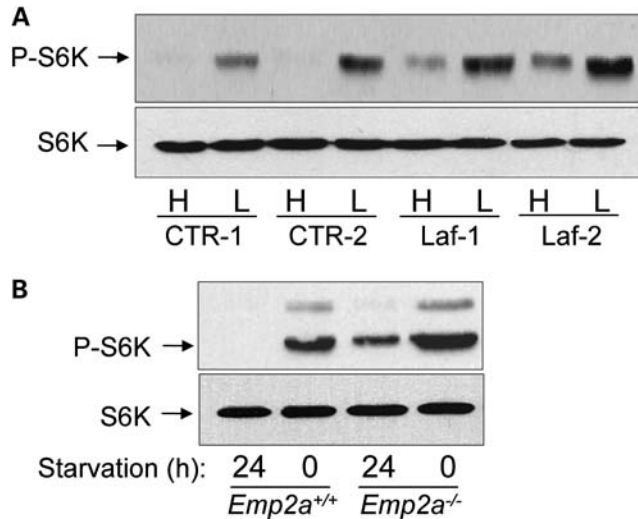


Figure 4. mTOR signalling pathway is upregulated in laforin-deficient cells. (A) Human fibroblasts from two control individuals (CTR-1 and CTR-2) and from two different LD patients (Laf-1 and Laf-2) were incubated for 2 h under conditions of high (H, Krebs–Henseleit medium) and low (L, full medium) proteolysis in the cells. Then, extracts (75 μ g) were prepared to determine the activation state of the down-stream target of mTOR p70S6 kinase (S6K) by immunoblot analysis, using antibodies which recognize phosphorylated Thr³⁸⁹ in p70S6 kinase (P-S6K) and total S6K. (B) mTOR activation was also analysed by the same procedures in liver extracts from 3-month-old control (*Epm2a*^{+/+}) and laforin-deficient (*Epm2a*^{-/-}) mice, starved for 24 h (high proteolysis conditions) or fed (0, low proteolysis conditions).

that TSC2 is a negative regulator of mTOR. Some studies have suggested that laforin dephosphorylates GSK3- β at Ser 9, thereby activating this enzyme (38). Lafora bodies are poorly branched polyglucosans, which could arise from an excess of glycogen synthase activity relative to glycogen branching enzyme activity. Glycogen synthase kinase 3 (GSK3) is the principal inhibitor of glycogen synthase. Thus, if laforin were a key GSK3 phosphatase, then one would expect that the loss of activity of this phosphatase would decrease GSK3 activity, increase glycogen synthase activity and thereby increase the formation of Lafora bodies. Unfortunately, this model is not supported by all studies, including analyses of Laforin null mice (34).

The exact mechanism for the laforin effect on autophagy is still elusive and may remain so for some time pending identification of authentic substrates. It is quite possible that laforin may have many substrates (such as other phosphatases), particularly since it is a dual specificity phosphatase that can hydrolyze phosphotyrosine as well as phosphoserine/threonine substrates *in vitro* (reviewed in 16). Thus the identification of the specific substrates relevant to autophagy will be the challenge. However, the consequences of its lack of activity in LD patients are easier to predict. Previous analyses of mice that lack laforin revealed progressive changes in the properties and structure of glycogen that paralleled the formation of Lafora bodies. One of the features observed was a progressive accumulation of glycogen, which also became more phosphorylated and insoluble (18). Since Lafora bodies are composed of poorly branched, water-insoluble, glycogen-like polymers that are also decorated with antiubiquitin antibodies,

we believe that it is likely that decreased autophagy caused by laforin deficiency will enhance their accumulation, given that autophagy compromise leads to the accumulation of glycogen, ubiquitinated proteins and aggregate-prone proteins (27,39) (Fig. 7). Indeed, it appears that glycogen autophagy is regulated via the mTOR signalling pathway, which is perturbed by the loss of laforin activity. In addition to enhancing formation of Lafora bodies, which may be a toxic entity in LD, autophagy deficiency also increases cellular susceptibility to various toxic insults, which may further exacerbate the problem (40).

The situation we have described has similarities and differences when compared with lysosomal storage diseases associated with hydrolase deficiencies. Both sets of conditions exhibit impaired autophagic flux and the accumulation of various substrates in the affected tissues. However, there are important differences. In lysosomal storage diseases, autophagy is impaired not by reduced autophagosome synthesis, as occurs in laforin deficiency, but due to a partial block in the delivery of autophagosomes to lysosomes (22). Indeed, lysosomal storage diseases are frequently characterized by the accumulation of autophagosomes (22). Also, in lysosomal storage diseases, it is likely that there is an initial loss of lysosomal enzyme function that then leads to impaired autophagic flux, while in laforin deficiency the impaired autophagic flux may be a direct consequence of enzyme activity, since we saw increased autophagy with laforin overexpression. Currently, we do not know how early the autophagy defect is in the pathogenic cascade of LD. While it is likely that the Lafora bodies contribute to pathology, our data do raise the possibility that autophagy compromise may be an important driver of pathology in its own right (independent of the Lafora bodies), but also that the formation of these inclusions may be enhanced as a consequence of impaired clearance.

MATERIALS AND METHODS

Plasmids

EGFP-HDQ74 construct was described previously (41). Constructs received as kind gifts were EGFP-LC3 (42) and Myc-Laforin (43).

Mammalian cell culture and transfection

Human fibroblasts were obtained from two patients with two different mutations (Y86X (Laf-1) and R241X (Laf-2)) in the laforin gene and with the clinical features of LD. Control fibroblasts were obtained from healthy subjects matched by sex and age. Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere in MEM supplemented with 15% foetal bovine serum, 1% MEM amino acids, 0.5% MEM non-essential amino acids, 1% glutamine, 1% vitamins, 100 units/ml penicillin and 100 μ g/ml streptomycin (all from Invitrogen Life Technologies). For starvation conditions (high proteolysis medium, H), cells were switched, after washing, from full growth medium (low proteolysis medium, L) to Krebs–Henseleit medium (118.4 mM NaCl, 4.75 mM KCl, 1.19 mM KH₂PO₄, 2.54 mM MgSO₄, 2.44 mM CaCl₂·2H₂O,

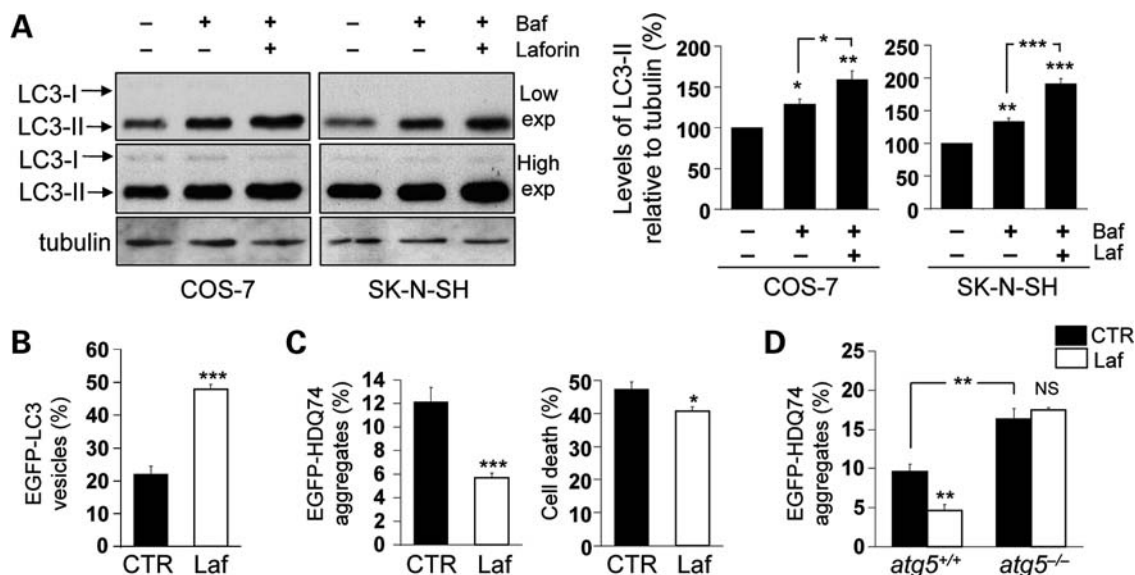


Figure 5. Wild-type laforin induces autophagy and facilitates the clearance of autophagy substrates. (A) COS-7 or SK-N-SH cells, transfected with 2 μ g pcDNA3.1 (empty vector) or Myc-Laforin for 4 h, were treated with or without 400 nM bafilomycin A₁ in the last 4 h of the 24 h post-transfection period. Overexpression of wild-type laforin increased autophagosome synthesis, as analysed by immunoblotting with anti-LC3 antibody (upper gels: low exposure (exp.), lower gels: high exposure) and densitometric analysis of LC3-II levels relative to tubulin. (B) COS-7 cells, transfected with 0.5 μ g EGFP-LC3 and either 1.5 μ g pcDNA3.1 (empty vector) or Myc-Laforin for 4 h, were fixed and analysed for EGFP-LC3 vesicles at 24 h post-transfection. Overexpression of wild-type laforin increased the proportion of transfected (EGFP-positive) cells with EGFP-LC3 vesicles. (C) COS-7 cells, transfected with 0.5 μ g EGFP-HDQ74 and either 1.5 μ g pcDNA3.1 (empty vector) or Myc-Laforin for 4 h, were fixed and analysed for EGFP-HDQ74 aggregates and cell death at 48 h post-transfection. Overexpression of wild-type laforin reduced the percentages of transfected (EGFP-positive) cells with mutant huntingtin aggregates and cell death, assessed by apoptotic nuclear morphology (see Materials and Methods). (D) *atg5*^{+/+} and *atg5*^{-/-} MEFs, transfected with 0.5 μ g EGFP-HDQ74 and either 1.5 μ g pcDNA3.1 (empty vector) or Myc-Laforin for 4 h, were fixed and analysed for EGFP-HDQ74 aggregates at 48 h post-transfection. Overexpression of wild-type laforin reduced mutant huntingtin aggregates in *atg5*^{+/+} MEFs, but not in *atg5*^{-/-} MEFs. *atg5*^{-/-} MEFs had increased aggregates compared with *atg5*^{+/+} MEFs.

28.6 mM NaHCO₃, 10 mM glucose) containing 10 mM HEPES, pH 7.4 and incubated for 2 h at 37°C.

COS-7 cells, SK-N-SH cells, *atg5*^{+/+} and *atg5*^{-/-} MEFs (44) and *tsc2*^{+/+} and *tsc2*^{-/-} MEFs (32) were maintained in DMEM supplemented with 10% foetal bovine serum, 100 units/ml penicillin/streptomycin and 2 mM L-glutamine (all from Sigma-Aldrich) in 37°C, 5% CO₂ humidified incubator. Cells were transfected with DNA constructs for 4 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Laforin-deficient mice

Laforin-deficient *Epm2a*^{-/-} mice (4) were kindly provided by Dr Antonio V. Delgado-Escueta. Male C57BL/6 wild-type and isogenic *Epm2a*^{-/-} mice were maintained at the Centro de Investigaciones Biológicas (CSIC, Madrid) on a light-dark 12:12 cycle under constant temperature (23°C) with free access to food and water. In some experiments, mice were treated with leupeptin (0.2 mg/10 g body weight, intraperitoneally) 1 h before being sacrificed. MEFs were isolated from 14-day-old mouse embryos. *Epm2a*^{+/+} and *Epm2a*^{-/-} MEFs were grown in DMEM supplemented with 15% inactivated foetal bovine serum, 1% MEM non-essential amino acids, 1% glutamine, 1% vitamins, 1% sodium pyruvate, 10⁻⁴ M mercaptoethanol, 100 units/ml penicillin and 100 μ g/ml streptomycin. One-, 3- and 12-month-old knockout, or control mice of the same age were sacrificed by cervical dislocation and livers were dissected for preparation of tissue extracts.

Western blot

Cell pellets were lysed on ice in Laemmli's buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate, 5% β -mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) for 30 min in the presence of complete protease inhibitor cocktail (Roche Diagnostics), boiled for 5–7 min at 100°C and subjected to western blot analysis, as previously described (45,46). Mouse monoclonal antibodies which recognize human laforin have been described previously (12). Other primary antibodies include anti-LC3 (1:4,000 dilution; NB100-2220, Novus Biologicals and 1:500 dilution; clone 5F10, Nanotools), anti-p62 (1:1000 dilution; ab-56416, Abcam), anti-ubiquitinated proteins (1:5000 dilution; PW-8805 and PW-8810, BIOMOL), anti-p70S6 kinase (9202), anti-phospho-p70S6 kinase (Thr389) (9206), anti-S6 ribosomal protein (2217) and anti-phospho-S6 ribosomal protein (Ser235/236) (2211) (all 1:1000 dilutions from Cell Signalling Technology), anti-Myc (1:3000 dilution; 11667203001, Roche Diagnostics), anti-tubulin (1:4,000 dilution; T-6199) and anti-actin (1:5000 dilution; A-2066) (Sigma-Aldrich). Blots were probed with anti-mouse or anti-rabbit IgG-HRP secondary antibody (1:4000 dilutions) and visualized using ECL detection kit (GE Healthcare). Phospho-specific antibodies were always used in the first round of immunoblotting. After treating the membrane with stripping buffer (0.1 M glycine, pH 2.3), it was probed using the antibodies that recognize the total amount of the specific protein of interest.

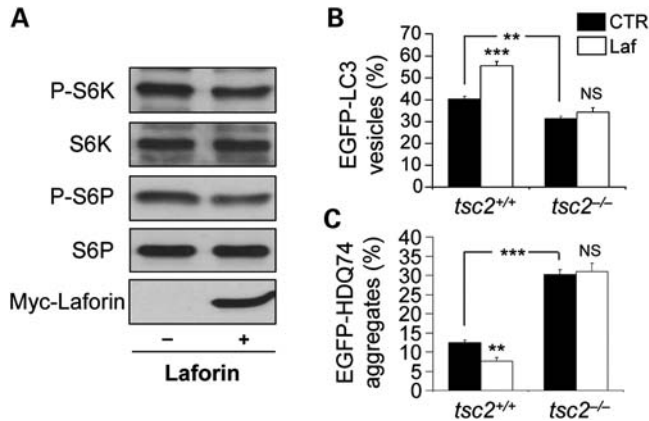


Figure 6. Wild-type laforin reduces mTOR activity to regulate autophagy. (A) COS-7 cells, transfected with 2 μ g pcDNA3.1 (empty vector) or Myc-Laforin for 4 h, were analysed for mTOR activity at 24 h post-transfection by immunoblotting with anti-phospho-S6 kinase (P-S6K, Thr389) and anti-phospho-S6 ribosomal protein (P-S6P, Ser235/236) antibodies. Overexpression of wild-type laforin (detected with anti-myc antibody) reduced phosphorylation of S6K and S6P relative to the total proteins. (B) *tsc2*^{+/+} and *tsc2*^{-/-} MEFs, transfected with 0.5 μ g EGFP-LC3 and either 1.5 μ g pcDNA3.1 (empty vector) or Myc-Laforin for 4 h, were fixed and analysed for EGFP-LC3 vesicles at 24 h post-transfection. Overexpression of wild-type laforin increased the proportion of cells with EGFP-LC3 vesicles in *tsc2*^{+/+} MEFs, but not in *tsc2*^{-/-} MEFs. *tsc2*^{-/-} MEFs had a lower proportion of cells with EGFP-LC3 vesicles compared with *tsc2*^{+/+} MEFs. (C) *tsc2*^{+/+} and *tsc2*^{-/-} MEFs, transfected with 0.5 μ g EGFP-HDQ74 and either 1.5 μ g pcDNA3.1 (empty vector) or Myc-Laforin for 4 h, were fixed and analysed for EGFP-HDQ74 aggregates at 48 h post-transfection. Overexpression of wild-type laforin reduced mutant huntingtin aggregates in *tsc2*^{+/+} MEFs, but not in *tsc2*^{-/-} MEFs. *tsc2*^{-/-} MEFs had increased aggregates compared with *tsc2*^{+/+} MEFs.

Autophagy analysis in mammalian cell culture

Pulse-chase experiments and inhibition of autophagy. Human control and LD patient fibroblasts or *Epm2a*^{+/+} and *Epm2a*^{-/-} MEFs were incubated for 48 h in fresh full medium with 2 μ Ci/ml [³H]valine (Hartmann Analytic GmbH), followed by a 24 h chase in fresh full medium containing 10 mM L-valine to degrade short-lived proteins (47). Then, all cultures were incubated for 4 h under high proteolysis conditions in Krebs–Henseleit medium with 10 mM Hepes, pH 7.4, containing 10 mM valine and the indicated additions. Protein degradation, which was analysed 1 h later to ensure maximal effects of the various additions and for only 3 h to avoid possible secondary effects, was calculated at intervals of 1.5 h by measuring the net release of trichloroacetic acid-soluble radioactivity from the labelled cells into the culture medium and expressed as percentage of protein degraded in 1 h. The contribution of macroautophagy was calculated using 10 mM 3-methyladenine (Sigma-Aldrich) as previously described (47).

Assessment of autophagic flux by LC3-II levels with bafilomycin A₁. Endogenous LC3-II levels, which directly correlate with autophagosome numbers (42), were detected with anti-LC3 antibody and densitometric analysis relative to actin. To assess autophagic flux, LC3-II was measured in the presence of 400 nM bafilomycin A₁ (treated in the last 4 h),

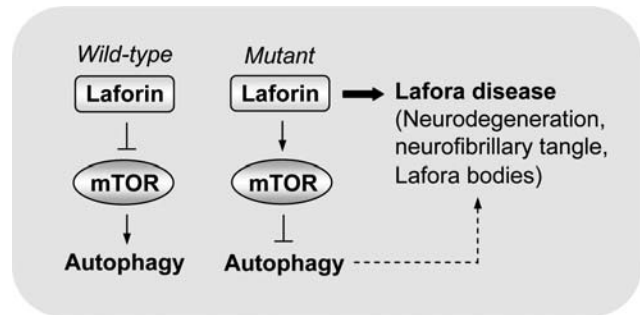


Figure 7. Schematic representation of regulation of autophagy by laforin and the disease consequences. Wild-type laforin induces autophagy by inhibiting mTOR in a TSC2-dependent manner. Loss of laforin in LD activates mTOR and inhibits autophagy. An impairment of autophagy in LD may contribute to the disease pathogenesis (indicated in dashed line).

which clamped LC3-II/autophagosome degradation (48). This assay has been established previously with various autophagy modulators (46,49–51).

Assessment of autophagy by EGFP-LC3 vesicles. The percentage of EGFP-positive cells with >5 EGFP-LC3 vesicles were assessed with a fluorescence microscope, as previously described (50). Alternatively, the number of fluorescent dots per cell was counted.

Quantification of mutant huntingtin aggregates and cell death

The percentage of EGFP-positive cells with EGFP-HDQ74 aggregates was assessed with a Nikon Eclipse E600 fluorescence microscope (plan-apo 60X/1.4 oil immersion lens at room temperature) (Nikon, Inc.), as previously described (46,50). Cells were counted as aggregate positive if one or several aggregates of any size were visible within a cell. Cell death was assessed as we have done previously (40,50,51) by determining if there was apoptotic nuclear morphology using DAPI staining and was considered abnormal if the nucleus was fragmented or condensed to a small size resulting in a much stronger fluorescence signal compared with normal nuclei.

Statistical analyses

Densitometric analysis on the immunoblots was done by Image J software or with an Image Quant ECL (GE Healthcare), and the *P*-values were determined by factorial ANOVA test using STATVIEW v4.53 (Abacus Concepts), where the control condition was set to 100%. The *y*-axis values are shown in percentage (%) and the error bars denote standard error of mean. The *P*-values for assessing EGFP-HDQ74 aggregation or EGFP-LC3 vesicles were determined by unconditional logistical regression analysis, using the general log-linear analysis of SPSS 9 software (SPSS, Chicago), as previously described (46,50,51). The convention we have used for *P* values in all figures is: ****P* < 0.001; ***P* < 0.01; **P* < 0.05; NS, non-significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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