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# The laforin/malin E3-ubiquitin ligase complex ubiquitinates members of the Hsp70 and Hsp90 protein family

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**Article**

**Keywords:**

**Posted Date:** July 31st, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-10069748/v1

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**Additional Declarations:** No competing interests reported.

**Version of Record:** A version of this preprint was published at Scientific Reports on October 29th, 2026.
See the published version at https://doi.org/10.1038/s41598-026-78451-z.

# Abstract

The presence of misfolded and aggregated proteins presents a problem in cellular protein homeostasis. To avoid this, cells have evolved powerful protein quality control systems that ensure proper folding or disposal of misfolded and aggregated proteins. Some of these systems are the ATP-dependent chaperones, such as Hsp70s and Hsp90s. By cycles of binding to substrates and ATP hydrolysis, and with the help of additional co-chaperones, these systems participate in the proper folding of misfolded proteins. The activity of the Hsp70s and Hsp90s is regulated by post-translational modifications, one of them being ubiquitination. In this work, we present evidence of the participation of the laforin/malin complex in the ubiquitination of several components of the Hsp70s and Hsp90s systems, such as HspA1L, Hsp90, Hsp90 and the co-chaperone Hop/Stip1. In addition, we show that there is a physical interaction between HspA1L and both laforin and malin. In the case of Hsp90, we observed a direct interaction with malin but not with laforin, and in the case of Hop/Stip1, both laforin and malin are needed for the interaction with this co-chaperone. Our work identifies the laforin/malin complex as a new E3-ubiquitin ligase that introduces post-translational modifications in these proteins, which may regulate their activity.


# INTRODUCTION

Around 30% of the proteins that are synthesized *de novo* do not fold properly, so they are targeted for degradation. Misfolded proteins (nascent proteins, folding intermediates) tend to aggregate due to the exposure of hydrophobic residues and unpaired b-strands, leading to the formation of initial fibrils that eventually aggregate, resulting in the formation of amorphous protein inclusions [1]. To avoid this problem, cells have evolved powerful protein quality control systems that ensure proper folding or disposal of misfolded and aggregated proteins [2]. The main systems related to proper folding include ATP-independent chaperones, such as small heat shock proteins (sHSPs), and ATP-dependent chaperones such as Hsp70s and Hsp90s, which function with the help of additional co-chaperones. The two main protein degradation pathways that eliminate misfolded proteins are the ubiquitin-proteasome system (UPS) and autophagy [3], [4].

The 70 kDa heat shock protein chaperone system (Hsp70s) plays a central role in protein folding. By an ATP-dependent mechanism, it participates in the unfolding of misfolded proteins [5]. Hsp70s act as dimers and have two conformations: the open ATP-bound state, which recognizes the misfolded protein substrate, and the closed ADP-bound state, which traps the substrate. They contain an N-terminal nucleotide binding domain (NTD) ligated to the C-terminal substrate binding domain (SBD) by a flexible linker [4, 6]. Hsp70s never work alone since their interaction with the substrate is facilitated by the collaboration of other chaperones such as J-domain proteins (JDPs; e.g., members of the Hsp40 protein family), which bind directly to substrates, recruit them to the Hsp70s in their ATP-bound state, and promote ATP hydrolysis for substrate capture. In addition, nucleotide exchange factors [NEFs; e.g., members of the Bcl-2 associated athanogene (BAG) protein family], promote substrate release from

Hsp70s by facilitating ADP dissociation, and accelerate the reset of Hsp70s to the open ATP-bound state [4, 6]. Fine tuning of the activity of Hsp70s come from factors such as the Hsp70-interacting protein (HIP/St13), which maintains Hsp70s in the closed ADP-bound state, thereby antagonizing in this way the action of NEFs; Hsp70/Hsp90-organizing protein (Hop/Stip1), which participates in the transfer of the unfolded substrate from the Hsp70s to the Hsp90s (see below); and the E3-ubiquitin ligase C-terminus of Hsp70-interacting protein (CHIP), which mediates the ubiquitination of proteins that have not succeeded in folding by the action of Hsp70s, targeting them form degradation at the proteasome [4, 6].

Important family members of Hsp70s are: i) HSPA1A and HSPA1B, which are inducible forms related to stress; ii) HSPA1L, which is similar to HSPA1A but of constitutive expression; iii) HSPA5 (also known as BIP/GRP78), which is located at the endoplasmic reticulum (ER) and mediates the unfolded protein response mechanism (UPR) in this organelle; and iv) HSPA8, involved in the process of chaperone mediated autophagy [7, 8]. The Hsp70s play a protective role in different neurological conditions. In this sense, HSPA1A interacts with Toll-like receptor 4 (TLR4), preventing microglia activation and the spread of seizure activity. Hsp70s also exert anti-inflammatory action by reducing NF-kB activity, playing in this way a neuroprotective role in neurodegenerative diseases [9].

The 90 kDa heat shock protein chaperone system (Hsp90s) are ATP-dependent heat shock proteins. They are very abundant, accounting for 1-2% of total cellular proteins. These chaperones collaborate with Hsp70s in the refolding of misfolded proteins. They also act as dimers and contain three domains: the N-terminal ATP-binding domain (NTD), the middle domain (MD) that associates with substrates, and the C-terminal dimerization domain (CTD) [10]. Hsp90s stay in an NTD-open conformation, forming dimers through the CTD domain. The substrate is loaded at the MD domain, and binding of ATP at the NTD induces a closed conformation of Hsp90. Upon ATP hydrolysis, ADP and the folded substrate are released from Hsp90s [11]. Hsp90s are involved in maintaining the stability and activity of more than 300 proteins (see a list of Hsp90 interactors in https://picard.ch/downloads/Hsp90interactors.pdf), indicating that more than 10% of the human proteome are known interactors of Hsp90s [6 10]. There are two main cytosolic isoforms of Hsp90s: Hsp90a, which is inducible under stress conditions, and Hsp90b, which has a constitutive expression. Although they share 85% homology, some substrates and functions are specific for each isoform: e.g., Hsp90a participates in processes related to adaptation to stress conditions, whereas Hsp90b participates in the maintenance of standard cellular functions [12].

Hsp90s interact with other chaperones and co-chaperones. As indicated above, Hop/Stip1 facilitates the substrate handover between Hsp70s and Hsp90s. This monomeric protein binds Hsp70s and Hsp90s at the same time, forming a ternary complex [13]. Hop/Stip1 has a diffused cytosolic localization, but under stress conditions, it can be found in perinuclear foci [13].

Since both Hsp70s and Hsp90s play a role in proteostasis, in this work, we have analyzed their role in a particular neurological condition that is affected by proteostatic stress, namely Lafora disease (LD, OMIM#254789, ORPHA 501), a rare and fatal form of progressive myoclonus epilepsy. LD is

characterized by the accumulation of aberrant forms of poorly branched glycogen (polyglucosans, PGs), in the brain and peripheral tissues [14]. LD starts in late childhood or early adolescence with the onset of seizures, myoclonus, and visual hallucinations. The disease progresses rapidly with the appearance of tonic-clonic seizures, which become continuous. Eventually, the patient dies approximately 10 years after the onset of the first symptoms [15]. LD is an autosomal recessive condition caused by mutations in the *EPM2A* or *NHLRC1/EPM2B* genes, encoding respectively laforin, a glucan phosphatase, and malin, an E3-ubiquitin ligase. Laforin and malin form a functional complex involved in the regulation of glycogen synthesis. In the absence of a functional complex, glycogen synthesis becomes dysregulated, leading to the accumulation of PGs. In addition to glycogen synthesis, the laforin/malin complex is also involved in the regulation of crucial pathways in cell physiology [16]. Among them, the laforin/malin complex plays a role in cellular proteostasis, and the absence of a functional complex results in alterations in proteasome function, the unfolded protein response at the ER, altered mitophagy, and dysfunctional autophagy (see [17] for review).

Another reason to study the role of the laforin/malin complex in the regulation of the proteostasis mediated by Hsp70s and Hsp90s, is that these proteins appeared in the list of the most differentially ubiquitinated proteins mediated the activity of malin, using the $^{bio}$Ub strategy [18].

In addition, it has been already described that laforin and malin form a functional complex with Hsp70, this interaction being essential for the protective effect of Hsp70 overexpression against the toxic effect of misfolded proteins. Laforin and malin interact with misfolded proteins and promote their proteasomal degradation. This may explain why laforin- or malin-deficient cells are more sensitive to the toxic effect of protein aggregates [19], [20], [21]. Laforin and malin also form a ternary complex with the carboxy-terminus of Hsc70 Interacting Protein (CHIP). This complex is involved in the heat shock factor 1 (HSF1) mediated heat shock response. Under heat stress conditions, laforin, malin, CHIP, and HSF1 translocate to the nucleus, where HSF1 performs its transcriptional function [22].

All these pieces of evidence prompted us to study in this work the relationship between the laforin/malin complex and the Hsp70s and Hsp90s chaperone systems. We present evidence that the laforin/malin complex ubiquitinates HspA1L, Hsp90a, Hsp90b, and Hop/Stip1 proteins. This identifies the laforin/malin complex as a new E3-ubiquitin ligase that introduces post-translational modifications in these proteins. In addition, we present evidence of the physical interaction of HspA1L with both laforin and malin. In the case of Hsp90a, we observed a direct interaction with malin but not with laforin, and in the case of Hop/Stip1, both laforin and malin are needed for the interaction with this co-chaperone.

# RESULTS

**1) The laforin/malin complex ubiquitinates different components of the heat shock machinery.**

Recently, we described a strategy to identify possible substrates of the laforin/malin complex. This was based on the use of the $^{bio}$Ub approach described in previous reports [23], [24], [25], applied to cultures of

HEK293 cells expressing wild-type malin or a mutated version (malin-P69A) with no enzymatic activity, which is the most prevalent pathological mutation in the *NHLRC1/EPM2B* gene [18]. Among the biological processes covered by the differentially ubiquitinated proteins, response to heat shock and protein folding were the most noticeable, and the most repeated molecular functions were heat shock proteins and ubiquitin ligases [18]. A STRING analysis (https://string-db.org/) of the identified proteins indicated that many of them clustered in the heat shock protein group [18]. In Fig. 1, we show a STRING analysis of the heat shock-related proteins, which belonged to the Hsp70s, Hsp90s, Hsp110s, and Hsp40s protein families, the co-chaperone family, and the protein phosphatase family (see Table 1). In all the cases, the differential ubiquitination of the selected proteins was higher than two-fold.

**2) HspA1L, a *bona fide* substrate of the laforin/malin complex.**

To confirm that some of the identified proteins were *bona fide* substrates of the laforin/malin complex, we performed a direct analysis of the ability of the complex to ubiquitinate them [26]. First, we analyze the ubiquitination of HspA1L, an isoform of the Hsp70 protein family. We transfected HEK293 cells with the plasmids indicated in Fig. 2A, and after purifying the ubiquitinated proteins by metal affinity chromatography, we observed a clear ubiquitination of exogenously expressed HA-HspA1L by the laforin/malin complex (Fig. 2A, upper panel, lane 4), which was almost absent when an inactive form of malin (P69A) was present in the assay (Fig. 2A, upper panel, lane 5). The expression of malin alone (Fig. 2A, upper panel, lane 3) also ubiquitinated HspA1L but to a lower extent, and the expression of laforin alone (Fig. 2A, upper panel, lane 2) did not produce any ubiquitination. The analysis of the crude extracts confirmed that the observed differences in the ubiquitination of HspA1L were not due to alterations in the expression of the corresponding proteins. These results indicated that a functional laforin/malin complex efficiently ubiquitinates HspA1L protein.

We also studied the action of the laforin/malin complex on endogenous levels of HspA1L. As shown in Fig. 2B, the functional complex was able to ubiquitinate endogenous levels of HspA1L protein (Fig. 2B, upper panel, lane 2). In the presence of the inactive form of malin (P69A), no ubiquitination was observed (Fig. 2B, upper panel, lane 3).

Next, we studied the topology of the ubiquitin chains introduced by the laforin/malin complex in HspA1L. To this end, we repeated the direct ubiquitination assay in the presence of mutated versions of ubiquitin: Ub-K48R, which prevents the formation of K48-linked ubiquitin chains, and Ub-K63R, which prevents the formation of K63-linked ubiquitin chains. As shown in Supplementary Fig. S1A, the laforin/malin complex mainly introduced K63-linked polyubiquitin chains in HspA1L, which was in agreement with previous reports that indicated that this is the type of modification produced by the laforin/malin complex in other substrates ([27], [28], [29]).

To understand the ability of the laforin/malin complex to ubiquitinate HspA1L, we analyzed the capacity of the members to interact physically with HspA1L. With this aim, we performed GFP-trap analysis using CFP-tagged laforin and GFP-tagged malin versions of these proteins. As shown in Fig. 3A,

upper panel, HspA1L was able to interact physically with CFP-Laforin, both when expressed alone (Fig. 3A, upper panel, lane 3) or when CFP-Laforin was forming part of a complex with functional (Fig. 3A, upper panel, lane 4) or inactive malin (Fig. 3A, upper panel, lane 5). The middle panel of the bound material in Fig. 3A confirms the interaction between CFP-Laforin and both forms of malin (lanes 4 and 5). Similar results were obtained when we used GFP-Malin in the assay (Fig. 3B, upper panel). Again, HspA1L was able to interact with GFP-Malin when expressed alone (lane 3), and when it was forming part of the complex with laforin (lane 4). When using a GFP-Malin P69A version, the interaction with HspA1L was maintained (Fig. 3B, upper panel, lane 5).

Overall, these results indicate that both laforin and malin can interact physically with HspA1L protein, which accounts for the ability of the laforin/malin complex to ubiquitinate HspA1L protein.

**3) Hsp90, a *bona fide* substrate of the laforin/malin complex.**

Secondly, we analyzed the ubiquitination of the two isoforms of Hsp90, Hsp90a and Hsp90b. As shown in Fig. 4A (upper panel), the expression of a functional laforin/malin complex produced an extensive ubiquitination of Hsp90a (Fig. 4A, upper panel, lane 4), which was absent in the presence of the inactive form of malin-P69A (Fig. 4A, upper panel, lane 5). The expression of malin alone produced a minor ubiquitination of Hsp90a (Fig. 4A, upper panel, lane 3), probably because of a reduced expression of malin in this case (Fig. 4A, middle panel of cell extracts, lane 3); the expression of laforin alone (Fig. 4A, upper panel, lane 2) did not produce any ubiquitination. We also studied the ability of the laforin/malin complex to ubiquitinate endogenous levels of Hsp90 isoforms. In Fig. 4B, upper panels, we observed that a functional laforin/malin complex (lane 4) but not an inactive one (lane 5) was able to ubiquitinate endogenous Hsp90a (upper panel) and Hsp90b (lower panel) isoforms. Curiously, the expression of a functional malin alone (lane 3) was able to ubiquitinate the endogenous levels of both isoforms.

We also studied the topology of the ubiquitin chains introduced by the laforin/malin complex in Hsp90, and observed a reduction in its ubiquitination when the Ub-K63R was present in the assay (Supplementary Fig. S1B), suggesting that the main topology of the introduced ubiquitin moieties was through the acquisition of K63-linked ubiquitin chains.

Next, we examined the physical interaction between laforin, malin, and Hsp90a. When we used CFP-Laforin, we observed only a clear interaction with Hsp90a when malin was also expressed (Fig. 5A, upper panel, lane 3). In the absence of malin, no direct interaction between CFP-Laforin and Hsp90a was detected (Fig. 5A, upper panel, lane 2). When using GFP-Malin in the assay, Hsp90a was able to interact with GFP-Malin when expressed alone (Fig. 5B, upper panel, lane 2) or when expressed in combination with laforin (Fig. 5B, upper panel, lane 3). Interestingly, malin also interacted with endogenous levels of Hsp90a (Fig. 5B, upper panel, lanes 2 and 3). The interaction between malin and Hsp90a was maintained when an inactive form of malin was expressed (Supplementary Fig. S2, Bound material, upper panel, lanes 5 and 6).

In summary, these results indicate that only malin can interact physically with Hsp90a, either alone or in combination with laforin. Thus, the observed interaction of Hsp90a with CFP-Laforin should be due to the presence of malin in the assay (Fig. 5A, upper panel, lane 3). This could explain why malin alone can efficiently ubiquitinate endogenous Hsp90a (Fig. 4B, upper panel, lane 3).

**4) Hop/Stip1, a *bona fide* substrate of the laforin/malin complex.**

Since the co-chaperone Hop/Stip1, which works in the Hsp70-Hsp90 folding system, was present in the list of the malin-differentially ubiquitinated proteins [18] (Table 1), we analyzed the ubiquitination of the Hop/Stip1 protein by the laforin/malin complex. As we were unable to express Hop/Stip1 exogenously, we analyzed the action of the laforin/malin complex on the endogenous levels of Hop/Stip1. As shown in Fig. 6, upper panel, the functional laforin/malin complex was able to ubiquitinate endogenous Hop/Stip1 (lane 4). In the presence of an inactive form of malin, we were not able to detect any modification (lane 5), which was also absent if laforin or malin were expressed alone (lanes 2 and 3).

When we analyzed the physical interaction of endogenous Hop/Stip1 protein with laforin and malin, we observed that CFP-Laforin alone was not able to pull down Hop/Stip1 (Fig. 7A, upper panel, lane 3). However, in the presence of malin, either active or inactive, we observed a clear interaction (Fig. 7A, upper panel, lanes 4 and 5). When GFP-Malin was used in the assay, we were not able to recover endogenous Hop/Stip1 unless laforin was present in the assay (Fig. 7B, upper panel, lane 3). The same was true when we used the inactive form of malin (Fig. 7B, upper panel, lane 5). These results suggest that the combination of laforin and malin is essential for the interaction with Hop/Stip1.

**5) Physiological outcome of the ubiquitination of HspA1L, Hsp90 and Hop/Stip1 by the laforin/malin complex**.

Next, we studied the outcome of the ubiquitination of the different substrates analyzed in this study by the laforin/malin complex. As shown in the previous figures, the overexpression of a functional laforin/malin complex did not affect the levels of endogenous HspA1L (Fig. 2B), Hsp90a and Hsp90b (Fig. 4B), and Hop/Stip1 (Fig. 6 and Fig. 7) in HEK293 cells. Then, we tested whether there was any change in the amount of these proteins in brain samples from a mouse model deficient in malin (*Epm2b-/-*) of 16 months of age. As shown in Supplementary Fig. S3, by western blot analysis of crude extracts from brain samples from wild type and *Epm2b-/-* mice, we did not detect major differences in the levels of HspA1L, Hsp90a, Hsp90b, and Hop/Stip1. These results suggest that the ubiquitination of these proteins by the laforin/malin complex does not have a major effect on their protein levels. Since the ubiquitination mediated by the laforin/malin complex is related to the introduction of K63-linked ubiquitin chains, this type of modification may confer alternative functions that will require further work to be elucidated.

# DISCUSSION

As indicated in the Introduction, Hsp70s and Hsp90s systems play a key role in protein folding. With the help of different co-chaperones, they decide whether a particular substrate is folded or targeted to the proteasome or autophagy systems for degradation. The folding process initiates with the recognition of misfolded proteins by the J-domain proteins and the recruitment of the substrate to Hsp70s for unfolding. If the substrate is still misfolded, then the E3-ubiquitin ligase CHIP is in charge of its ubiquitination to target it to proteasomal degradation. On the contrary, if the substrate is correctly folded, the Hop/Stip1 chaperone transfers it to the Hsp90s to continue with the folding of the substrate [6]. The function of the Hsp70s and Hsp90s system is regulated by stress-induced gene expression and also by post-translational modifications (PTMs). In the case of Hsp70s, they suffer a great number of PTMs, such as phosphorylation, acetylation, AMPylation, methylation, prenylation, and ubiquitination. This last modification is particularly interesting since it may modify the activity of the Hsp70s. E3-ubiquitin ligases that participate in this modification are CHIP and Parkin [8, 30]. For Hsp90s, similar PTMs have also been reported [11]. Regarding ubiquitination, several E3-ubiquitin ligases have been described for Hsp90s: HECTD1 (HECT domain E3 ubiquitin ligase 1), which introduces K63-linked polyubiquitin chains, regulating in this way the subcellular localization of the Hsp90s [31]; and FBXL6 (F-box Skp1-Cul1 ligase), which also participates in the attachment of K63-linked polyubiquitin chains to Hsp90s, making them more stable [32].

In this work, we present evidence of the ubiquitination of HspA1L, Hsp90a, Hsp90b and the co-chaperone Hop/Stip1 by the laforin/malin E3-ubiquitin ligase complex. The expression of this complex allowed the ubiquitination of endogenous levels of these Hsps, and this modification was dependent on the activity of malin, since no ubiquitination was observed in the presence of an inactive form of the E3-ligase (malin P69A). Importantly, the modification was produced by the attachment of K63-linked polyubiquitin chains, as in the case of HECTD1 and FBXL6 for their action on Hsp90s. We could hypothesize that the laforin/malin-dependent ubiquitination of Hsp70s, Hsp90s, and Hop/Stip1 could improve the stability of these chaperones. However, when we expressed laforin and malin in HEK293 cells, we did not observe changes in the endogenous levels of the analyzed Hsps. Perhaps, the putative stabilization effect of the laforin/malin complex is masked by the huge amount of the Hsp70s and Hsp90s proteins in the cell. We then analyzed the levels of Hspa1L, Hsp90a, Hsp90b and Hop/Stip1 in crude extracts from the brain of a 16-month-old mouse model deficient in malin. Again, we did not observe major differences in the levels of these proteins when compared to samples from wild-type mice. Our results are in contrast with those reported recently, which indicated that in samples from 8-month-old malin-KO mice, there was a reduction in the levels of Hsp70 proteins and an increase in the levels of Hsp90 proteins [33]. Perhaps differences in the genetic background of the mice may account for these differences.

In addition to the confirmed ubiquitination of Hsp70s, Hsp90s and Hop/Stip1 by the laforin/malin complex, we would like to point out the fact that in the list of malin-dependent differentially ubiquitinated proteins (Table 1), there are proteins that participate actively in the activity of the Hsp70s system, such as DNAJA2, a J-domain protein belonging to the small Hsp40 protein family that recruits substrates to

the Hsp70 [34]; HIP/St13, a protein that antagonizes the role of NEFs [35]; NEFs themselves such as BAG2 and BAG5 [36]; and the catalytic subunit of the protein phosphatase 5 (PP5), which binds to the C-terminal domain of Hsp70s [37]. Moreover, the list contains proteins that affect the activity of the Hsp90 system, like FKBP5, a prolyl cis/trans isomerase that modifies the action of Hsp90s on AMPA receptors [38]. Taken together, this information suggests a role of the laforin/malin complex in regulating the function of heat shock chaperones and co-chaperones.

We have checked for physiological consequences related to the absence of a laforin/malin-dependent ubiquitination of Hsp70s or Hsp90s by using samples from malin-deficient mice. In previous reports, we demonstrated that these mice presented different abnormalities related to proteostasis dysfunction, such as a decrease in proteasomal activity, enhanced unfolded protein response at the ER, and autophagy dysfunction (reviewed in [17]). We suggest that, although additional factors may be responsible for these abnormalities, the lack of modification of Hsp70s and Hsp90s chaperone systems by the absence of a functional laforin/malin complex could be responsible for these deficiencies. In fact, it has been reported that the overexpression of laforin and malin reduced the proteotoxicity of misfolded proteins, whereas models deficient in laforin or malin showed enhanced proteotoxicity [19]. The same authors indicated that the overexpression of Hsp70 alleviates the toxicity of misfolded proteins but only in the case that a functional laforin/malin complex is present [19]. All these data suggest that the activity of the laforin/malin complex plays a role in the cellular response to misfolded proteins.

# MATERIALS AND METHODS

### 1.- Ethical statement, animal care, mice, and husbandry.

This study was carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the Consejo Superior de Investigaciones Cientificas (CSIC, Spain) and approved by the Consellería de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural from the Generalitat Valenciana. Mouse procedures were approved by the animal Ethics committee of the Instituto de Biomedicina de Valencia-CSIC [Permit Number: IBV-51, 2019/VSC/PEA/0271]. All efforts were made to minimize animal suffering. Male and female homozygous *Epm2b−/−* in a pure C57BL/6JRccHsd background [39] and the corresponding control WT mice (from ENVIGO, Barcelona, Spain), were used in this study. Mice were maintained in the IBV-CSIC facility on a 12/12 light/dark cycle under constant temperature (23°C) with food and water provided ad libitum. Mice of 16 months of age were euthanized by cervical dislocation, a procedure that is pain free and no harm is caused to the animals (according to the Spanish Regulation for the Care and Use of Laboratory Animals, no anesthetic is necessary prior cervical dislocation in the case of animals that weight less than 150 grams). Whole brains were rinsed twice with cold PBS and frozen immediately in liquid nitrogen.

### 2.- Mammalian cell culture

Human embryonic kidney cells (HEK293) (HPA Culture Collection, #851820602) were used for transfection experiments. Cells were grown in Dulbecco's modified Eagle medium (Lonza, Barcelona, Spain), supplemented with 10% inactivated fetal bovine serum (FBS) (Invitrogen, Madrid, Spain), 1% L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37°C and 5% (vol/vol) of CO2.

**3.- Plasmid constructs**

The following plasmids were described in reference [18]: pFLAG-Laforin, pECFP-Laforin, pEGFP-Malin, pEGFP-Malin-P69A, pFLAG-Malin, pFLAG-Malin P69A, pCMV-6xHisUbiq, pCMV-6xHis-Ubiq-K48R, and pCMV-6xHis-Ubiq-K63R. Plasmid HA-HspA1L was constructed as follows: plasmid pcDNA5-GFP-HspA1L (#19484; Addgene, Watertown, MA, USA) was digested with NcoI/blunted with Klenow and XhoI to release the HspA1L ORF and subcloned into pGADT7 (#630442; Clontech, Madrid, Spain) digested with SmaI/XhoI. Then, the resulting plasmid was digested with SfiI/XhoI and subcloned into plasmid pCMV-HA (#K6003; Clontech, Madrid, Spain), to obtain pCMV-HA-HspA1L (HA-HspA1L). Plasmid pCHERRY-Hsp90a was from Addgene, Watertown, MA, USA (#108222).

**4.- Analysis of protein ubiquitination**

The method described in Kaiser et al., (2005) [26] was used to confirm the ubiquitination of the proteins described in this study. For this purpose, HEK293 cells were transfected with the plasmids indicated in each experiment using X-treme GENE HP transfection reagent according to the manufacturer's protocol (Roche Diagnostics, Barcelona, Spain). After 24 h of transfection, cells were lysed using a 25-gauge needle 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 endogenous deubiquitinases. Protein extracts were clarified after centrifugation (12.000 × g, 15 min), and protein concentration was measured through the Bradford technique. 1.5 mg of protein were incubated with 150 µl of a TALON cobalt resin (Clontech, Madrid, Spain) equilibrated in buffer B containing 10 mM imidazole, 6 M guanidinium-HCl, 0.1 M sodium phosphate, 0.1 M Tris-HCl, pH 8.0. To purify His-tagged proteins, incubation was carried out for 2 h at room temperature on a rocking platform. Then, the resin was 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 at 95°C for 5 min in 50 µl of 2×Laemmli's sample buffer and analyzed by Western blotting using the appropriate antibodies. To determine the topology of the ubiquitin chains, when indicated, plasmids pCMV-6xHis-Ubiq-K48R and pCMV-6xHis-Ubiq-K63R were used in the assay instead of pCMV-6xHis-Ubiq wild type.

**5.- GFP-trap analysis of protein-protein interactions**

GFP-trap analysis was performed according to the manufacture’s instructions (https://www.ptglab.com/results?q=gta). HEK293 cells were transfected with specific constructs of laforin, malin, and the protein of interest. Cells were washed twice with cold phosphate-buffered saline (PBS) and scraped on ice in lysis buffer [10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% (v/v) Nonidet P-40, complete protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain), 1 mM

PMSF, 2.5 mM NaF, 0.5 mM NaVO4, and 2.5 mM Na4P2O7]. The lysates were collected in an Eppendorf tube, and further lysis was performed using a 25-gauge needle. Cell lysates were then centrifuged at 13.000 × g for 10 min at 4°C. Supernatants (1.5 mg of total protein) were incubated with Chromotek GFP-trap beads (Chromotek, Planegg-Martinsried, Germany) for 1 h on a rocking platform at 4°C. Beads were washed two times with 1 mL of lysis buffer and one time with the lysis buffer containing 300 mM NaCl. Bound proteins were boiled at 95°C for 5 min in 30 µl of 2×Laemmli's sample buffer. The GFP- and CFP-fused proteins were pelleted and visualized by immunoblotting using specific antibodies. As a negative control, a construct expressing CFP or GFP proteins (plasmid pECFP-C1 and pEGFP-C1, respectively; Clontech, Madrid, Spain) was used to confirm the specificity of the interaction.

**6.- Western blot analyses**

Western blot analysis was performed as in Towbin et al. (1979) [40]. 30 µg of total protein from the soluble fraction of cell lysates were analyzed by SDS-PAGE, and proteins were transferred to PVDF membranes (Millipore, Madrid, Spain). In the case of mouse brain homogenates, they were lysed in RIPA buffer [50 mM Tris-HCl, pH 8; 150 mM NaCl; 0.5% sodium deoxycholate; 0.1% SDS; 1% Nonidet P40; 1mM PMSF; 100 µM leupeptin and complete protease inhibitor cocktail (Roche, Barcelona, Spain)] for 30 min at 4°C with occasional vortexing. The homogenates were passed ten times through a 25-gauge needle in a 2 ml syringe and centrifuged at 13,000xg for 15 min at 4ºC. Supernatants were collected, and a total of 30 µg protein was subjected to SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline Tween20 buffer [TBS-T: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween20] for 1 hr at room temperature and incubated overnight at 4°C with the corresponding primary antibodies: mouse anti-HA (ab18181; Abcam, Cambridge, UK), mouse anti-FLAG (F3165. Sigma-Aldrich, Madrid, Spain), polyclonal anti-HspA1L (13970-1-AP, Proteintech, Planegg, Germany), polyclonal anti-mCherry (ab167453, Abcam, Cambridge, UK), mouse anti-Hsp90a (ab79849, Abcam, Cambridge, UK), mouse anti-Hsp90b (ab53497, Abcam, Cambridge, UK), anti-Hop (DS14F5, Enzo, Lausen, Switzerland), and rabbit anti-GFP (210-PS-1GFP. Inmunokontackt, Madrid, Spain), Mouse anti-Gapdh (sc-32233. Santa Cruz Biotechnologies, Madrid, Spain), mouse anti-Tubulin (T6199. Sigma-Aldrich, Madrid, Spain), and rabbit anti-Actin (A2066. Sigma-Aldrich, Madrid, Spain), were used as loading controls. After washing, membranes were incubated with the corresponding HRP-conjugated secondary antibodies for 1 hr at room temperature. Signals were visualized using Lumi-Light Western Blotting Substrate (Roche Applied Science, Barcelona, Spain) or ECL Prime Western Blotting Detection Reagent (GE Healthcare, Barcelona, Spain), and analyzed by chemiluminescence using the FujiLAS400 (GE Healthcare, Barcelona, Spain) image reader.

# Declarations

**ACKNOWLEDGEMENTS:** We thank Dr. Ugo Mayor and Dr. Juan Manuel Ramirez (Department of Biochemistry and Molecular Biology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Leioa, Spain), for their collaboration in running the $^{bio}$Ub strategy, and for the critical reading of the manuscript.

**FUNDING:**

This work was supported by grants from the Spanish Ministry of Science and Innovation PID2023-1481023OB-I00, and the Prometeo program from Generalitat Valenciana (CIPROM2022/42) to P.S. and M.A.G.G; P.G.R. holds a predoctoral fellowship from CIBERER.

**AUTHOR CONTRIBUTIONS:** P.G.R., L.K., D.T., and B.L performed the experiments. M.A.G-G. and P.S. analyzed the data. P.S. participated in the writing of the manuscript. All authors have read and approved the final version of the manuscript.

**DATA AVAILABILITY STATEMENT**: The data presented in this study are available from the first author upon request.

**CONFLICTS OF INTEREST:** The authors declare no conflicts of interest.

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# Tables

**Table 1:** List of heat shock-related proteins differentially ubiquitinated by malin [18]. Gene and protein names are according to the human gene database (https://www.genecards.org/). Fold change is also indicated.

| HSP-related families | Gene name | Protein name | Fold change |
|---|---|---|---|
| HSP70 family | HSPA1L | HspA1L | 7.11 |
| | HSPA8 | Hsc70 | 3.32 |
| | HSPA1B | HspA1B | 3.01 |
| HSP90 family | HSP90AA1 | Hsp90a | 3.69 |
| | HSP90AB1 | Hsp90b | 3.29 |
| HSP110 family | HSPA4 | Hsp70-4 | 8.59 |
| | HSPH1 | Hsp105 | 5.11 |
| | HSPA4L | Hsp70-4L | 4.66 |
| HSP40 family | DNAJA2 | DnajA2 | 2.30 |
| Co-chaperon family | FKBP5 | Fkbp5 | 7.80 |
| | HOP/STIP1 | Hop/Stip1 | 4.42 |
| | CRYAB | a-crystallin B | 3.66 |
| | ST13 | Hip | 2.83 |
| | BAG5 | Bag5 | 2.41 |
| | BAG2 | Bag2 | 2.32 |
| Protein phosphatase | PPP5C | PP5 | 2.07 |

# Figures

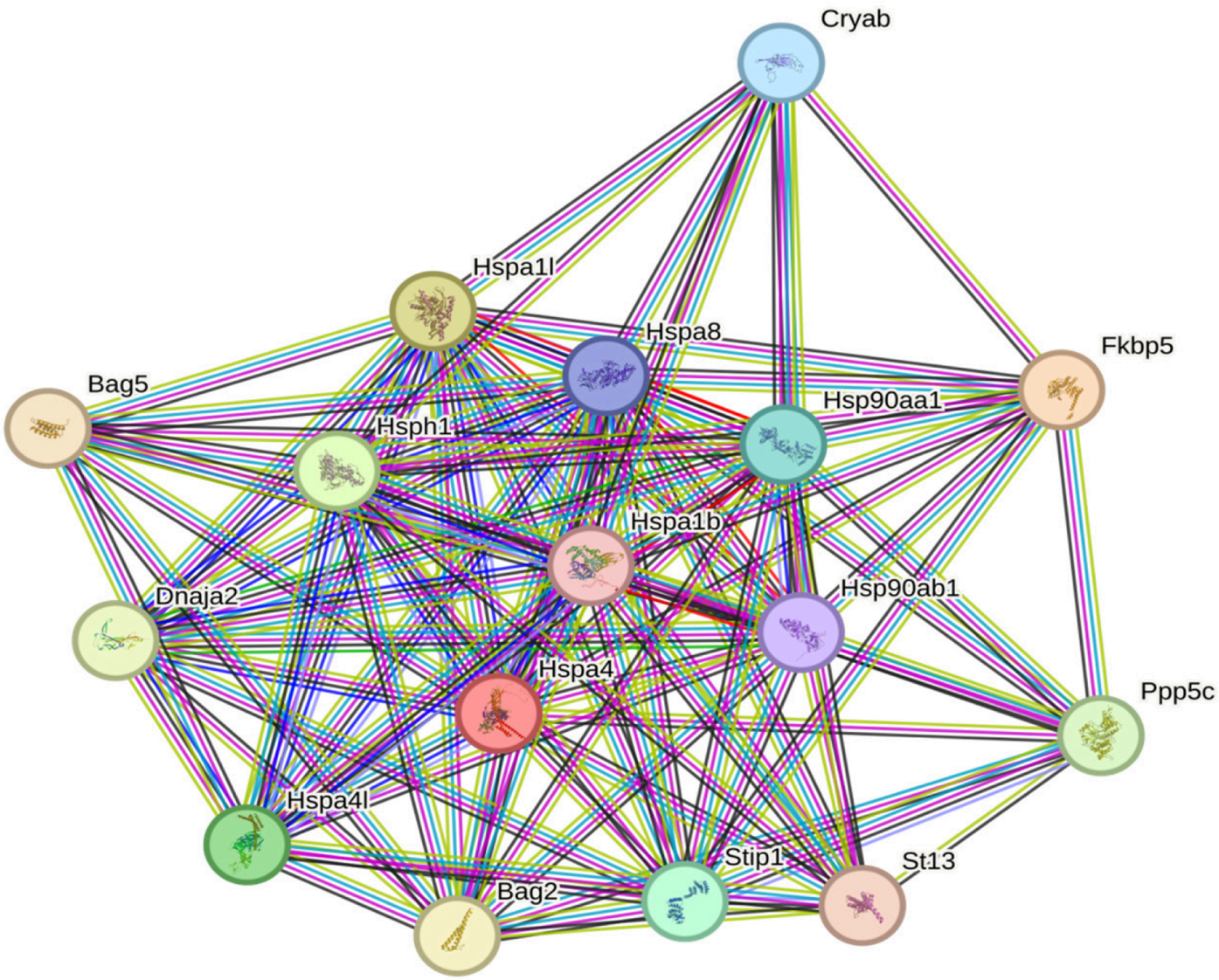



**Figure 1**

STRING analysis of the heat shock-related proteins differentially ubiquitinated by malin described in Table 1.

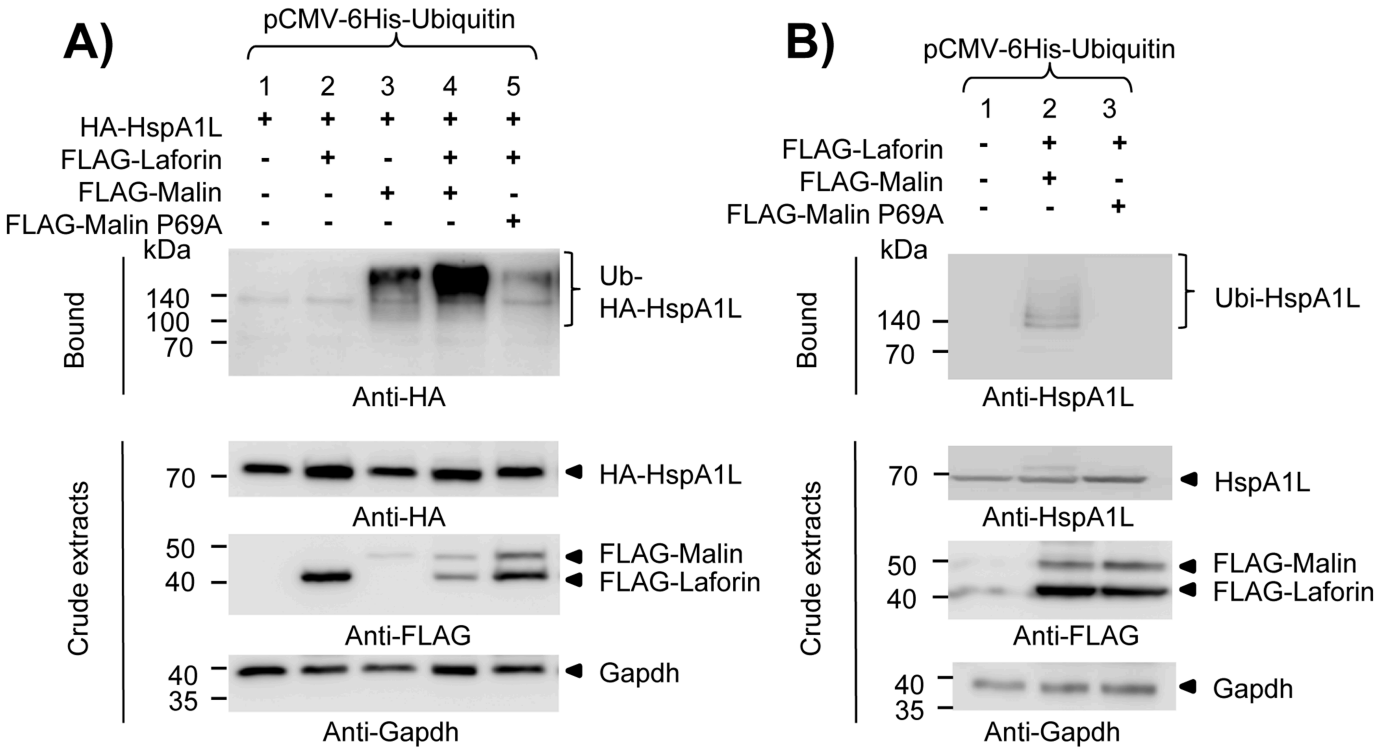



**Figure 2**

The functional laforin/malin complex promotes the ubiquitination of Hspa1L. Analysis of ubiquitination of overexpressed (A) or endogenous (B) HA-HspA1L mediated by wild-type or catalytically inactive (P69A) malin. HEK293 cells were co-transfected with the indicated plasmid combinations. Cells were lysed, and 1.5 mg of total protein was subjected to immobilized metal affinity chromatography (IMAC) using cobalt resin, as described in Materials and Methods. Proteins present in the bound fraction (Bound: proteins retained in the metal affinity resin) or in the crude cell extract (30 µg) were analyzed by Western blotting using the indicated antibodies. Representative blots of three independent experiments are shown.

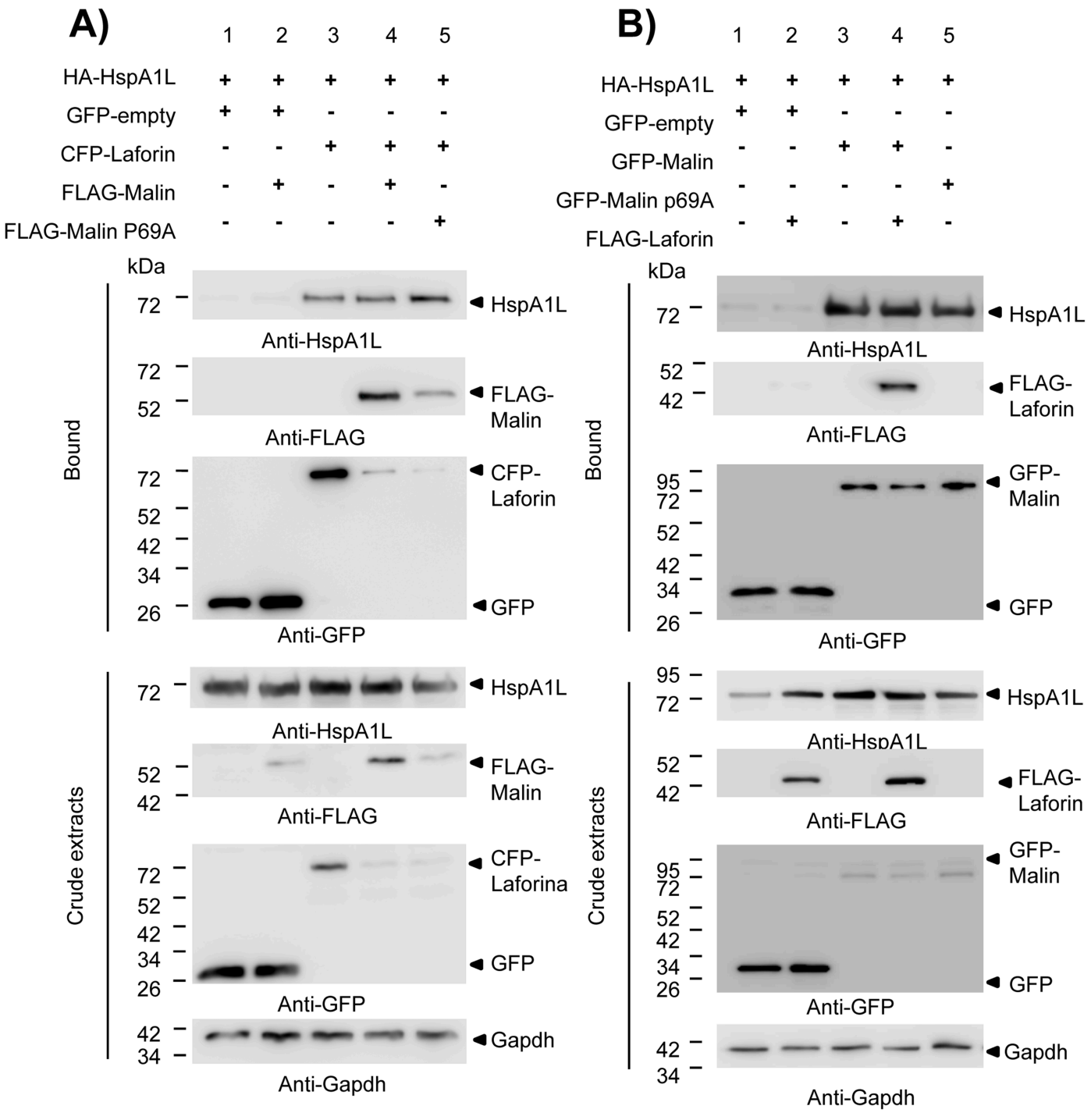



**Figure 3**

The laforin/malin complex interacts physically with HA-HspA1L. CFP-Laforin (A) and GFP-Malin (B) pull-down HA-HspA1L. HEK293 cells were co-transfected with the indicated combination of plasmids. Cells were lysed, and 1.5 mg of proteins were subjected to GFP-trap affinity purification. After washing, beads were boiled in loading buffer, and the purified proteins were analyzed by SDS PAGE and Western blot using anti-HspA1L, anti-GFP, anti-FLAG, and anti-Gapdh antibodies, as indicated adjacent to the corresponding panels. Bound: proteins retained on the resin; Cell Extract: 30 µg of protein were loaded

for the total fraction. Representative blots of three independent experiments are shown. See text for details.

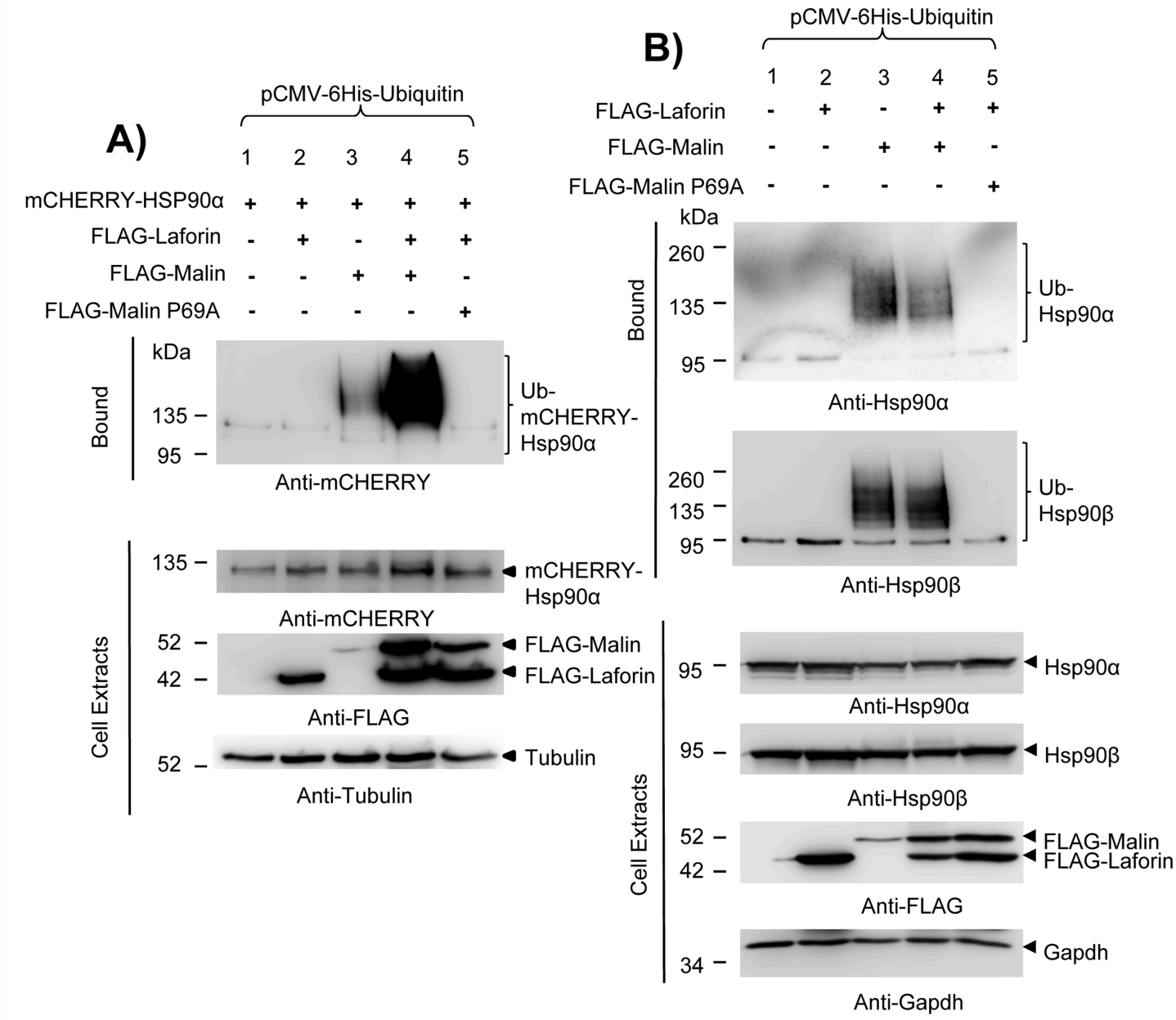



**Figure 4**

The functional laforin/malin complex promotes the ubiquitination of Hsp90a. Analysis of ubiquitination of overexpressed mCHERRY-Hsp90a (A) or endogenous Hsp90a (B) mediated by wild-type or catalytically inactive (P69A) malin. HEK293 cells were co-transfected with the indicated plasmid combinations. Cells were lysed, and 1.5 mg of total protein was subjected to immobilized metal affinity chromatography (IMAC) using cobalt resin, as described in Materials and Methods. Proteins present in the bound fraction (Bound: proteins retained in the metal affinity resin) or in the crude cell extract (30 µg) were analyzed by Western blotting using the indicated antibodies. Representative blots of three independent experiments are shown.

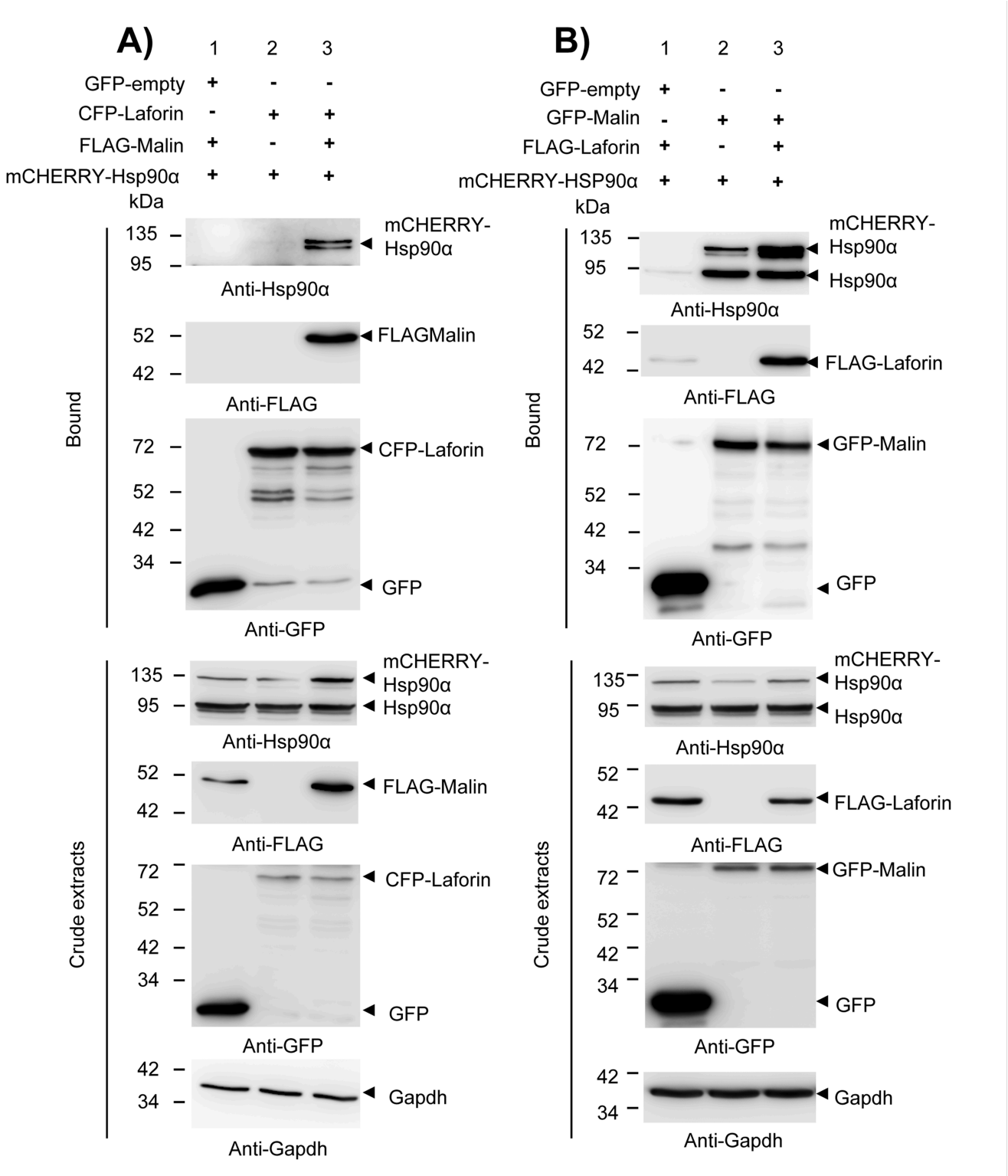



**Figure 5**

The laforin/malin complex interacts physically with mCHERRY-Hsp90a. CFP-Laforin (A) and GFP-Malin (B) pull-down mCHERRY-Hsp90a. HEK293 cells were co-transfected with the indicated combination of plasmids. Cells were lysed, and 1.5 mg of proteins were subjected to GFP-trap affinity purification. After washing, beads were boiled in loading buffer, and the purified proteins were analyzed by SDS PAGE and Western blot using anti-Hsp90a, anti-GFP, anti-FLAG, and anti-Gapdh antibodies, as indicated adjacent to

the corresponding panels. Bound: proteins retained on the resin; Cell Extract: 30 µg of protein were loaded for the total fraction. Representative blots of three independent experiments are shown. See text for details.

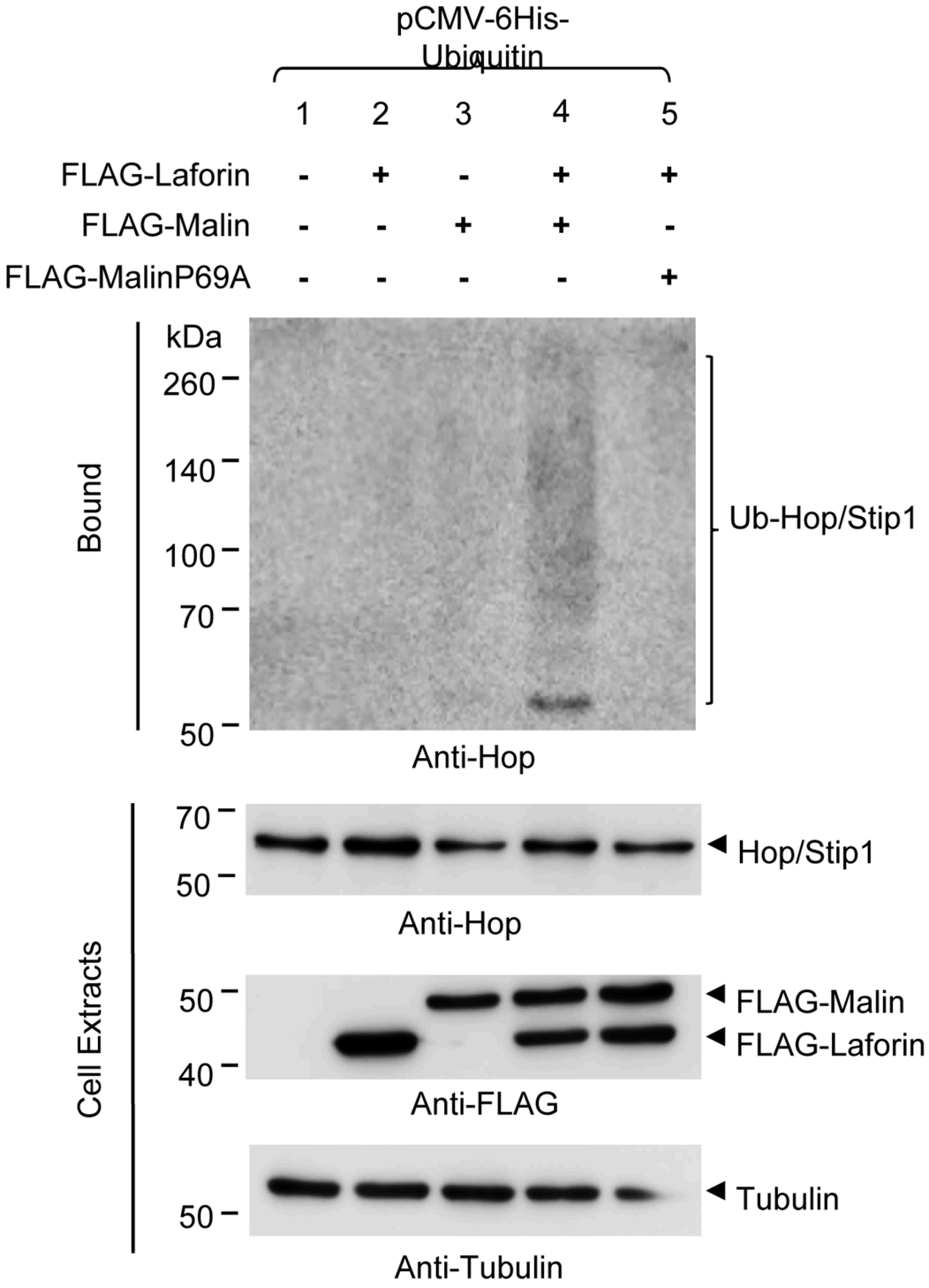



**Figure 6**

Analysis of ubiquitination of endogenous Hop/Stip1 mediated by wild-type or catalytically inactive (P69A) malin. HEK293 cells were co-transfected with the indicated plasmid combinations. Cells were lysed, and 1.5 mg of total protein was subjected to immobilized metal affinity chromatography (IMAC) using cobalt resin, as described in Materials and Methods. Proteins present in the bound fraction (Bound: proteins retained in the metal affinity resin) or in the crude cell extract (30 µg) were analyzed by Western blotting using the indicated antibodies. Representative blots of three independent experiments are shown.

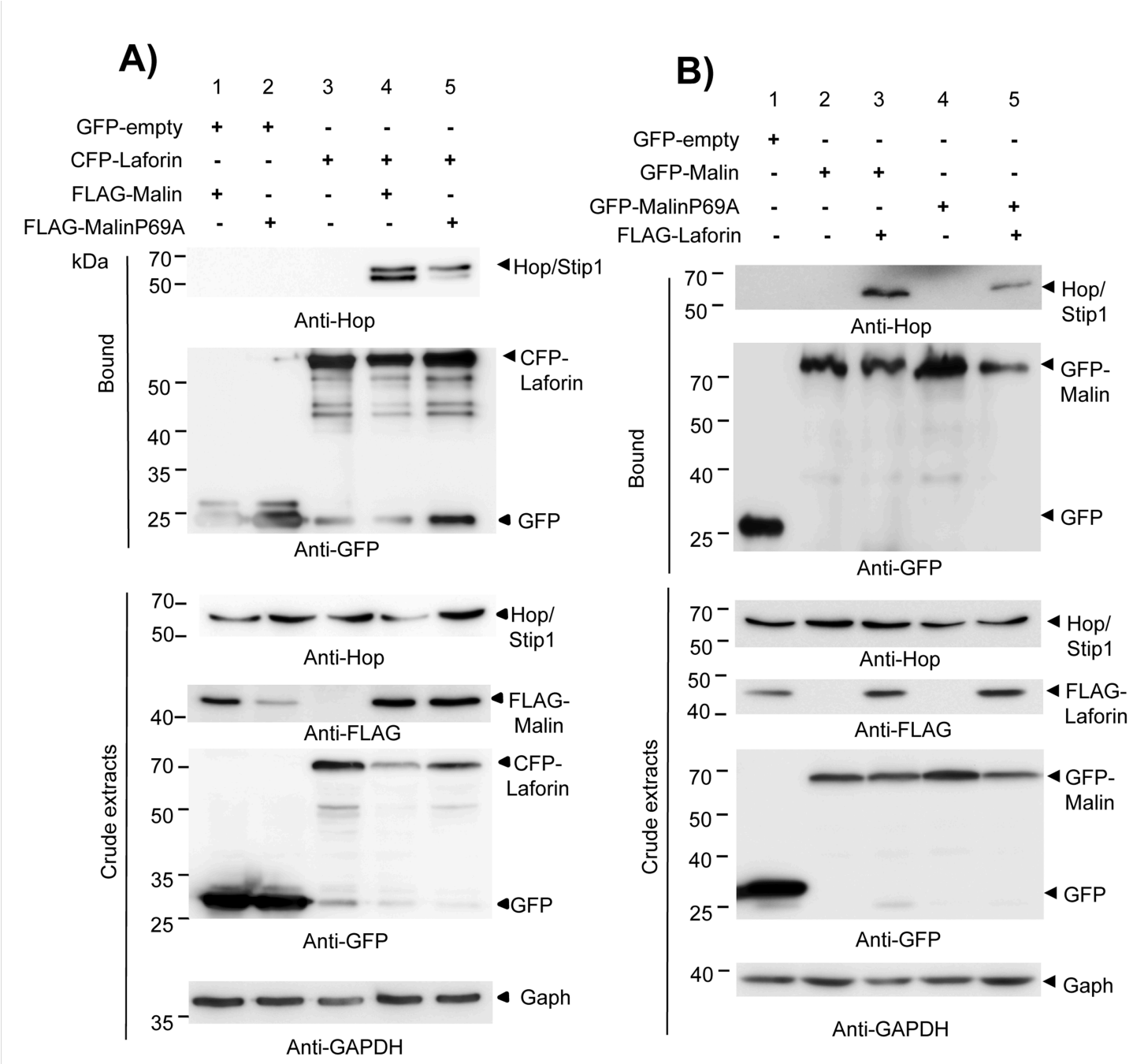



**Figure 7**

The laforin/malin complex interacts physically with Hop/Stip1. CFP-Laforin (A) and GFP-Malin (B) pull-down analysis of endogenous Hop/Stip1. HEK293 cells were co-transfected with the indicated combination of plasmids. Cells were lysed, and 1.5 mg of proteins were subjected to GFP-trap affinity purification. After washing, beads were boiled in loading buffer, and the purified proteins were analyzed by SDS PAGE and Western blot using anti-Hop, anti-GFP, anti-FLAG, and anti-Gapdh antibodies, as indicated adjacent to the corresponding panels. Bound: proteins retained on the resin; Cell Extract: 30 µg of protein were loaded for the total fraction. Representative blots of three independent experiments are shown. See text for details.

## Supplementary Files

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