# Ubiquitin conjugating enzyme E2-N and sequestosome-1 (p62) are components of the ubiquitination process mediated by the malin-laforin E3-ubiquitin ligase complex

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**Short title:** UBE2N, SQSTM1 and the malin-laforin E3-ligase complex

**ABSTRACT**

Lafora disease (LD, OMIM254780, ORPHA501) is a rare neurodegenerative form of epilepsy related to mutations in two proteins: laforin, a dual specificity phosphatase, and malin, an E3-ubiquitin ligase. Both proteins form a functional complex, where laforin recruits specific substrates to be ubiquitinated by malin. However, little is known about the mechanism driving malin-laforin mediated ubiquitination of its substrates. In this work we present evidence indicating that the malin-laforin complex interacts physically and functionally with the ubiquitin conjugating enzyme E2-N (UBE2N). This binding determines the topology of the chains that the complex is able to promote in the corresponding substrates (mainly K63-linked polyubiquitin chains). In addition, we demonstrate that the malin-laforin complex interacts with the selective autophagy adaptor sequestosome-1 (p62). Binding of p62 to the malin-laforin complex allows its recognition by LC3, a component of the autophagosomal membrane. In addition, p62 enhances the ubiquitinating activity of the malin-laforin E3-ubiquitin ligase complex. These data enrich our knowledge on the mechanism of action of the malin-laforin complex as an E3-ubiquitin ligase and reinforces the role of this complex in targeting substrates towards the autophagy pathway.

**Key words:** UBE2N, E2-conjugase, p62 (SQSTM1), ubiquitination, E3-ubiquitin ligase, malin, laforin, autophagosome, LC3.

**Abbreviations:** LD, Lafora disease; RING, really interesting new gene; SDS-PAGE, sodium dodecylsulfate electrophoresis, SQSTM1, sequestosome-1; UBE2D2, ubiquitin conjugating enzyme E2-D2; UBE2N, ubiquitin conjugating enzyme E2-N.

## 1.- INTRODUCTION

Lafora disease (LD; OMIM 254780, ORPHA501) is a rare form of progressive myoclonus epilepsy, first described in 1911 by the Spanish neurologist Gonzalo R. Lafora (Lafora and Glueck, 1911). The first symptoms of the disease appear at early adolescence, producing myoclonus, tonic-clonic seizures and epileptic crises, and a progressive dementia, apraxia, aphasia and visual loss (Monaghan and Delanty, 2010). Histologically, the most characteristic hallmark of LD is the accumulation of aberrant glycogen forms named Lafora bodies (LBs) (Minassian, 2001). However, at present, it is still not clear whether the accumulation of LBs is the cause of the disease or a consequence of previous alterations. In the vast majority of cases, the disease is produced by mutations either in the *EPM2A* gene, which encodes the dual specificity phosphatase laforin (Minassian et al., 1998, Serratosa et al., 1999), or in the *EPM2B* gene, encoding the E3 ubiquitin ligase malin (Chan et al., 2003). Patients with mutations in either *EPM2A* or *EPM2B* genes are histologically and neurologically indistinguishable, suggesting that both proteins are involved in the same physiological pathways (Gómez-Abad et al., 2005). It has been described that laforin and malin form a functional complex in which laforin targets malin to the appropriate substrates for their ubiquitination. Several substrates of the malin-laforin complex have been described (Romá-Mateo et al., 2012), including proteins involved in glycogen synthesis. Thereby, the loss of either laforin or malin results in a dysregulation of glycogen metabolism, leading to the formation of LBs (Lohi et al., 2005, Rubio-Villena et al., 2013, Solaz-Fuster et al., 2008, Worby et al., 2008). Other processes have been shown to be affected by mutations in laforin and malin. For instance, the malin-laforin complex participates in the clearance of misfolded proteins through the ubiquitin-proteasome pathway: dysfunction of the malin-laforin complex results in a reduction of proteasomal activity leading to increased sensitivity to endoplasmic reticulum stress (Garyali et al., 2009, Vernia et al., 2009). In addition, a clear impairment in autophagy has also been described both in malin and laforin knock-out mice (Aguado et al., 2010, Criado et al., 2012), as well as the appearance of oxidative stress (Romá-Mateo et al., 2015a). Taken together, these evidences suggest a broad impairment of the protein clearance systems when the malin-laforin complex is not functional, indicating that there are more processes implicated in the disease other than glycogen synthesis and accumulation (Romá-Mateo et al., 2015b).

Ubiquitination is a highly conserved process in eukaryotic organisms, which depends on the sequential activity of three different enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). E1s use ATP to bind a molecule of ubiquitin to the Cys residue in their active site. Then, this ubiquitin is transferred to the Cys residue of an E2. The third step depends on the type of E3 involved: E3s with a HECT domain incorporate themselves the ubiquitin from the E2, before transferring it to the

substrate. However, E3s with a RING domain, like malin, act as scaffolds bringing together the substrate and an activated E2, so the ubiquitin can be transferred directly from the E2 to a Lys residue of the substrate (Ye and Rape, 2009). In this type of E3 ubiquitin ligases, the specific combination of the E2 and the E3 determines the topology and length of the ubiquitin chains conjugated to the substrate [see (Deshaies and Joazeiro, 2009, Metzger et al., 2014, Ye and Rape, 2009) for review]. The function of the ubiquitin chains depends on their topology, which might either label proteins for proteasomal degradation or target them to participate in alternative cellular functions (DNA repair, cell signaling, etc.) (Deshaies and Joazeiro, 2009, Ye and Rape, 2009). For instance, poly-ubiquitin chains linked through Lys63 direct the substrate to autophagy (Pickart and Fushman, 2004).

Autophagy is a degradation pathway existing in all eukaryotic organisms. It is the main system for the degradation of bulk cytoplasmic components in cells (Feng et al., 2014), but it also plays a crucial role in other processes, as immunity (Wileman, 2013), mitochondrial (Dengjel and Abeliovich, 2014) and peroxisomal (Till et al., 2012) degradation and clearance of misfolded protein aggregates (Lamark and Johansen, 2012). These pathways of selective autophagy, in contrast to the more or less random sequestration of cytosol components produced by the bulk autophagy, are regulated by the participation of specific adaptors. The first protein reported to have this adaptor function was p62 (also named sequestosome-1, SQSTM1) (Pankiv et al., 2007), which, in addition to its implication in cell growth and proliferation, was detected in ubiquitinated protein aggregates (Moscat and Diaz-Meco, 2009). p62 possesses, among other interacting motifs, a C-terminal ubiquitin-binding domain (UBA) which allows its interaction with ubiquitinated substrates [containing mainly K63-linked polyubiquitin chains (Seibenhener et al., 2004)] and a short LIR (LC3-interacting region) sequence (Ciani et al., 2003) which allows its interaction with LC3 (Pankiv et al., 2007), a protein anchored in the autophagosomal membrane (Kirisako et al., 1999). In addition, p62 contains an N-terminal PB1 domain that allows homopolymerization of the protein (Johansen and Lamark, 2011). It has been reported that once p62 binds to ubiquitinated proteins, it oligomerizes serving these oligomers as a nucleating factor for autophagosome formation (Lippai and Low, 2014).

In this work, we aimed to deepen our knowledge on the functional regulation of the malin-laforin complex. First, we focused our attention on the E2-conjugase that participates in the ubiquitination reaction. We found that the ubiquitin conjugating enzyme E2-N (UBE2N; Ubc13), one of the more than 38 different E2 conjugating enzymes which are encoded in the human genome (Ye and Rape, 2009), interacts with the malin-laforin complex and modulates its function. Second, we have also found a physical and functional interaction between p62 and the malin-laforin complex, highlighting the relationship between this complex and autophagy.

## 2.- MATERIALS AND METHODS

### 2.1.- Plasmids

Plasmids pCMV-myc-laforin, pCMV-HA-laforin, pFLAG-laforin, pcDNA3-HA-malin, pFLAG-malin and pCMV-myc-R5/PTG are described in (Solaz-Fuster et al., 2008). Plasmid pCMV-6xHisUbiq was kindly provided by Dr. Manuel Rodriguez (Proteomics Unit, CIC-BioGUNE, Vizcaya, Spain), plasmids pCMV-6xHis-Ubiq-K48R and pCMV-6xHis-Ubiq-K63R were a generous gift of Dr. Ch. Blattner (Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Karlsruhe, Germany), plasmid pcDNA3-myc-p62 was kindly provided by Dr. Jorge Moscat (Sanford-Burnham Medical Research Institute, La Jolla, CA 92037, USA), plasmid pEGFP-LC3 was donated by Dr. Erwin Knecht (Centro de Investigación Principe Felipe, Valencia, Spain) and plasmids pV5-DEST-UBE2D2 and pV5-DEST-UBE2D2-C85A were a generous gift from Dr. Peter Tontonoz (Howard Hughes Medical Institute, UCLA, Los Angeles, CA 90095, USA). Plasmid pECFP-laforin was obtained by digesting plasmid pEG202-laforin [described in (Solaz-Fuster et al., 2008)] with BglII/BamHI and subcloning into plasmid pECFP-N1 (BD Biosciences, Madrid, Spain).

UBE2N cDNA was acquired from the Mammalian Gene Collection (cDNA clone MGC:8489; IMAGE:2822013) and amplified by PCR using the following oligonucleotides: forward 5'-GGCGAATTCATGGCCGGGCTGCCCCGC-3' and reverse 5'-CCGCTCGAGTTAAATATTATTCATGGCATATAGCCT-3'. The amplified fragment was digested with EcoRI/XhoI and subcloned into pGEM-T-Easy vector, digested with the same set of enzymes. The construct was sequenced to ensure that no mutations were introduced during amplification. An EcoRI/XhoI fragment containing UBE2N ORF was then subcloned into vector pGADT7 (Clontech, Madrid, Spain) to obtain plasmid pGADT7-UBE2N. An SfiI/XhoI fragment from the latter was subcloned into pCMV-myc and pCMV-HA vectors (BD Biosciences, Madrid, Spain), obtaining plasmids pCMV-myc-UBE2N and pCMV-HA-UBE2N, respectively. An EcoRI/BamHI fragment from pGADT7-UBE2N was subcloned into vector pECFP-N1 to obtain plasmid pECFP-N1-UBE2N. UBE2N-C87A was obtained by site directed mutagenesis using pCMV-myc-UBE2N as template, the Quick Change kit (Stratagene) and the following mutagenic oligonucleotides: forward 5'-GTAGACAAGTTGGGAAGAATAGCTTTAGATATTTTGAAAGATAAG-3' and reverse 5'-CTTATCTTTCAAAATATCTAAAGCTATTCTTCCCAACTTGTCTAC-3'. The amplified fragment was digested with EcoRI/XhoI and subcloned into pGEM-T-Easy vector, digested with the same set of enzymes. The construct was sequenced to ensure that no other mutations were introduced during amplification. The pGEM-T-easy-UBE2N C87A plasmid was digested as described above for wild type UBE2N to obtain the corresponding plasmids based on the following vectors: pGADT7, pCMV-myc, pCMV-HA and pECFP-N1.

**2.2.- Cell culture and immunodetection**

Human embryonic kidney (HEK293) cells were grown in DMEM (Lonza, Barcelona, Spain) supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 10% inactivated fetal bovine serum (GIBCO, Madrid, Spain) in a humidified atmosphere at 37ºC with 5% $CO_2$. Cells were transfected with 3 µg of each plasmid using X-treme GENE HP transfection reagent (Roche Diagnostics, Barcelona, Spain), according to the manufacturer's instructions. Twenty-four hours after transfection, cells were scraped on ice in lysis buffer 1 [25 mM Tris-HCl pH 8, 5 mM NaF, 5 mM $Na_4P_2O_7$, 0.5% Triton X100, 100 mM KCl, protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain) and 1 mM PMSF]. Cells were lysed by repeated passage through a 25-gauge needle and clarified by centrifugation at 13,000xg for 10 min at 4ºC. 50 µg of total protein from the soluble fraction of cell lysates were analyzed by SDS-PAGE and Western blotting using appropriated antibodies: anti-actin, anti-myc, anti-Flag, anti-HA (all from Sigma-Aldrich, Madrid, Spain), anti-laforin (Solaz-Fuster et al., 2008), anti-malin (NeuroMab, California, USA), anti-GFP (ImmunoKontact, Oxon, UK), anti-V5 (Invitrogen, Madrid, Spain) and anti-p62 (Abcam, Cambridge, UK) antibodies.

**2.3.- GFP-trap analysis**

HEK293 cells were transfected with specific constructs of laforin, malin, and the protein of interest (UBE2N, UBE2N-C87A or p62). Cells were scraped on ice in phosphate-buffered saline (PBS), washed twice in PBS and then lysed using a 25-gauge needle in lysis buffer 2 [10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 2.5% glycerol, 0.5% nonidet P-40 (NP-40), complete protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain), 1 mM PMSF, 2.5 mM NaF, 0.5 mM $NaVO_4$ and 2.5 mM $Na_4P_2O_7$]. Cell lysates were then centrifuged at 13,000×g for 10 min at 4ºC. CFP-fused proteins were immunoprecipitated from supernatants (1.5 mg of total protein) by incubating with Chromotek GFP-trap beads (Chromotek, Planegg-Martinsried, Germany) for ten minutes in a rocking platform at 4ºC and visualized by immunoblotting using specific antibodies. As a negative control, a construct expressing CFP alone (plasmid pECFP-N1) was used to confirm the specificity of the interaction.

**2.4.- Analysis of ubiquitination**

To study ubiquitination, we followed the method described in (Kaiser and Tagwerker, 2005). Briefly, HEK293 cells were transfected with plasmid pCMV-6xHis-Ubiq (encoding a modified ubiquitin tagged with 6xHis residues), and plasmids encoding laforin, malin and the protein of interest (R5/PTG, UBE2N, UBE2N-C87A, UBE2D2, UBE2D2-C85A or p62). After

24 hours of transfection, cells were lysed in buffer A (6 M guanidinium-HCl, 0.1 M sodium phosphate, 0.1 M Tris-HCl, pH 8.0) to inhibit the action of deubiquitinases. 1.5 mg of protein of a clarified extract (12,000xg, 15 min) were incubated with 150 µl of a TALON cobalt resin (Clontech, Barcelona, Spain) in buffer A in the presence of 10 mM imidazole (buffer B), for 3 hours at room temperature on a rocking platform, to purify His-tagged proteins. The resin was then successively washed with 1 mL of buffer B and four times with buffer C (buffer B, but with 8 M urea instead of 6 M guanidinium-HCl). Bound proteins were boiled in 40 µl of 2xLaemmli's sample buffer and analyzed by Western blotting using appropriate antibodies. When indicated, plasmids pCMV-6xHis-Ubiq-K48R and pCMV-6xHis-Ubiq-K63R were used in the assay instead of pCMV-6xHis-Ubiq wild type, to determine the topology of the ubiquitin chains.

**2.5.- siRNA UBE2N silencing**

HEK293 cells were treated, in parallel, with a control siRNA (Ambion. Austin, TX, USA) or a specific siRNA against UBE2N (sense, 5'- CCUCUUUGUUUGCAUUUAA[dT][dT]-3'; antisense, 5'- UUAAAUGCAAACAAAGAGG[dT][dT]-3') (Sigma-Aldrich, Madrid, Spain). Cells were transfected at a confluence of 70% using Lipofectamine 2000 (Invitrogen, Carlsbad, CA. USA) according to manufacturer's instructions. Twenty-four hours after transfection, cells were transfected with the indicated plasmids and the analysis of ubiquitination was performed as described above.

**2.6.- Immunofluorescence and confocal microscopy**

Human osteosarcoma U2OS cells were grown as indicated in the case of HEK293 cells. They were transfected with the indicated plasmids and grown on 12-well plates containing coverslips. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Then cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min. Cells were washed three times with PBS and blocked one hour with 10% fetal bovine serum, 5% nonfat dried milk, 0.5% BSA and 0.1% Triton X-100 in PBS. Cells were then incubated overnight at 4 ºC with 1/500 dilution of anti-HA or anti-myc (Sigma–Aldrich, Madrid, Spain) in blocking solution. Samples were washed three times with PBS and incubated with a 1/1000 dilution of anti-mouse Texas Red or anti-rabbit Alexa-Fluor 633 (Invitrogen, Madrid, Spain), respectively. Finally, samples were washed three times with PBS and mounted on slices using Aqua-Poly/Mount coverslipping medium (Polysciences, Inc. Eppelheim, Germany). The co-localization assays were performed with a Leica TCS SP8 confocal microscope (Leica,Wetzlar, Germany) using a HCX PL APO 63x 1.4 NA oil objective. Images were treated with the ImageJ 1.49 software (Wayne Rasband, National Institutes of Health, USA).

## 3.- RESULTS

### 3.1.- The E2-conjugase UBE2N modulates the E3-ubiquitin ligase activity of malin in human HEK293 cells.

Malin is an E3-ubiquitin ligase of the RING type (Gentry et al., 2005, Solaz-Fuster et al., 2008). It is assumed that in RING-type ubiquitin ligases, the topology of the polyubiquitin chains they are able to promote depends on the specific interaction between the E3 and the corresponding E2-conjugases [see (Deshaies and Joazeiro, 2009, Metzger et al., 2014, Ye and Rape, 2009) for review]. Although it is known that malin forms a functional complex with laforin (a dual specificity phosphatase) and mediates the formation of K63-linked polyubiquitin chains into their corresponding substrates (Moreno et al., 2010, Romá-Mateo et al., 2011, Rubio-Villena et al., 2013), little is known about the E2-conjugases that participate in this reaction. So far, it is only known that malin may use the E2-conjugases UBE2H (UbcH2), UBE2D (UbcH5) and UBE2E1 (UbcH6) (in brackets the second name of the same enzyme), in *in vitro* polyubiquitination assays (Gentry et al., 2005, Solaz-Fuster et al., 2008). However, there are no reports on the specific E2-conjugase that associates with malin *in vivo*. As indicated above, malin promotes the formation of K63-linked polyubiquitin chains; thus, we wondered whether UBE2N (Ubc13), an E2 that specifically promotes the formation of this type of polyubiquitin linkages (Eddins et al., 2006, Petroski et al., 2007), associated with malin *in vivo*. To test this possibility we followed an approach based on the fact that when the active cysteine residue of a particular E2 is mutated to alanine, the conjugation activity is no longer functional and the overexpression of such a mutant acts as dominant negative for the particular E2-dependent ubiquitination (Slotman et al., 2012, Zhang et al., 2013). Thus, we decided to overexpress UBE2N-C87A inactive mutant and check the efficiency of the malin-laforin dependent polyubiquitination of laforin. As shown in Fig. 1A and in agreement with previous reports, malin promoted the ubiquitination of laforin (compare lanes 1 and 2). Overexpression of UBE2N did not increase significantly the efficiency of the ubiquitination reaction (Fig. 1A, lane 3), probably because HEK293 cells may have enough endogenous UBE2N to assist the malin-laforin dependent ubiquitination. However, overexpression of the inactive UBE2N-C87A clearly reduced the ubiquitination of laforin (compare lanes 3 and 4). These data indicated that malin required a functional UBE2N to promote the incorporation of ubiquitin chains into laforin.

We extended our analysis to another substrate of the malin-laforin complex such as R5/PTG, a regulatory subunit of type 1 protein phosphatase (Solaz-Fuster et al., 2008), observing again a dramatic reduction in the ubiquitination of this protein when the inactive UBE2N-C87A form was present (Fig. 1B, compare lanes 3 and 4). Interestingly, the overexpression of UBE2D2-C85A (an inactive form of UBE2D2 enzyme, another type of E2

conjugase) had no effect on the rate of ubiquitination of the malin-laforin substrates (see Fig. 1B, lanes 5 and 6), highlighting the specificity of the effect observed with the UBE2N-C87A mutant form. These results collectively suggested the participation of UBE2N in the *in vivo* malin-dependent ubiquitination of substrates.

To confirm the role of UBE2N in the malin-dependent polyubiquitination reaction, we silenced the expression of UBE2N by siRNA. Silencing efficiency was estimated around 73% as demonstrated by qRT-PCR analysis under the assayed conditions (not shown). We then overexpressed the malin-laforin complex in the silenced cells. As shown in Fig. 2A, specific downregulation of UBE2N expression diminished the efficiency of the malin-dependent ubiquitination of laforin (compare lanes 2 and 4). We repeated the same analysis for the modification of R5/PTG (Fig. 2B; compare lanes 2 and 4) with similar results. Therefore, a decrease in the expression of UBE2N impairs the malin-dependent ubiquitination of its substrates.

Finally we decided to check if there was any physical interaction between UBE2N and the malin-laforin complex. We performed yeast two-hybrid analysis between UBE2N, malin and laforin but we did not observe any positive reaction. As the putative interaction could be too transient to be recorded by the two-hybrid analysis, we decided then to make a protein fusion of UBE2N to CFP and use the GFP-trap assay (a particular kind of pull-down assay) to check the putative physical interaction. HEK293 cells were transfected with plasmids expressing malin and laforin and with plasmids expressing CFP-UBE2N-WT, CFP-UBE2N-C87A or CFP alone. Crude extracts were obtained, pulled down with anti-GFP-beads (GFP-trap) and proteins in the pellet were analyzed by immunoblotting using specific antibodies. As observed in Fig. 3, CFP-UBE2N was able to specifically pull down both malin and laforin, as these proteins were absent in the samples that expressed CFP alone (compare lanes 1 and 2). Since the yield of the purification was higher for malin than for laforin, we suggest that UBE2N interacts better with malin, and probably the laforin species that are detected come down because of the interaction between malin and laforin (Fig. 3, lane 2). Surprisingly, the efficiency of the interaction with malin decreased dramatically when the UBE2N-C87A mutant was expressed (Fig. 3, compare lanes 2 and 3). As this mutant is unable to bind ubiquitin, this might reflect that the binding of malin to UBE2N is higher if this E2 is loaded with ubiquitin, as reported previously in the binding of other E2-E3 complexes (Eddins et al., 2006).

Taking all these results together, we suggest that UBE2N participates in the *in vivo* ubiquitination of malin-dependent substrates.

### 3.2.- The selective autophagy modulator p62 (SQSTM1) enhances the malin-dependent ubiquitination reaction.

It has been described that p62 (SQSTM1), a scaffolding protein involved in selective autophagy (Johansen and Lamark, 2011, Shaid et al., 2013, Stolz et al., 2014), interacts with the E3-ubiquitin ligase TRAF6 (which directs the synthesis of K63-linked polyubiquitin chains on specific substrates) and regulates its K63-polyubiquitination and oligomerization (Wooten et al., 2005). TRAF6 also associates with the E2 UBE2N in the polyubiquitination reaction (Deng et al., 2000). Due to the similarities in the mechanisms of action of TRAF6 and malin, we decided to check whether p62 could affect the efficiency of the polyubiquitination reaction mediated by malin. With this aim, we overexpressed p62 in HEK293 cells and compared the efficiency of the malin-laforin dependent ubiquitination of substrates in the presence or absence of this protein. As observed in Fig. 4, the expression of p62 enhanced both the malin-dependent ubiquitination of laforin (compare lanes 3-4 in first panel) and malin itself (compare lanes 1-2, and 3-4, in second panel). p62 was also ubiquitinated when overexpressed, but this reaction was strongly increased when both malin and laforin were present (compare lane 2 and lane 4 in third panel).

We next determined whether the malin-laforin dependent ubiquitination of p62 was dependent on UBE2N. As shown in Fig. 5A, we observed a drastic reduction in the ubiquitination of p62 when the catalytically inactive UBE2N C87A was present in the assay (compare lanes 3 and 4), indicating a role of UBE2N in this modification. In addition we analyzed the topology of the ubiquitin chains that the malin-laforin complex was able to promote on p62. As shown in Fig. 5B, p62 was still fully polyubiquitinated when a modified K48R form of ubiquitin (which prevented the formation of K48-linked ubiquitin chains) was present in the assay (compare lanes 1 and 2), whereas p62 ubiquitination was drastically reduced when a modified K63R form of ubiquitin (which prevented the formation of K63-linked ubiquitin chains) was present (compare lane 1 and 3). These results clearly indicate that the malin-laforin complex promotes the formation of K63-linked ubiquitin chains into p62.

To understand the enhancer property of p62 on malin-dependent ubiquitination, we checked whether there was a physical interaction between p62 and the malin-laforin complex. As observed in Fig. 6, when crude extracts from HEK293 cells expressing CFP-laforin, HA-malin and myc-p62 were pulled down with anti-GFP-beads (GFP-trap), both malin and p62 were recovered in the pellet (compare lanes 2 and 4). However, if malin was not overexpressed (lane 3), CFP-laforin was not able to pull down p62. These data suggested that malin was required to allow the interaction of p62 with laforin. We also observed that this tertiary complex (laforin-malin-p62) was still able to maintain a physical interaction with UBE2N (Fig. 6, lane 5), as this E2 was recovered in the pulldown pellet. Next, we wanted to assess if the interaction between p62 and the malin-laforin complex was dependent on the ubiquitination of these proteins, since as p62 possesses an ubiquitin-binding domain (UBA), the reported interaction could be mediated by the ubiquitin moieties. However, the

overexpression of UBE2N-C87A, which, as demonstrated above greatly diminished the malin-laforin complex ubiquitination activity, did not affect the interaction between p62 and the malin-laforin complex (Fig. 6 lane 6).

The interaction between the malin-laforin complex and p62 was confirmed by immunofluorescence. These experiments were performed with human osteosarcoma U2OS cells since they are larger and allow a better observation of the subcellular compartments [similar malin-laforin dependent ubiquitination of p62 was observed in these cells (not shown)]. U2OS cells were transfected with the corresponding plasmids and the associated fluorescence to each protein visualized by confocal microscopy. As shown in Fig. 7 (panel 1), in cells expressing HA-malin and myc-p62 alone, malin showed a mainly nuclear localization of the protein, as previously described (Sengupta et al., 2011) and p62 showed a punctuated distribution; however no major co-localization of both proteins was observed. Interestingly, when U2OS cells co-expressed CFP-laforin at the same time, all the proteins (laforin, malin and p62) localized in similar punctuated structures and malin was not longer localized inside the nucleus (Fig. 7, panel 2). When CFP-laforin was co-expressed only with HA-malin (Fig. 7, panel 3) or only with myc-p62 (Fig. 7, panel 4) similar punctuated structures were observed that contained the two proteins under study. However, the expression of CFP-laforin alone did not produce these structures (Fig. 7, panel 5). These results indicate that the combined expression of CFP-laforin with any of the other proteins results in punctuated structures which recruit malin and p62.

Since p62 is a selective adaptor of autophagy that interacts with LC3 (Pankiv et al., 2007), a protein anchored in the autophagosomal membrane (Kirisako et al., 1999), we decided to check whether the punctuated structures that contained laforin, malin and p62 were related to autophagy. With this aim we transfected U2OS cells with a plasmid expressing GFP-LC3 and checked whether the expression of laforin, malin and p62 co-localized with this autophagy marker. As shown in Fig. 8 (panel 1), GFP-LC3 clearly co-localized with p62 as already known. However, cells expressing laforin and malin did not show any co-localization with GFP-LC3 (Fig. 8, panel 2). The co-expression of p62 in these cells resulted in a co-localization of LC3, p62, malin and laforin in punctuated structures (Fig. 8, panel 3). If cells expressed GFP alone instead of GFP-LC3, the punctuated structures containing p62, malin and laforin were still visible, although they did not co-localize with GFP fluorescence, confirming the results presented in Fig. 7 panel 2. These results indicate that the expression of laforin, malin and p62 produces punctuated structures that are recognized by the autophagy machinery (LC3).

Taking together, these results suggest that there is a physical and functional interaction between the malin-laforin complex, p62 and the autophagosome.

## 4.- DISCUSSION

Pathophysiology of LD involves alterations in many cellular processes, among which glycogen metabolism and protein homeostasis (proteostasis) mechanisms seem to play a central role. In the last years, growing evidence of numerous and particularly diverse molecular pathways such as the unfolded protein and heat-shock responses (Sengupta et al., 2011), antioxidant defense systems (Romá-Mateo et al., 2015a) and autophagy (Aguado et al., 2010, Criado et al., 2012) have been reported to be altered as a consequence of mutations in either malin and laforin, the two proteins related to the disease. Most of these alterations correlate with a dysfunction of the E3-ubiquitin ligase complex formed by malin and laforin; however, there is scarce evidence reporting the specific molecular determinants that participate in the ubiquitination reaction mediated by the complex and how it takes place.

Ubiquitination is a versatile modification that can lead substrates to a wide variety of destinations. Knowing the modulators that participate in the malin-laforin mediated ubiquitination will allow us to better understand the multiple physiological roles of these proteins, providing novel insights and deeper knowledge of the pathophysiology of LD as well as widening our understanding of the complex interactions that drive specific intracellular signaling pathways. In the present work, we have identified novel partners that affect the E3-ubiquitin ligase activity of the malin-laforin complex. First, we have studied which E2-conjugase interacts with the complex. Since there are more than 38 genes encoding for E2 enzymes in humans, and our group previously found evidence showing that the malin-laforin complex incorporates K63-linked ubiquitin chains (Moreno et al., 2010, Romá-Mateo et al., 2011, Rubio-Villena et al., 2013), we centered our study in UBE2N, the only E2 with a known specificity for the incorporation of these type of chains (Eddins et al., 2006, Petroski et al., 2007). Two complementary approaches were performed to assess the relevance of UBE2N on the malin-laforin complex mediated ubiquitination. First, it is known that mutation of the catalytic cysteine of an E2 enzyme to alanine provides a stable form which can no longer bind ubiquitin, but its overexpression has a dominant negative effect, impeding endogenous functional E2s to bind their putative E3s and transfer the ubiquitin. This approach has been successfully employed to study the interaction of UBE2N with other E3s (Slotman et al., 2012), and for other E2 enzymes such as UBE2D2 (Gonen et al., 1999). Our results show that overexpression of the catalytically inactive version of UBE2N (UBE2N-C87A) drastically impairs the ubiquitination of different substrates by the malin-laforin complex. However, taking into account that the overexpression of this dominant negative mutant could affect the whole cellular ubiquitination machinery, we performed a complementary approach based on the specific silencing of the expression of the endogenous UBE2N. Again, when HEK293 cells were silenced with specific siRNA against UBE2N, the activity of the malin-laforin

complex was diminished. Therefore, both approaches confirmed the participation of UBE2N in the malin-laforin complex mediated ubiquitination of different substrates.

Despite the weak and transient nature of E2-E3 interactions, this binding is required for the transfer of ubiquitin to the substrate. The low affinity characteristics of this interaction may be due to the fact that E2s use the same surface to interact with E1s and E3s, and hence while binding to an E3 enzyme, they cannot be recharged with ubiquitin by an E1. Thus, binding of E2 to E3 is necessarily weak in order to allow E2 to be involved in repeated cycles of interaction with E1 and E3 (Ye and Rape, 2009). In this sense, traditional interaction analysis methodologies, such as the yeast two-hybrid system, may not be sensitive enough to detect the E2-E3 interaction, and perhaps for this reason we did not detect any two-hybrid interaction between UBE2N and laforin or malin. We obtained better results when using GFP-trap, a pull-down assay based on the use of anti-GFP antibodies which has been previously employed to detect this kind of weak interactions (Hoxhaj et al., 2012). Using this technique, we observed that UBE2N interacted physically with the malin-laforin complex, and our results also suggested a stronger interaction between UBE2N and malin. The interaction also occurred when the UBE2N-C87A form was used in the assay, although with reduced intensity. This latter result may suggest that binding between UBE2N and malin is increased if ubiquitin is attached to the E2, as already reported for other E2-E3 complexes (Eddins et al., 2006).

K63-linked ubiquitination of substrates has been related to autophagic degradation (Deshaies and Joazeiro, 2009, Tan et al., 2008). During this process, cargos are bound by specific adaptors which recognize both the ubiquitin chains and components in the autophagosome membrane, recruiting the substrates for their degradation (Lippai and Low, 2014). Previous publications have shown a relationship between LD and autophagy (Aguado et al., 2010, Criado et al., 2012). Here, we show that one of the specific adaptors that mediates selective autophagy, p62 (SQSTM1), interacts with the malin-laforin complex. This interaction was not dependent on the ubiquitination state of the complex, since in the presence of UBE2N-C87A, which is unable to bind ubiquitin, the interaction of p62 with the complex was maintained. Remarkably, binding of p62 to the malin-laforin complex enhanced its E3-ubiquitin ligase activity. As the malin-laforin complex generates K63-linked polyubiquitin chains (Moreno et al., 2010, Romá-Mateo et al., 2011, Rubio-Villena et al., 2013) and p62 seems to have a preference for this type of modifications (Seibenhener et al., 2004), the interaction between the malin-laforin complex and p62 is in agreement with the clearance of malin-dependent substrates by autophagy. In addition, the fact that p62 is ubiquitinated by the malin-laforin complex with K63-ubiquitin linked chains might create a positive feedback loop where the modified p62 could recruit more p62, facilitating in this way its oligomerization, which would induce autophagy, as reported in other cases (Johansen and

Lamark, 2011, Shaid et al., 2013). We also present evidence indicating that malin-laforin complex and p62 form multimeric structures that are recognized by LC3 which would target them to the autophagosome (Fig. 8).

In summary, we describe here two proteins that interact with the malin-laforin complex and modulate its ubiquitination activity. These new partners again highlight a link between proteins involved in LD and autophagy. Presumably, as we propose in Fig. 9, UBE2N interacts with the malin-laforin complex in order to promote the incorporation of K63-linked ubiquitin chains in the corresponding substrates. These chains would be recognized by p62, which would direct them to degradation via autophagy. This hypothesis would be in agreement with the previous observation that there is autophagy dysfunction in laforin- and malin-deficient mice (Aguado et al., 2010, Criado et al., 2012) and further corroborates the relevance of a functional malin-laforin complex in the maintenance of cellular protein homeostasis.

## 5.- ACKNOWLEDGEMENTS

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## 6.- AUTHOR CONTRIBUTION

P.S-M., C.R-M. and R.V. carried out the experimental work. P.S-M., C.R-M. and P.S. analyzed the data. P.S-M. and P.S. wrote the paper.

**7.- REFERENCES**

Aguado C, Sarkar S, Korolchuk VI, Criado O, Vernia S, Boya P, et al. Laforin, the most common protein mutated in Lafora disease, regulates autophagy. Hum Mol Genet. 2010;19:2867-2876.

Ciani B, Layfield R, Cavey JR, Sheppard PW, Searle MS. Structure of the ubiquitin-associated domain of p62 (SQSTM1) and implications for mutations that cause Paget's disease of bone. J Biol Chem. 2003;278:37409-37412.

Criado O, Aguado C, Gayarre J, Duran-Trio L, Garcia-Cabrero AM, Vernia S, et al. Lafora bodies and neurological defects in malin-deficient mice correlate with impaired autophagy. Hum Mol Genet. 2012;21:1521-1533.

Chan EM, Young EJ, Ianzano L, Munteanu I, Zhao X, Christopoulos CC, et al. Mutations in NHLRC1 cause progressive myoclonus epilepsy. Nat Genet. 2003;35:125-127.

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, et al. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell. 2000;103:351-361.

Dengjel J, Abeliovich H. Musical chairs during mitophagy. Autophagy. 2014;10:706-707.

Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. Annu Rev Biochem. 2009;78:399-434.

Eddins MJ, Carlile CM, Gomez KM, Pickart CM, Wolberger C. Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. Nat Struct Mol Biol. 2006;13:915-920.

Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell research. 2014;24:24-41.

Garyali P, Siwach P, Singh PK, Puri R, Mittal S, Sengupta S, et al. The malin-laforin complex suppresses the cellular toxicity of misfolded proteins by promoting their degradation through the ubiquitin-proteasome system. Hum Mol Genet. 2009;18:688-700.

Gentry MS, Worby CA, Dixon JE. Insights into Lafora disease: malin is an E3 ubiquitin ligase that ubiquitinates and promotes the degradation of laforin. Proc Natl Acad Sci U S A. 2005;102:8501-8506.

Gómez-Abad C, Gómez-Garre P, Gutiérrez-Delicado E, Saygi S, Michelucci R, Tassinari CA, et al. Lafora disease due to EPM2B mutations. A clinical and genetic study. Neurology. 2005;64:982-986.

Gonen H, Bercovich B, Orian A, Carrano A, Takizawa C, Yamanaka K, et al. Identification of the ubiquitin carrier proteins, E2s, involved in signal-induced conjugation and subsequent degradation of IkappaBalpha. J Biol Chem. 1999;274:14823-14830.

Hoxhaj G, Najafov A, Toth R, Campbell DG, Prescott AR, MacKintosh C. ZNRF2 is released from membranes by growth factors and, together with ZNRF1, regulates the Na+/K+ATPase. J Cell Sci. 2012;125:4662-4675.

Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy. 2011;7:279-296.

Kaiser P, Tagwerker C. Is this protein ubiquitinated? Methods in enzymology. 2005;399:243-248.

Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, et al. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. The Journal of cell biology. 1999;147:435-446.

Lafora GR, Glueck B. Beitrag zur histogpathologie der myoklonischen epilepsie. Gesamte Neurol Psychiatr. 1911;6:1-14.

Lamark T, Johansen T. Aggrephagy: selective disposal of protein aggregates by macroautophagy. Int J Cell Biol. 2012;2012:736905.

Lippai M, Low P. The role of the selective adaptor p62 and ubiquitin-like proteins in autophagy. Biomed Res Int. 2014;2014:832704.

Lohi H, Ianzano L, Zhao XC, Chan EM, Turnbull J, Scherer SW, et al. Novel glycogen synthase kinase 3 and ubiquitination pathways in progressive myoclonus epilepsy. Hum Mol Genet. 2005;14:2727-2736.

Metzger MB, Pruneda JN, Klevit RE, Weissman AM. RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. Biochim Biophys Acta. 2014;1843:47-60.

Minassian BA. Lafora's disease: towards a clinical, pathologic, and molecular synthesis. Pediatr Neurol. 2001;25:21-29.

Minassian BA, Lee JR, Herbrick JA, Huizenga J, Soder S, Mungall AJ, et al. Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. Nat Genet. 1998;20:171-174.

Monaghan TS, Delanty N. Lafora disease: epidemiology, pathophysiology and management. CNS drugs. 2010;24:549-561.

Moreno D, Towler MC, Hardie DG, Knecht E, Sanz P. The laforin-malin complex, involved in Lafora disease, promotes the incorporation of K63-linked ubiquitin chains into AMP-activated protein kinase beta subunits. Molecular biology of the cell. 2010;21:2578-2588.

Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. Cell. 2009;137:1001-1004.

Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem. 2007;282:24131-24145.

Petroski MD, Zhou X, Dong G, Daniel-Issakani S, Payan DG, Huang J. Substrate modification with lysine 63-linked ubiquitin chains through the UBC13-UEV1A ubiquitin-conjugating enzyme. J Biol Chem. 2007;282:29936-29945.

Pickart CM, Fushman D. Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol. 2004;8:610-616.

Romá-Mateo C, Aguado C, Garcia-Gimenez JL, Ibanez-Cabellos JS, Seco-Cervera M, Pallardo FV, et al. Increased oxidative stress and impaired antioxidant response in lafora disease. Mol Neurobiol. 2015a;51:932-946.

Romá-Mateo C, Aguado C, Luis Garcia-Gimenez J, Knecht E, Sanz P, Pallardo FV. Oxidative stress, a new hallmark in the pathophysiology of Lafora progressive myoclonus epilepsy. Free Radic Biol Med. 2015b;88:30-41.

Romá-Mateo C, Moreno D, Vernia S, Rubio T, Bridges TM, Gentry MS, et al. Lafora disease E3-ubiquitin ligase malin is related to TRIM32 at both the phylogenetic and functional level. BMC evolutionary biology. 2011;11:225.

Romá-Mateo C, Sanz P, Gentry MS. Deciphering the role of malin in the lafora progressive myoclonus epilepsy. IUBMB Life. 2012;64:801-808.

Rubio-Villena C, Garcia-Gimeno MA, Sanz P. Glycogenic activity of R6, a protein phosphatase 1 regulatory subunit, is modulated by the laforin-malin complex. Int J Biochem Cell Biol. 2013;45:1479-1488.

Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, Wooten MW. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol. 2004;24:8055-8068.

Sengupta S, Badhwar I, Upadhyay M, Singh S, Ganesh S. Malin and laforin are essential components of a protein complex that protects cells from thermal stress. J Cell Sci. 2011;124:2277-2286.

Serratosa JM, Gomez-Garre P, Gallardo ME, Anta B, de Bernabe DB, Lindhout D, et al. A novel protein tyrosine phosphatase gene is mutated in progressive myoclonus epilepsy of the Lafora type (EPM2). Hum Mol Genet. 1999;8:345-352.

Shaid S, Brandts CH, Serve H, Dikic I. Ubiquitination and selective autophagy. Cell death and differentiation. 2013;20:21-30.

Slotman JA, da Silva Almeida AC, Hassink GC, van de Ven RH, van Kerkhof P, Kuiken HJ, et al. Ubc13 and COOH terminus of Hsp70-interacting protein (CHIP) are required for growth hormone receptor endocytosis. J Biol Chem. 2012;287:15533-15543.

Solaz-Fuster MC, Gimeno-Alcaniz JV, Ros S, Fernandez-Sanchez ME, Garcia-Fojeda B, Criado Garcia O, et al. Regulation of glycogen synthesis by the laforin-malin complex is modulated by the AMP-activated protein kinase pathway. Hum Mol Genet. 2008;17:667-678.

Stolz A, Ernst A, Dikic I. Cargo recognition and trafficking in selective autophagy. Nat Cell Biol. 2014;16:495-501.

Tan JM, Wong ES, Kirkpatrick DS, Pletnikova O, Ko HS, Tay SP, et al. Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Hum Mol Genet. 2008;17:431-439.

Till A, Lakhani R, Burnett SF, Subramani S. Pexophagy: the selective degradation of peroxisomes. Int J Cell Biol. 2012;2012:512721.

Vernia S, Rubio T, Heredia M, Rodriguez de Cordoba S, Sanz P. Increased endoplasmic reticulum stress and decreased proteasomal function in lafora disease models lacking the phosphatase laforin. PloS one. 2009;4:e5907.

Wileman T. Autophagy as a defence against intracellular pathogens. Essays in biochemistry. 2013;55:153-163.

Wooten MW, Geetha T, Seibenhener ML, Babu JR, Diaz-Meco MT, Moscat J. The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. J Biol Chem. 2005;280:35625-35629.

Worby CA, Gentry MS, Dixon JE. Malin decreases glycogen accumulation by promoting the degradation of protein targeting to glycogen (PTG). J Biol Chem. 2008;283:4069-4076.

Ye Y, Rape M. Building ubiquitin chains: E2 enzymes at work. Nature reviews. 2009;10:755-764.

Zhang L, Xu M, Scotti E, Chen ZJ, Tontonoz P. Both K63 and K48 ubiquitin linkages signal lysosomal degradation of the LDL receptor. J Lipid Res. 2013;54:1410-1420.

**8.- FIGURE LEGENDS**

**Fig. 1. E3 ubiquitin ligase activity of the malin-laforin complex depends on the activity of the E2-conjugase UBE2N.** HEK293 cells were transfected with the indicated plasmids and ubiquitination analysis of different substrates carried out as described in Materials and Methods. A) Ubiquitination assays of laforin; B) Ubiquitination assays of R5/PTG. When indicated catalytically inactive forms of UBE2N (C87A) or UBE2D2 (C85A) were expressed. Proteins present in the bound fraction (Bound: proteins retained in the metal affinity resin) or in the crude extract (CE, 50 µg) were analyzed by Western blotting using the indicated antibodies (the fuzzy bands in the CE are probably due to the presence of 6 M guanidinium hydrochloride in the extracts). Molecular size markers are indicated. The position of ubiquitinated forms of the corresponding substrates is also indicated. A representative blot of three independent experiments is shown.

**Fig. 2. UBE2N silencing by siRNA diminishes the E3 activity of the malin-laforin complex.** HEK293 cells were transfected with siRNA against UBE2N (+) or with negative control siRNA (-). Twenty-four hours after transfection, cells were transfected again with the corresponding plasmids and ubiquitination assays performed as indicated in Materials and Methods. A) Effect of UBE2N silencing on laforin ubiquitination; B) Effect of UBE2N silencing on R5/PTG ubiquitination (the two panels correspond to different areas of the same gel). Proteins present in the bound fraction (Bound: proteins retained in the metal affinity resin) or in the crude extract (CE, 50 µg) were analyzed by Western blotting using the indicated antibodies (the fuzzy bands in the CE are probably due to the presence of 6 M guanidinium hydrochloride in the extracts). Molecular size markers are indicated. The position of ubiquitinated forms of the corresponding substrates is also indicated. A representative blot of three independent experiments is shown.

**Fig. 3. The malin-laforin complex interacts physically with UBE2N.** HEK293 cells were co-transfected with plasmids expressing HA-laforin, HA-malin and CFP (empty, UBE2N WT or UBE2N C87A). Cells were lysed and 1.5 mg of proteins were incubated with GFP-trap beads (Chromotek), which bind CFP forms with high affinity. After washing, beads were boiled in loading buffer and the purified proteins analyzed by SDS-PAGE and Western blot using anti-GFP and anti-HA antibodies. GFP-trap beads: proteins retained in the resin; C.E: crude extracts (50 µg of protein). A representative blot of three independent experiments is shown.

**Fig. 4. Overexpression of p62 (SQSTM1) enhances the ubiquitination activity of the malin-laforin complex.** HEK293 cells were transfected with the indicated plasmids and

ubiquitination analysis of different substrates carried out as described in Materials and Methods. Proteins present in the bound fraction (Bound: proteins retained in the metal affinity resin) or in the crude extract (CE, 50 µg) were analyzed by Western blotting using the indicated antibodies (the fuzzy bands in the CE are probably due to the presence of 6 M guanidinium hydrochloride in the extracts). Molecular size markers are indicated. The position of ubiquitinated forms of the corresponding substrates is also indicated. A representative blot of three independent experiments is shown.

**Fig. 5. The E2 conjugase UBE2N participates in the malin-laforin dependent ubiquitination of p62, which is modified with K63-linked ubiquitin chains. A)** Involvement of UBE2N in the ubiquitination of p62; **B)** Topology of the ubiquitination reaction. HEK293 cells were transfected with the indicated plasmids and ubiquitination analysis of different substrates carried out as described in Materials and Methods. Proteins present in the bound fraction (Bound: proteins retained in the metal affinity resin) or in the crude extract (CE, 50 µg) were analyzed by Western blotting using the indicated antibodies (the fuzzy bands in the CE are probably due to the presence of 6 M guanidinium hydrochloride in the extracts). Molecular size markers are indicated. The position of ubiquitinated forms of p62 is also indicated. A representative blot of three independent experiments is shown.

**Fig. 6. p62 interacts physically with the malin-laforin complex.** HEK293 cells were co-transfected with plasmids expressing CFP-laforin (or CFP alone), HA-malin, myc-p62 and myc-UBE2N (WT or C87A). Cells were lysed and 1.5 mg of proteins were incubated with GFP-trap beads (Chromotek), which bind CFP forms with high affinity. After washing, beads were boiled in loading buffer and the purified proteins analyzed by SDS-PAGE and Western blot using anti-GFP, anti-myc, anti-p62 and anti-HA antibodies. GFP-trap beads: proteins retained in the resin; C.E: crude extracts (50 µg of protein). A representative blot of three independent experiments is shown.

**Fig. 7. Immunofluorescence analyses confirm the interaction of the malin-laforin complex with p62.** U2OS cells were transfected with the indicated plasmids. Then the subcellular localization of the corresponding proteins was analyzed either by direct fluorescence (in the case of CFP-fused proteins) or by immunofluorescence using anti-HA and anti-myc antibodies, as described in Materials and Methods. Fluorescence associated to the corresponding proteins was analyzed by confocal microscopy. A merge analysis of two or more fluorescent dyes is also presented. Bar: 30 µm.

**Fig. 8. Punctuated structures containing the malin-laforin complex and p62 co-localize with LC3, an autophagosomal marker.** U2OS cells were transfected with the indicated plasmids. Then the subcellular localization of the corresponding proteins was analyzed either by direct fluorescence (in the case of GFP-fused proteins) or by immunofluorescence using anti-HA and anti-myc antibodies, as described in Materials and Methods. When indicated, cells also contained FLAG-laforin. Fluorescence associated to the corresponding proteins was analyzed by confocal microscopy. A merge analysis of two or more fluorescent dyes is also presented. Bar: 30 μm.

**Fig. 9. Modulators of the ubiquitinating activity of the malin-laforin complex.** The schematic representation shows the proposed physiological effects of UBE2N and p62 on the activity of the malin-laforin E3-ubiquitin ligase complex. UBE2N and p62 interact with the malin-laforin complex. UBE2N promotes the incorporation of the K63-linked ubiquitin chains into the corresponding substrate, being these chains recognized by p62. This modification will target the substrates for autophagy degradation, using p62 as a selective autophagy adaptor, which would interact with LC3, an autophagosomal marker. Ub, ubiquitin; UBA, ubiquitin associated binding domain; LIR, LC3-interacting region. See text for details.