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Protein Degradation and Quality Control in Cells from Laforin and Malin Knockout Mice*

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Background: Lafora disease involves impaired glycogen metabolism and protein degradation.
Results: Impaired proteasomal degradation and mTOR-dependent autophagy in the absence of laforin or malin but decreased LAMP1 and GABARAPL1, perhaps indicative of defective lysosomal glycogen trafficking, only in malin deficiency.
Conclusion: We speculate that defective protein degradation and quality control might be secondary to glycogen accumulation.
Significance: Identification of a malin function independent of laforin.

Lafora disease is a progressive myoclonus epilepsy caused by mutations in the *EPM2A* or *EPM2B* genes that encode a glycogen phosphatase, laforin, and an E3 ubiquitin ligase, malin, respectively. Lafora disease is characterized by accumulation of insoluble, poorly branched, hyperphosphorylated glycogen in brain, muscle, heart, and liver. The laforin-malin complex has been proposed to play a role in the regulation of glycogen metabolism and protein quality control. We evaluated three arms of the protein degradation/quality control process (the autophago-lysosomal pathway, the ubiquitin-proteasomal pathway, and the endoplasmic reticulum (ER) stress response) in mouse embryonic fibroblasts from *Epm2a*$^{-/-}$, *Epm2b*$^{-/-}$, and *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ mice. The levels of LC3-II, a marker of autophagy, were decreased in all knock-out cells as compared with wild type even though they still showed a slight response to starvation and rapamycin. Furthermore, ribosomal protein S6 kinase and S6 phosphorylation were increased. Under basal conditions there was no effect on the levels of ubiquitinated proteins in the knock-out cells, but ubiquitinated protein degradation was decreased during starvation or stress. Lack of malin (*Epm2b*$^{-/-}$ and *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ cells) but not laforin (*Epm2a*$^{-/-}$ cells) decreased LAMP1, a lysosomal marker. CHOP expression was similar in wild type and knock-out cells under basal conditions or with ER stress-inducing agents. In conclusion, both laforin and malin knock-out cells display mTOR-dependent autophagy defects and reduced proteasomal activity but no defects in the ER stress response. We speculate that these defects may be secondary to glycogen overaccumulation. This study also suggests a malin function independent of laforin, possibly in lysosomal biogenesis and/or lysosomal glycogen disposal.

Lafora disease (OMIM 254780) is a progressive fatal juvenile epileptic disorder characterized by stimulus-sensitive grand mal tonic-clonic, absence, visual, and myoclonic seizures (1). There is a gradual decline with dementia, cerebellar ataxia, dysarthria, and muscle wasting, and death normally occurs within 10 years of onset usually because of respiratory failure. Lafora bodies, pathognomonic of Lafora disease, are intracellular polyglucosan bodies containing poorly branched, hyperphosphorylated insoluble glycogen-like polymers and are present in neurons, muscle, heart, liver, and several other tissues (1–5). Lafora disease is a non-classical type of glycogen storage disease. Glycogen is a branched storage polymer of glucose and is present in many tissues such as muscle, liver, heart, and brain (6). The synthesis of the polymer is mediated by glycogen synthase and branching enzyme and the breakdown of glycogen to glucose takes place either in the cytosol by the action of glycogen phosphorylase and debranching enzyme (AGL)[2] or in the lysosomes via α-acid glucosidase activity (7). Aberrations in the synthesis or the degradation of glycogen could lead to an abnormal accumulation of the glycogen as seen in various glycogen storage diseases (8, 9) and also in Lafora disease.

Mutations in two genes are implicated in causation of Lafora disease, *EPM2A* encoding the laforin phosphatase (10) and *EPM2B,* which codes for malin, a potential E3 ubiquitin ligase (11). Both in patients and mouse models, defects in laforin and malin lead to clinically indistinguishable phenotypes (10–13). Extensive studies have been conducted using cell culture and animal models in attempts to understand the etiopathogenesis of Lafora disease. We have previously shown that laforin acts as a glycogen phosphatase *in vitro* and *in vivo*, and as a result of increased phosphorylation of glycogen there are disturbances in glycogen structure and defects in branching and water solubility, consistent with Lafora body formation (14, 15). Malin contains a RING finger domain characteristic of E3 ubiquitin ligases, and several proteins involved in glycogen metabolism, laforin (16), glycogen synthase (17), protein targeted to glycogen (PTG) (18), and AGL (19) have been proposed to be malin substrates. However, most of these conclusions are derived

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[2] The abbreviations used are: AGL, debranching enzyme; Atg, autophagy-related genes; CHOP, C/EBP homologous protein; EPM, epilepsy progressive myoclonus; ER, endoplasmic reticulum; GABARAPL1, γ-amino butyric acid receptor-associated protein like 1; LAMP1, lysosomal-associated membrane protein 1; LC3, microtubule-associated protein 1A-light chain 3; MEF, mouse embryonic fibroblasts; mTOR, mammalian target of rapamycin; PTG, protein targeted to glycogen; S6, ribosomal protein S6; S6K, S6 kinase; Stbd1, Starch-binding domain protein 1; LKO, Epm2a$^{-/-}$; MKO, Epm2b$^{-/-}$; DKO, Epm2a$^{-/-}$ Epm2b$^{-/-}$.

from experiments performed *in vitro* (16) or using cell culture overexpression systems (17–19). Furthermore in neither *Epm2a*$^{-/-}$ nor *Epm2b*$^{-/-}$ mice did we find any evidence for alterations in the levels of PTG, AGL, or glycogen synthase activity (13) arguing against these proteins being malin substrates. Several studies have also shown that laforin protein level follows glycogen accumulation independently of whether *Epm2b* is deleted or not (13, 20, 21) suggesting that, contrary to previous proposals (16), laforin too is not a malin substrate.

The laforin-malin complex has also been proposed to play a role in protein clearance. It was suggested that laforin and malin form a functional complex with the cellular chaperone Hsp70 and suppress the toxicity of misfolded proteins by promoting their degradation through the ubiquitin-proteasomal pathway (22). Aguado *et al.* (23) reported that laforin inactivates mTOR and thus activates autophagy. Therefore, with defects in laforin, activation of mTOR would inhibit autophagy to cause the Lafora disease phenotype. Criado *et al.* (24) reported that impairment in autophagy in the absence of malin was independent of mTOR and proposed defects in autophagy as the primary cause of Lafora disease. In the present study, we analyzed the effect of defects in laforin and malin on protein degradation and quality control by investigating the three different arms of the cellular quality control process (the ubiquitin-proteasomal pathway, the autophago-lysosomal pathway, and the ER stress response) in mouse primary embryonic fibroblasts (MEFs) derived from *Epm2a*$^{-/-}$, *Epm2b*$^{-/-}$, and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ mice. The rationale for using MEFs is that they are amenable to treatments with drugs and other agents that are challenging or difficult using whole animals. At the same time, the MEFs allow for a powerful comparison among the different genotypes that does not rely on knockdown or overexpression approaches. Although loss of laforin or malin led generally to similar phenotypes, we found a novel potential function of malin on lysosomes not shared by laforin.

EXPERIMENTAL PROCEDURES

Chemicals, Antibodies, and Other Reagents—Chloroquine was from Sigma. Rapamycin was from LC Laboratories. Thapsigargin, tunicamycin, and MG132 were from Cayman Chemicals. Antibody sources were as follows: Anti-LC3, anti-phospho-S6K, anti-S6K, anti-phospho-S6, and anti-S6 were from Cell Signaling Technology; anti-GABARAPL1 and anti-CHOP were from Protein Tech Group; anti-LAMP1 and the anti-S5a subunit of the 26 S proteasome were from Iowa Hybridoma; anti-ubiquitin was from Millipore; anti-glyceraldehyde-3-phosphate dehydrogenase was from Biodesign International; anti-Stbd1 has been previously described (25). Other chemicals and reagents were from Sigma, Bio-Rad, Invitrogen, or New England Biolabs.

Animals—The *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mice have been previously described (13, 26). *Epm2a*$^{-/-}$ and *Epm2b*$^{-/-}$ mice were intercrossed to generate double heterozygous *Epm2a*$^{+/-}$ *Epm2b*$^{+/-}$ mice, which were crossed to generate the double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ mice. All mice were maintained in temperature- and humidity-controlled conditions with a 12:12-h light-dark cycle, fed a standard chow (Harlan Teklad global diet 2018SX), and allowed food and water *ad libitum*. All studies were conducted in accordance with federal guidelines and were approved by the Institutional Animal Use and Care Committee of Indiana University School of Medicine.

Mammalian Cell Culture and Treatments—MEFs were isolated from 13.5-day-old embryos from wild type, *Epm2a*$^{-/-}$, *Epm2b*$^{-/-}$, and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ mice as described previously (27). Embryos were generated by crossing appropriate homozygous parents, and the genotype of the resulting MEFs was confirmed by PCR. MEFs were maintained in DMEM-F-12 (Hyclone) medium supplemented with 10% fetal bovine serum (ThermoScientific), 100 units/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) at 37 °C with 5% CO_2 in a humidified incubator. MEFs were seeded at a cell density of around 1.5×10^6 cells/100-mm plate, grown till 85–90% confluence, and then passaged in a 1:3 ratio for propagation. MEFs between passages 3 and 8 were used for all experiments.

To culture cells under starvation conditions, cells were washed with phosphate-buffered saline (PBS) twice at room temperature and switched from full growth medium to Earle's balanced salt solution (Sigma). The cells were incubated in Earle's balanced salt solution for 2 h at 37 °C with 5% CO_2 in a humidified incubator. Cell extracts were prepared at the time of harvest as described below.

For inhibition of lysosomes, MEFs were incubated with medium that contained 20 μM chloroquine for 2 h. MEFs were also treated with 10 nM rapamycin, an inhibitor of mTOR (mammalian target of rapamycin) for 2 h. For inhibition of the proteasomal function, MEFs were incubated with medium that contained 20 μM MG132 for 2 h. To measure ER stress responses, MEFs were incubated with medium that contained 1 μM thapsigargin or 2 μg/ml tunicamycin for 18 h. In control experiments, MEFs were incubated in medium that contained vehicles, dimethyl sulfoxide, or sterile distilled water as appropriate. Cell extracts were prepared at the time of harvest as described below.

Cell Extraction and Western Blotting—Cells were washed three times with 10 ml of ice-cold PBS and then lysed with ice-cold radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, and 1% nonidet-P40) for 15 min in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-p-tosyl-1-lysine chloromethyl ketone (TLCK), 1 mM benzamidine, 1 μg/ml aprotinin, pepstatin, and leupeptin). The cell lysates were centrifuged at 10,000 $\times$ *g* for 15 min at 4 °C to pellet insoluble materials. For detection of LC3 and GABARAPL1, cells were lysed with ice-cold radioimmune precipitation assay buffer with 0.5% sodium dodecyl sulfate for 15 min in the presence of the protease inhibitors described above. The supernatants of the lysates or total lysates (for LC3 and GABARAPL1) were used for Western blotting analyses. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (28).

Samples of 10 μg of protein from MEFs were separated by 10% SDS-PAGE and transferred onto a 0.45-μm nitrocellulose membrane (Bio-Rad) at 15 V overnight. For detection of LC3 and GABARAPL1, 10 μg of protein were separated by 15% SDS-PAGE and transferred onto a 0.22-μm polyvinylidene fluoride (PVDF from Millipore) membrane at 100 V for 90 min at 4 °C. After transfer, the nitrocellulose membranes were

stained with Ponceau S to monitor loading followed by blocking in 5% nonfat milk powder in 1× Tris-buffered saline (TBS) with 0.1% Tween 20 (for nitrocellulose) or 5% BSA in 1 × TBS with 0.1% Tween 20 (for PVDF) and subsequently incubated with primary antibodies. The primary antibodies were diluted according to the manufacturer's protocol. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Detection was performed by enhanced chemiluminescence using Pierce ECL Western blot substrate (Thermo Scientific). Phospho-specific antibodies were used first during immunoblotting, and then membranes were treated with stripping buffer (62.5 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 0.7% β-mercaptoethanol) and probed for the total protein of interest.

Quantitation of Proteasomal Activity—Proteasomal activity was determined using the luminogenic proteasome substrate from Promega according to manufacturer's protocol. Proteasome-Glo™ Assay reagent was incubated for 15 min with 10 μg of soluble MEF protein extracts prepared as described above. Chymotrypsin-like proteasomal activity was detected by measuring the relative luminescence units using SpectraMax M5 Luminometer (Molecular devices).

Glycogen Determination—Glycogen content in the MEFs was measured by modifications of Suzuki *et al.* (29). MEFs were seeded in 100-mm plates as described above and grown till 100% confluence. The cells were grown for 7 days after reaching confluence and supplemented with fresh medium daily. Total lysates from MEFs were hydrolyzed in 30% potassium hydroxide in a boiling water bath for 30 min. After repeated ethanol precipitations, the glycogen was digested with 2 N hydrochloric acid for 2 h in a boiling water bath and neutralized with 2 N sodium hydroxide. The glucose equivalents were determined by the method of Bergmeyer (30). Glycogen content is expressed as μmol of glucose/mg of protein.

Statistical Analysis—Densitometric analysis of the immunoblots was performed using Carestream Molecular Imaging Software. Each experiment was performed in duplicate and repeated at least three times. The differences between groups were analyzed with one-way analysis of variance followed by Student-Newman-Keul's post hoc test. A p value <0.05 is considered statistically significant.

RESULTS

Glycogen Levels in MEFs from Mouse Models of Lafora Disease—Because Lafora disease is characterized by the accumulation of glycogen in patients and the different mouse models (10–13), glycogen content was analyzed in MEFs from wild type (WT), *Epm2a*$^{-/-}$ (LKO), *Epm2b*$^{-/-}$ (MKO), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (DKO) mice. Glycogen was ~70% higher in all the knock-out MEFs as compared with the wild type control cells (Fig. 1), indicating that the MEFs have similar phenotypes to the corresponding mouse models with regard to glycogen accumulation. The double knock-out cells showed the same increase as the single knock-out consistent with the involvement of a similar pathway.

Proteasomal Activity in MEFs from Mouse Models of Lafora Disease—Using MEFs from wild type (WT), *Epm2a*$^{-/-}$ (LKO), *Epm2b*$^{-/-}$ (MKO), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (DKO) mice, we assessed proteasomal function by measuring the chymotrypsin-like activity of the proteasome using the Suc-LLVY-aminoluciferin substrate. The absence of laforin and/or malin significantly decreased proteasomal activity in the knock-out MEFs as compared with the wild type controls (Fig. 2*A*). However, the protein level of the S5a subunit of the 26 S proteasome was not different between genotypes (Fig. 2*B*), suggesting that the observed difference in the chymotrypsin-like activity is not due to the levels of proteasome but, rather, to intrinsic activity.

Autophago-Lysosomal Pathway in MEFs from Mouse Models of Lafora Disease—Upon induction of autophagy, microtubule-associated protein 1 light chain 3 (LC3-I) is lipidated to LC3-II, which displays increased electrophoretic mobility. LC3-II is then incorporated into the inner and outer membranes of the newly formed autophagosomes. The autophagosomes fuse with the lysosomes to form autolysosomes where the engulfed components are degraded by the lysosomal enzymes. Thus, the level

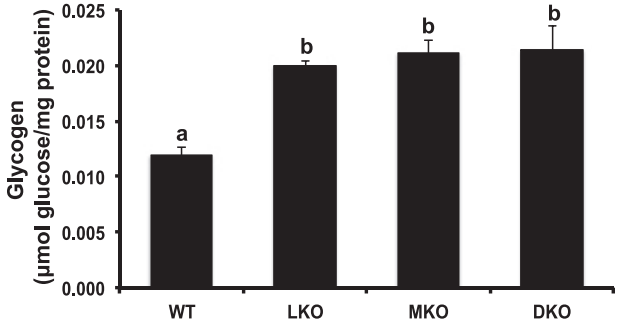

FIGURE 1. **Glycogen levels in MEFs from mouse models of Lafora disease.** Glycogen levels in MEFs WT, *Epm2a*$^{-/-}$ (*LKO*), *Epm2b*$^{-/-}$ (*MKO*), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (*DKO*) mice were analyzed as described under "Experimental Procedures." The levels are expressed as glucose eq/mg of protein. Values represent the average of three independent experiments ±S.E. Values marked by the same letter are not statistically significant; different letters indicate $p < 0.05$.

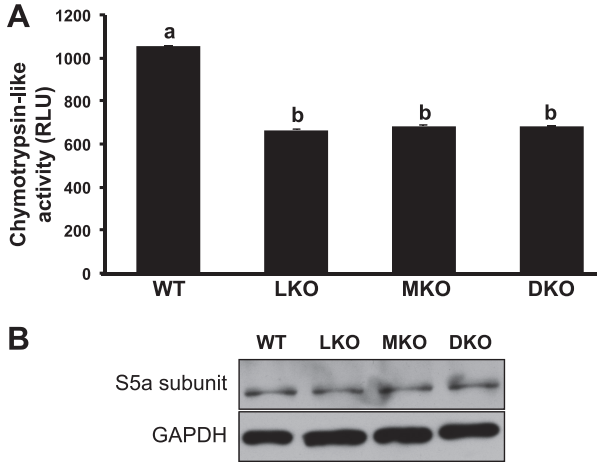

FIGURE 2. **Proteasomal activity in MEFs from mouse models of Lafora disease.** Soluble extracts from MEFs of WT, *Epm2a*$^{-/-}$ (*LKO*), *Epm2b*$^{-/-}$ (*MKO*), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (*DKO*) mice were analyzed. *A*, proteasomal activities were measured in soluble lysates (10 μg) using the Proteasome-Glo™ assay for chymotrypsin-like activity as described under "Experimental Procedures." Values represent the average ± S.E. of three independent experiments performed in duplicate. *RLU*, relative luminescence units. Values marked by the same letter are not statistically significant; different letters indicate $p < 0.05$. *B*, representative Western blots using antibody against S5a subunit of the 26 S proteasome. Loadings were 10 μg of protein.

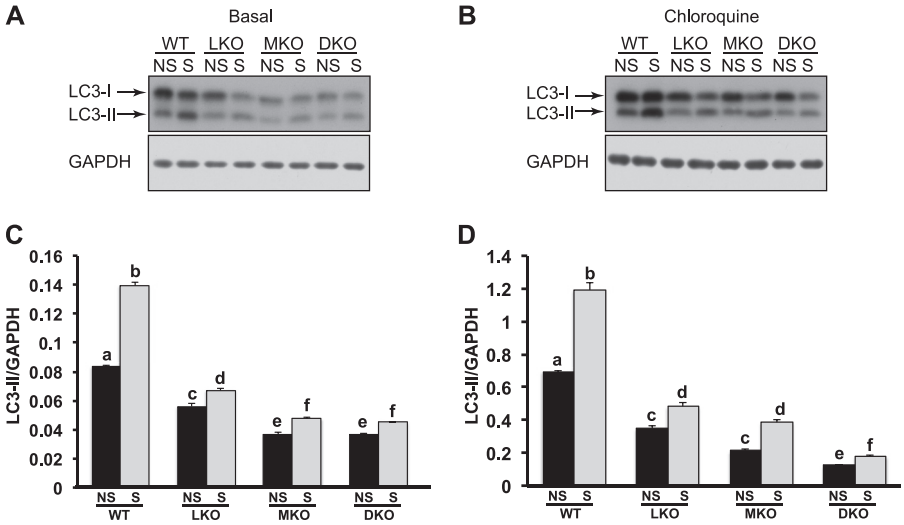

FIGURE 3. **Autophagy markers in MEFs from mouse models of Lafora disease.** MEFs from WT, *Epm2a*$^{-/-}$ (*LKO*), *Epm2b*$^{-/-}$ (*MKO*), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (*DKO*) mice were incubated without and with 20 μM chloroquine for 2 h under basal (*NS*) or starvation (*S*) conditions. Total lysates (10 μg) from MEFs were analyzed. *A* and *B*, representative Western blots using antibody against LC3 in the absence and presence of chloroquine, respectively. The positions of LC3-I and LC3-II are indicated on the *left*. *C* and *D*, LC3-II bands were quantitated by densitometry and normalized to the corresponding GAPDH bands. Values represent the average of three independent experiments ± S.E. Values marked by the same letter are not statistically significant; different letters indicate $p < 0.05$.

of LC3-II is commonly used as a marker to evaluate autophagosomes (31).

MEFs were cultured in complete medium to assess basal autophagy or in serum-free and amino acid-free medium (Earle's balanced salt solution) to induce autophagy by starvation (32). Under both basal and starved conditions, there was a significant decrease in the levels of LC3-II in knock-out MEFs compared with the wild type controls (Fig. 3, *A* and *C*). Starvation induced a significant increase in the LC3-II levels in the wild type cells, but the effect was much smaller in the knock-out cells. A decrease in the levels of LC3-II could either be due to impairment in the formation of autophagosomes or an increase in the degradation by autolysosomes. To differentiate between the two, MEFs were treated with chloroquine, a lysosomal inhibitor, to block the degradation of LC3-II. The changes in the levels of LC3-II upon lysosomal inhibition should, thus, reflect changes in the rate of formation of autophagosomes (31). In the presence of chloroquine, there was a significant decrease in the levels of LC3-II in knock-out MEFs indicating impairment in the formation of autophagosomes (Fig. 3, *B* and *D*). Combined starvation and chloroquine treatment still resulted in a small increase in the levels of LC3-II in knock-out MEFs, but the increase was significantly lower than in the wild type MEFs. This small increase was further decreased in the double knock-out cells. Thus, the absence of either laforin or malin dampens the autophagic process.

mTOR Signaling Pathway in MEFs from Mouse Models of Lafora Disease—mTOR negatively regulates autophagy. Nutritional deprivation inhibits mTOR, resulting in induction of autophagy. Conversely, nutrient replenishment activates mTOR and suppresses autophagy (33, 34). Activation of mTOR causes phosphorylation of its downstream targets, such as ribosomal protein S6 kinase (p70S6K) and ribosomal protein S6. Analysis of the levels of phosphorylated p70S6K and S6 protein indicated increased phosphorylation (Fig. 4, *A*–*D*) under basal conditions in the knock-out MEFs. Upon starvation, although there was a decrease in the phosphorylation of the S6 protein compared with the basal state, the phosphorylation in knock-out MEFs was still higher than in the wild type control cells (Fig. 4, *C* and *E*, high exposure). When MEFs were treated with rapamycin, the level of phosphorylated S6 protein in all MEFs decreased significantly as compared with the basal levels (Fig. 4*E*). Taken together, these observations suggest that mTOR signaling is up-regulated in knock-out cells, and impairment of autophagy may be mTOR-dependent.

Consistent with inhibition of mTOR, upon rapamycin treatment, LC3-II was increased in the wild type cells to a level similar to that of starvation. LC3-II in the knock-out MEFs was also increased to the same level in the starved and rapamycin-treated cells, although the effect did not reach the levels of the wild type cells (Fig. 4, *F* and *G*). These observations suggest that either the autophagy defect in knock-out MEFs is only partly mTOR-dependent or that the maximum capacity of the cells to form new autophagosomes is impaired in the absence of laforin and/or malin.

Effect of Malin Deficiency on Lysosome and Late Endosome Markers—LC3 belongs to the autophagy-related genes 8 (Atg8) family. Because the levels of LC3-II were lower in the knock-out MEFs, we also analyzed GABARAPL1, another member of the Atg8 family. Interestingly, the levels of GABARAPL1 in the MKO but not in the LKO MEFs were much lower than the wild type controls (Fig. 5, *A* and *B*). Similar to GABARAPL1, LAMP1, a marker of late endosomes and lysosomes, was unchanged in LKO but significantly lower in the MKO cells (Fig. 5, *A* and *C*). Additional loss of laforin in the DKO cells did not cause any further decrease in either GABARAPL1 or LAMP1 levels (Fig. 5*A*).

Starch binding domain protein 1 (Stbd1 or genethonin 1), a membrane and glycogen-binding protein, has been shown to interact with GABARAPL1 and abnormal glycogen and might

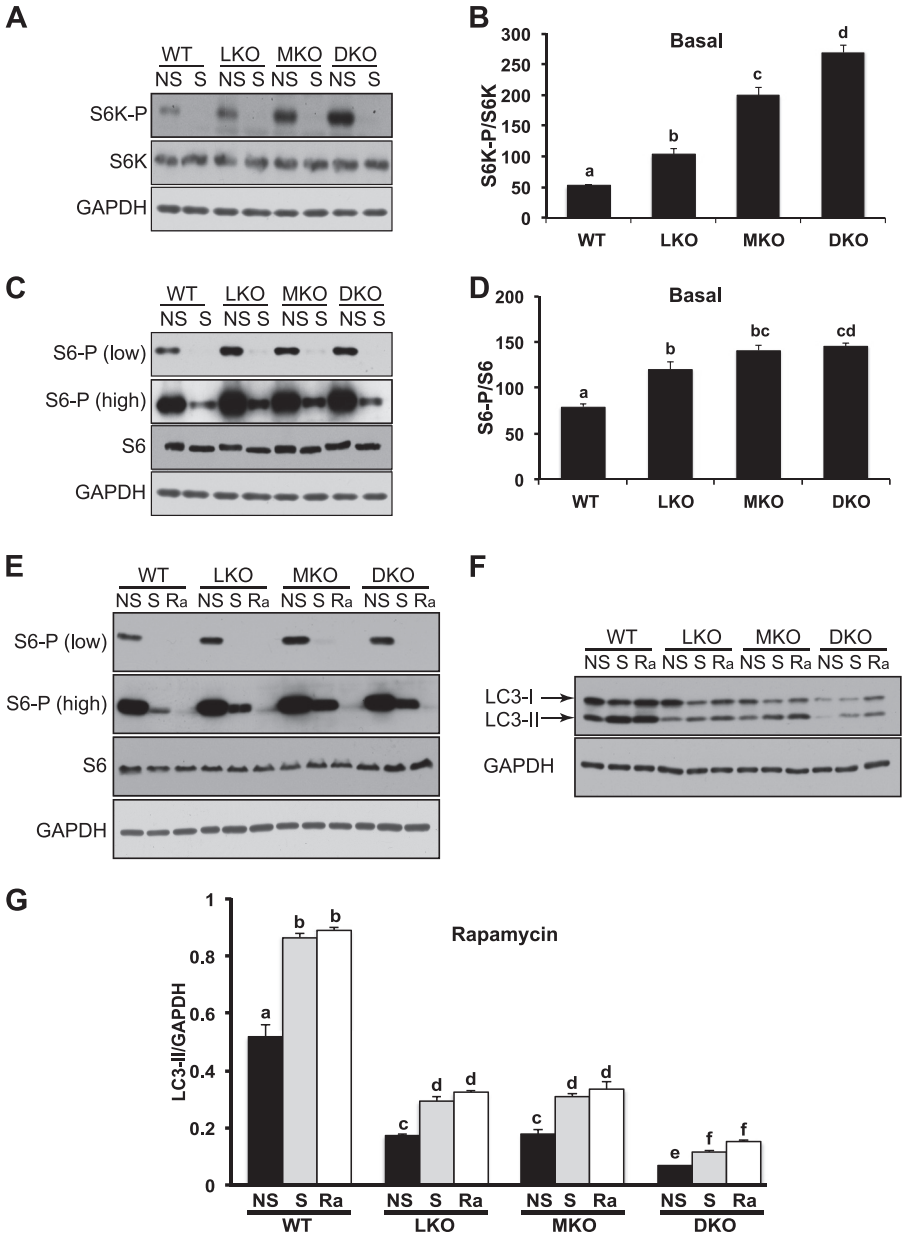

FIGURE 4. **mTOR signaling pathway in MEFs from mouse models of Lafora disease.** MEFs from WT, *Epm2a*$^{-/-}$ (*LKO*), *Epm2b*$^{-/-}$ (*MKO*), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (*DKO*) mice were incubated under basal (*NS*) or starvation (*S*) conditions or with 10 nM rapamycin (*Ra*) for 2 h. Soluble extracts (10 μg) from MEFs were analyzed. Representative Western blots are shown; phosphorylated Thr^{389} p70S6 kinase and total S6K (*panel A*), phosphorylated Ser$^{235/236}$ S6 and total S6 protein (*panels C* and *E*), LC3 (*panel F*). P-S6K and P-S6 bands were quantified by densitometry and normalized to the corresponding total S6K (*panel B*) and total S6 bands (*panel D*), respectively. LC3-II bands were quantified by densitometry and normalized to the corresponding GAPDH bands (*panel G*). Values represent the average of three independent experiments ± S.E. Values marked by the same letter are not statistically significant; different letters indicate $p < 0.05$.

be involved in the transfer of abnormal glycogen to lysosomes (25, 35). However, no difference in Stbd1 protein was found in the knock-out MEFs as compared with the wild type controls (Fig. 5*A*). These results suggest that there may not be a complete overlap in the functions of malin and laforin and that a unique function of malin might be related to lysosome biogenesis or lysosomal trafficking of glycogen.

Degradation of Ubiquitinated Proteins in Laforin and/or Malin-deficient Cells—Ubiquitinated proteins are degraded either via the ubiquitin-proteasomal or the autophago-lysosomal pathway depending on the lysine involved in the formation of the polyubiquitinated chains (36, 37). We compared the levels of ubiquitinated proteins in the knock-out MEFs with the wild type controls. Under basal conditions, there was no difference in the level of ubiquitinated proteins. However, upon starvation, there was a significant decrease in wild type cells, whereas in the knock-out MEFs the levels were no different from the basal state (Fig. 6, *A* and *D*). When the MEFs were treated with the proteasomal inhibitor MG132 (Fig. 6, *C* and *F*), or the lysosomal inhibitor chloroquine (Fig. 6, *B* and *E*), even under starvation conditions, neither the wild type nor the knock-out MEFs showed any decrease in levels of ubiquitinated proteins. These results indicate that loss of laforin and/or malin impairs the starvation- or stress-induced degradation of the ubiquitinated proteins and that the degradation could be either via the ubiquitin-proteasomal or the autophago-lysosomal pathway.

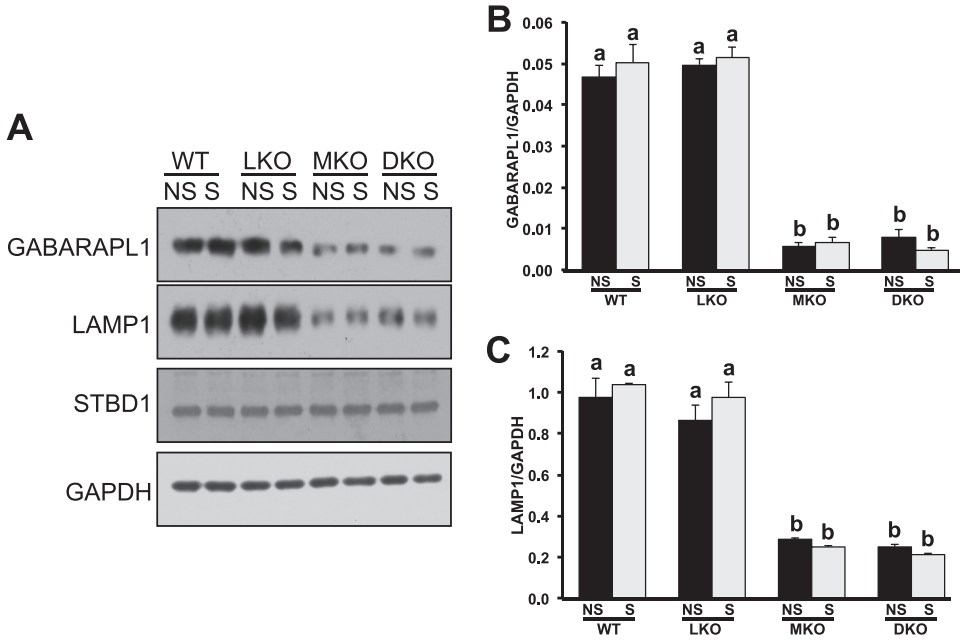

FIGURE 5. **Lysosome and late endosome markers in MEFs from mouse models of Lafora disease.** MEFs from WT, *Epm2a*$^{-/-}$ (*LKO*), *Epm2b*$^{-/-}$ (*MKO*), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (*DKO*) mice were incubated under basal conditions (*NS*) or starvation (*S*) for 2 h. Total lysates (10 μg) from MEFs were analyzed. *A*, representative Western blots using antibodies against GABARAPL1, LAMP1, Stbd1, and GAPDH. *B* and *C*, GABARAPL1 or LAMP1 bands were quantified by densitometry and normalized to the corresponding GAPDH bands. Values represent the average of three independent experiments ± S.E. Values marked by the same letter are not statistically significant; different letters indicate $p < 0.05$.

ER Stress Response in MEFs from Mouse Models of Lafora Disease—Impairment of proteasomal function might affect ER stress, and an earlier study by Vernia *et al.* (38) suggested that laforin deficiency leads to an increased sensitivity to ER stress-inducing agents. Therefore, we investigated the ER stress response in MEFs by analyzing the expression of the transcriptional factor CHOP (39), a widely used ER stress marker. MEFs were treated with two known ER stress-inducing agents, thapsigargin, an inhibitor of the ER calcium pump, and tunicamycin, an inhibitor of ER glycosylation. The levels of CHOP were higher upon treatment with thapsigargin or tunicamycin by comparison to the basal condition both in wild type and in the knock-out MEFs, with no differences between genotypes (Fig. 7, *A* and *B*). Thus, the ER stress response is not compromised in the absence of malin and/or laforin.

DISCUSSION

Recent studies on Lafora disease indicate that the Lafora disease proteins, laforin and malin, may be involved in the control of a number of cellular functions and/or pathways, including glycogen metabolism and protein quality control. Defects in either of these pathways have been proposed to play a role in the pathogenesis of Lafora disease. Previous work has shown that laforin dephosphorylates glycogen (14) and that the absence of laforin in *Epm2a*$^{-/-}$ mice causes hyperphosphorylated glycogen, which correlates with abnormalities in glycogen structure, decreased branching, and solubility in water (15), consistent with the formation of Lafora bodies. However the role of malin to reduce glycogen phosphorylation is not fully understood. The increased association of laforin with insoluble glycogen and depletion from the cytosol in the *Epm2b*$^{-/-}$ mice led us to propose that in the absence of malin, laforin is sequestered by insoluble glycogen where it may not be functional (13). However, laforin may have other functions independent of malin and may not always act as a complex with malin, as has been suggested (17–19). Based primarily on experiments using cell culture systems, several substrates of malin have been proposed, mainly relevant to glycogen metabolism; laforin (18), glycogen synthase (17), PTG (18), AGL (19), AMP-activated protein kinase (40), and neuronatin (41). In 3-month-old *Epm2b*$^{-/-}$ mice, we found no evidence for alterations in the levels of PTG, AGL, or glycogen synthase activity (13), arguing against these proteins being malin substrates.

The laforin-malin complex has been proposed to be involved in clearance of cytotoxic proteins via the proteasome (22) or autophagy (23). Defects in the protein clearance pathway have been implicated in the etiopathogenesis of Lafora disease by various studies (23, 42). In an effort to further understand the physiological roles of laforin and malin, we analyzed the protein degradation and quality control systems in *Epm2a*$^{-/-}$, *Epm2b*$^{-/-}$ and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ mice. Lack of laforin and/or malin impairs autophagy in an mTOR-dependent manner. Although there was an overall decrease in LC3-II, indicating reduced formation of autophagosomes, in all the knock-out MEFs, induction of autophagy upon starvation or rapamycin treatment was still observed, although to a much lower extent than in the wild type cells. This could be because the capacity of the knock-out MEFs to form new autophagosomes is limited. Criado *et al.* (24) reported that malin regulates autophagy in an mTOR-independent manner, which is inconsistent with the conclusion that laforin and malin regulate autophagy as a complex. However, in our studies, both laforin and malin are mTOR-dependent in their role to regulate autophagy.

In contrast to previous studies (24, 43), we found that in MEFs there was proteasomal dysfunction in the absence of

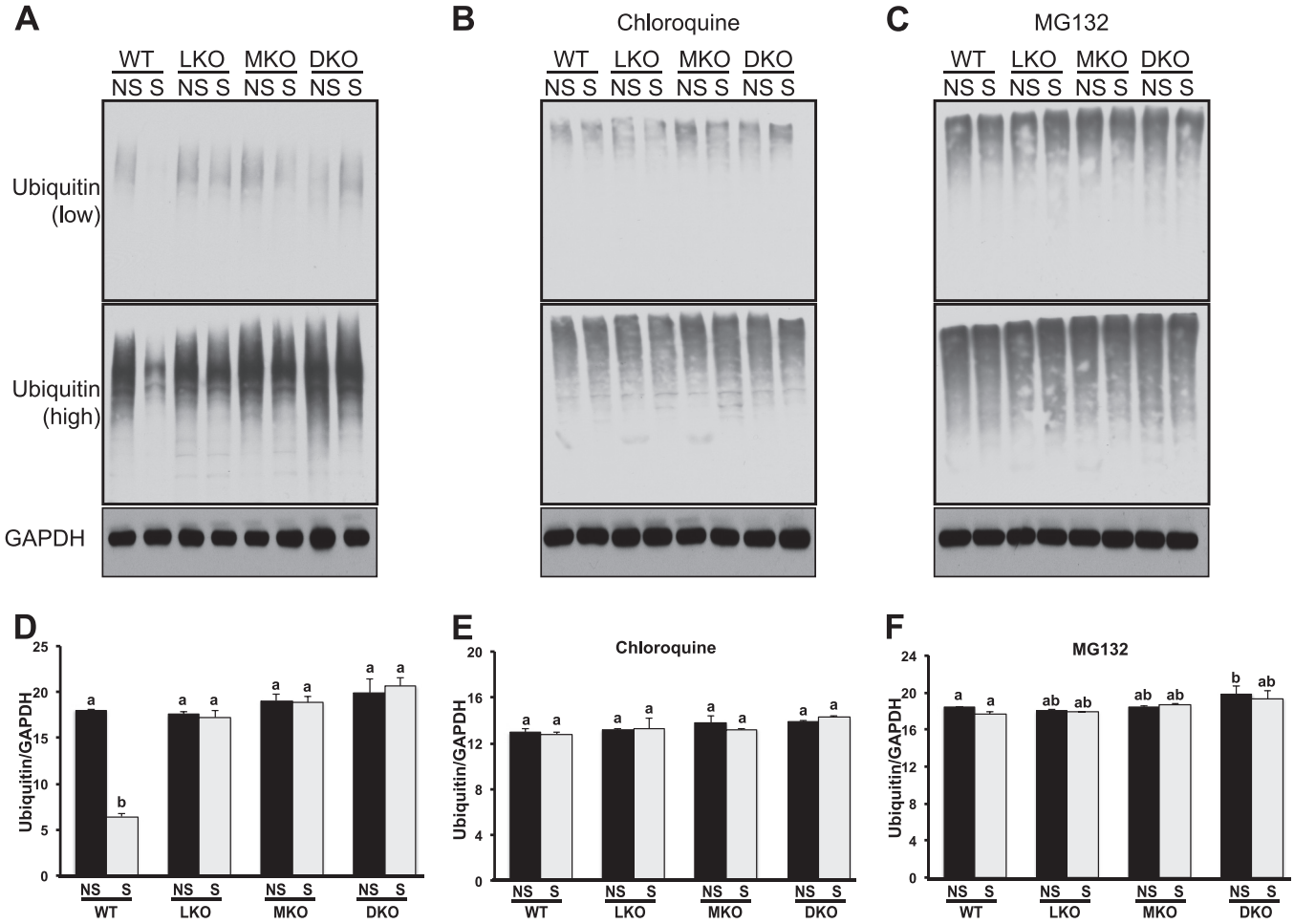

FIGURE 6. **Degradation of ubiquitinated proteins in MEFs from mouse models of Lafora disease.** MEFs from WT, *Epm2a*$^{-/-}$ (*LKO*), *Epm2b*$^{-/-}$ (*MKO*), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (*DKO*) mice were incubated under basal conditions (*NS*) or starvation (*S*) without or with 20 μM chloroquine or 20 μM MG132 for 2 h. Soluble extracts (10 μg) from MEFs were analyzed. Representative Western blots using antibody against ubiquitinated proteins without (*panel A*) and with chloroquine (*panel B*) or with MG132 (*panel C*) are shown. *D–F*, bands for ubiquitinated proteins were quantified by densitometry and normalized to the corresponding GAPDH bands. Values represent the average of three independent experiments ± S.E. Values marked by the same letter are not statistically significant; different letters indicate $p < 0.05$.

either laforin or malin. Deletion of both genes in *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ MEFs did not cause a further decrease in the proteasomal activity. The proteasomal activity was, however, not completely absent in the knock-out MEFs. Under basal conditions, the levels of ubiquitinated proteins were not altered in the knock-out MEFs as compared with the wild type cells. Upon starvation, ubiquitinated proteins in the wild type cells were degraded either via the proteasome or the lysosome because blocking either of the two pathways prevented the degradation of the proteins. However, the starvation-induced degradation of the ubiquitinated proteins was absent in all the knock-out MEFs, suggesting impairment of the protein clearance pathways. These results, therefore, suggest either an overlap in malin and laforin functions or an effect secondary to a common phenotype such as generalized cell stress caused by abnormal glycogen accumulation. An uncompromised ER stress response points toward a secondary effect that becomes enhanced under stress. Recent studies by Turnbull *et al.* (44, 45), Pederson *et al.* (46), and Duran *et al.* (20) have linked the overaccumulation of abnormal glycogen to the causation of Lafora disease as genetic reduction of the glycogen levels by eliminating either glycogen synthase or PTG in mice rescues not only Lafora body formation but also the neurological symptoms associated with the disease. Thus, the defect in protein clearance in Lafora disease may be secondary to the accumulation of abnormal glycogen and sequestration of cellular proteins in the Lafora bodies. This conclusion is supported by the work of Duran *et al.* (20) and DePaoli-Roach *et al.*[3] showing that decreased glycogen accumulation in *Epm2b*$^{-/-}$ mice restored autophagy markers and proteasomal activity.

Interestingly, we found that the levels of GABARAPL1 and LAMP1 were normal in *Epm2a*$^{-/-}$ MEFs but much lower in *Epm2b*$^{-/-}$ and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ MEFs, suggesting a function of malin independent of laforin. We have shown earlier that Stbd1 preferentially binds to abnormal and hyperphosphorylated glycogen (25). It has been proposed that Stbd1 may participate in the lysosomal trafficking of glycogen by anchoring glycogen to intracellular membranes. Stbd1 interacts with GABARAPL1, which may function as a novel cargo

[3] A. A. DePaoli-Roach, D. M. Segvich, C. J. Contreras, P. Garyali, and P. J. Roach, unpublished results.

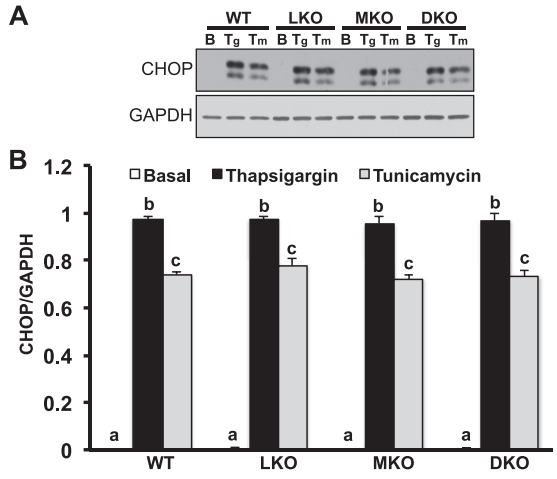

FIGURE 7. **ER stress response in MEFs from mouse models of Lafora disease.** MEFs from WT, *Epm2a*$^{-/-}$ (*LKO*), *Epm2b*$^{-/-}$ (*MKO*), and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ (*DKO*) mice were incubated without (*B*) or with 1 μM thapsigargin (*Tg*) or 2 μg/ml tunicamycin (*Tm*) for 18 h. Soluble extracts (10 μg) from MEFs were analyzed. *A*, representative Western blots using antibody against CHOP. *B*, bands for CHOP were quantified by densitometry and normalized to the corresponding GAPDH bands. Values represent the average of three independent experiments ± S.E. Values marked by the same letter are not statistically significant; different letters indicate $p < 0.05$.

binding protein that delivers glycogen to lysosomes (35). In knock-out MEFs, the protein level of Stbd1 is not altered, although GABARAPL1, an interacting partner of Stbd1, and LAMP1 are decreased in *Epm2b*$^{-/-}$ and double *Epm2a*$^{-/-}$ *Epm2b*$^{-/-}$ MEFs, suggesting a possible role of malin in glycogen trafficking to lysosomes and/or lysosome biogenesis.

In conclusion, in MEFs from mouse models of Lafora disease autophagy is impaired in an mTOR-dependent manner, there is impairment of the proteasomal activity, whereas the ER stress response is not compromised. We propose that this defect in protein clearance might be secondary to generalized cellular stress because of glycogen overaccumulation. Laforin and malin do not always function as a complex, and malin may play a role, independent of laforin, in lysosomal biogenesis or lysosomal trafficking of glycogen. Future experiments will need to focus on determining the precise physiological role of malin in glycogen trafficking to lysosomes and in the etiopathogenesis of Lafora disease.

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