Interdependence of laforin and malin proteins for their stability and functions could underlie the molecular basis of locus heterogeneity in Lafora disease

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Lafora disease (LD), an autosomal recessive and fatal form of neurodegenerative disorder, is characterized by the presence of polyglucosan inclusions in the affected tissues including the brain. LD can be caused by defects either in the *EPM2A* gene coding for the laforin protein phosphatase or the *NHLRC1* gene coding for the malin ubiquitin ligase. Since the clinical symptoms of LD patients representing the two genetic groups are very similar and since malin is known to interact with laforin, we were curious to examine the possibility that the two proteins regulate each other's function. Using cell biological assays we demonstrate here that (i) malin promotes its own degradation via auto-ubiquitination, (ii) laforin prevents the auto-degradation of malin by presenting itself as a substrate and (iii) malin preferentially degrades the phosphatase-inactive laforin monomer. Our results that laforin and malin regulate each other's stability and activity offers a novel and attractive model to explain the molecular basis of locus heterogeneity observed in LD.

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1. Introduction

Lafora disease (LD), one of the five forms of progressive myoclonus epilepsies, is an autosomal recessive and fatal form of neurodegenerative disorder (Delgado-Escueta *et al.* 2001; Ganesh *et al.* 2006; Singh and Ganesh 2009; Serratosa *et al.* 2012). Clinical symptoms of LD include adolescence onset stimulus-sensitive myoclonus seizures, progressive dementia, ataxia and psychosis (Ganesh *et al.* 2006; Singh and Ganesh 2009; Serratosa *et al.* 2012). The pathological hallmark of LD is the presence of abnormal glycogen aggregates as Lafora bodies in the affected tissues including neurons (Ganesh *et al.* 2006; Singh and Ganesh 2009; Serratosa *et al.* 2012). In addition, widespread neurodegeneration was also seen (Ganesh *et al.* 2006; Singh and Ganesh 2009). LD is caused by defects in the *EPM2A* gene encoding a protein phosphatase named laforin or the *NHLRC1* gene coding for an E3 ubiquitin ligase named malin (reviewed in Singh and Ganesh 2009). Laforin and malin, referred to hereafter as LD proteins, interact with each other, and are believed to function as a complex in regulating diverse cellular processes (Singh and Ganesh 2009). Indeed it has been shown that both laforin and malin negatively regulate glycogen synthesis by regulating the cellular glucose uptake (Singh *et al.* 2012). LD proteins were also shown to regulate the cellular level of protein targeting to glycogen (PTG/R5), a scaffold protein that binds to glycogen (Vilchez *et al.* 2007; Worby *et al.* 2008). Thus loss of laforin or malin would result in increased cellular levels of PTG and the accumulation of abnormal

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glycogen (*loc cit*). Besides glycogen metabolism, LD proteins were also implicated cellular quality control and cellular stress response mechanisms. For example, laforin and malin are recruited to the aggresome upon proteasomal blockade (Mittal *et al.* 2007), as a complex they interact with and ubiquitinate various forms of misfolded proteins and target them for degradation (Garyali *et al.* 2009), and regulate the heat-shock induced cellular stress response pathway (Sengupta *et al.* 2011). Loss of laforin or malin also results in defective autophagy (Aguado *et al.* 2010; Puri *et al.* 2012; Criado *et al.* 2012). Thus, defects in protein quality control processes are likely to contribute in the physiopathology of LD.

LD exhibit locus heterogeneity; besides *EPM2A* and *NHLRC1* genes, at least one more locus is expected to carry third LD gene (Singh and Ganesh 2009). Since the clinical symptoms of LD patients representing the two major genetic groups are very similar, it has been suggested that the protein products of these two genes might function together in the same physiological pathway(s) and hence loss of any one of these three proteins would result in LD (Ganesh *et al.* 2006; Singh and Ganesh 2009). This model also proposes a role for laforin in regulating the cellular functions of malin and vice versa (Ganesh *et al.* 2006). Indeed, malin is known to interact with laforin and promote its polyubiquitination and degradation (Gentry *et al.* 2005) although the physiological significance of this process was not well understood. Similarly, a regulatory role for laforin in the cellular functions of malin was suggested (Singh *et al.* 2006) but was not tested. In the present report we tested these possibilities using cell biological approaches and demonstrate that (i) malin promotes its own degradation, (ii) laforin prevents the autodegradation of malin by presenting itself as a substrate, and (iii) malin preferentially degrades the inactive form of laforin.

2. Materials and methods

Expression constructs: The expression vectors containing Myc-, FLAG-, or GFP-tagged wild-type or the mutant forms of laforin or malin, and the ubiquitin constructs were described previously (Mittal *et al.* 2007; Garyali *et al.* 2009). The RNAi constructs for laforin and malin (shRNAmir) were purchased from Open Biosystem, USA (Expression Arrest™ microRNA-adapted shRNA libraries). The efficiency of shRNA constructs was demonstrated in our previous studies (Garyali *et al.* 2009; Sengupta *et al.* 2011; Singh *et al.* 2012). The yeast two-hybrid bait vector pEG202 coding for the laforin and the prey vector pJG4-5 are described in our previous study (Ganesh *et al.* 2003). The malin coding sequence was cloned in-frame into the pEG202 and pJG4-5 for the yeast two hybrid assays.

Yeast two-hybrid assay: The Duplex-A yeast two-hybrid system of OrigiGene Technologies was performed as described previously (Ganesh *et al.* 2003), and the assays were carried out using the yeast strain EGY48. The transformants were plated on YNB (gal)-his-ura-trp-leu selective plates. After incubation at 30°C for 3–5 days, positive clones were further tested for galactose growth dependence.

Cell culture and transfections: COS-7 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich India Pvt Ltd) supplemented with 10% (vol/vol) foetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Transfections were performed using Lipofectamine 2000 (Invitrogen Inc, USA) and the cells were harvested at 24 h post-transfection, as recommended by the manufacturer.

DSS cross-linking and Immunoblotting analysis: DSS cross linking and immunbloting were carried out as described previously (Dubey and Ganesh 2008). The following antibodies were used in the present study; anti-ubiquitin, anti-Myc (both from Cell Signalling Technology, USA), anti-γ-tubulin, and anti-FLAG (both from Sigma-Aldrich). Secondary antibodies were obtained from Jackson Immuno Research Inc. For quantitation, the signal intensity in the digital images was measured using the Quantity One Discovery Series software of Bio-Rad Laboratories.

Pull-down experiments: To establish the physical interaction between two proteins, expression construct that code for Myc/His-tagged malin, or laforin was used as described previously (Dubey and Ganesh 2008; Garyali *et al.* 2009). Lysates of cells that had expressed His-tagged protein were incubated with Ni-affinity resin (Sigma-Aldrich India Pvt Ltd) for 2 h at 4°C and processed for pull-down assays as recommended by the manufacturer. Pulled-down products were detected by immunoblotting using specific antibodies.

In vivo ubiquitination assay: Cells were transfected with desired constructs, and subjected to pull-down using the ubiquitin enrichment kit as recommended by manufacturer (Thermo Scientific India).

3. Results

3.1 *Malin interacts with itself and facilitates its degradation via auto-ubiquitination*

A yeast two-hybrid screening carried out in our laboratory identified malin to interact with itself. To establish that malin indeed interact with itself, and which domain of malin is critical for this process, bait vectors coding for the full-length malin was co-expressed with a prey vector coding for the full-length malin or one of its two domains − the RING domain or the NHL repeat containing region (figure 1A) − and their functional interaction was assayed in a yeast two-

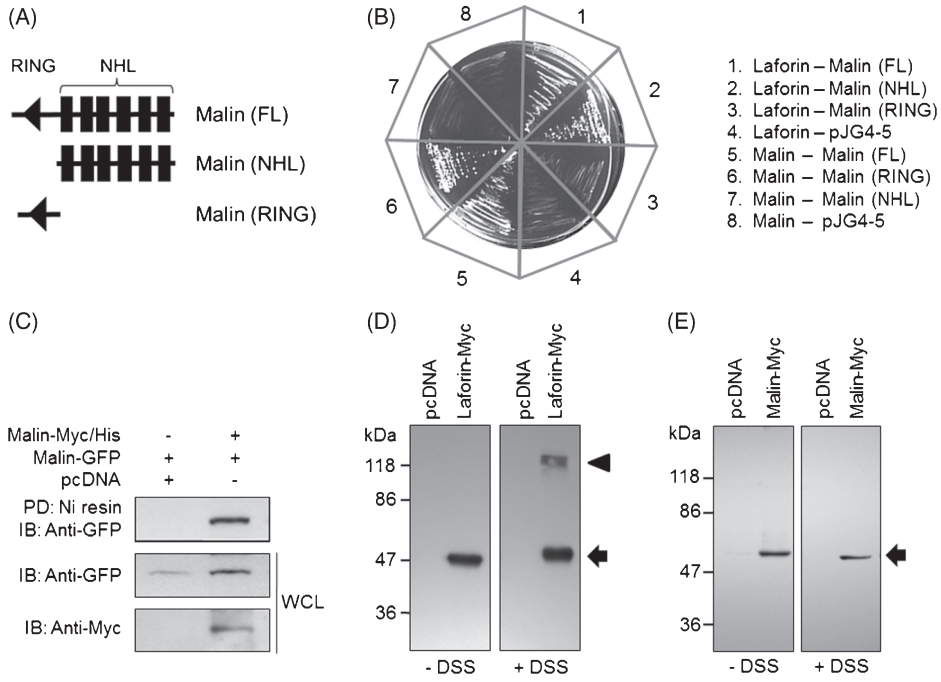

Figure 1. Malin interacts with itself but does not form dimers: (**A**) Schematic diagram showing the domain structure of full-length malin (malin-FL) or its truncated version having only the RING domain or the NHL repeats used in the yeast two-hybrid assay, as indicated. (**B**) Yeast two-hybrid assay to test the protein–protein interactions. Yeast cells were transformed with the indicated plasmid combinations (identified by numbers and explained on the left), plated on a selection medium, and assessed for their survival and growth. Laforin-malin interaction served as the positive control (Gentry *et al.* 2005) and the empty prey vector pJG4-5 served as the negative control. (**C**) GFP-tagged malin (wild-type) and Myc/His-tagged malin (wild-type) were co-expressed in COS-7 cells and processed for the pull-down assay using the Ni-affinity resin, as indicated. As a negative control, COS-7 cells expressing GFP malin with an empty vector (pcDNA) were processed in parallel. The pulled-down products (PD) and whole cell lysates (WCL) were immunoblotted (IB) and probed with anti-GFP or anti-Myc antibody. Malin-GFP was pulled-down with Myc-malin but not with empty vector, demonstrating the specificity of the pull-down assay. (**D, E**) Laforin (**D**) and malin (**E**) were overexpressed in COS-7 cells, treated (+) or not treated (−) with the cross-linker DSS, resolved in SDS–PAGE and detected by immunoblotting. Anti-Myc antibody detected a band at around 100 kDa, representing the dimeric form laforin (identified by an arrow head) which was absent for malin. The monomeric form (~45 kDa for laforin and ~55 kDa for malin; identified by an arrow) was present both in DSS-treated and DSS–untreated samples.

hybrid system. Here, a positive interaction would result in the growth of the cells on a conditional medium. As shown in figure 1B, yeast cells co-expressing the full-length malin both as bait and the prey have shown good growth. Similar observations were made between full-length malin and it truncated form with only the RING, but not with the NHL only form. The interaction between malin and laforin and or with the empty vector served as positive and negative controls respectively (figure 1B).

To further establish that malin does show self interaction, we co-expressed malin with two different tags (Myc/His-tagged malin and GFP-tagged malin) in COS-7 cells, carried out a pull-down assay and confirmed malin–malin interaction (figure 1A). We next explored whether this interaction results in the dimerization of malin – as demonstrated for laforin (Liu *et al.* 2006; Dubey and Ganesh 2008) and for several E3 ubiquitin ligases (Nikolay *et al.* 2004; Tang *et al.* 2007; Linke *et al.* 2008). For this, Myc-tagged malin was transiently expressed in COS-7 cells, treated or not treated with the chemical cross linker di-succinimidyl suberate (DSS), and then analysed by immunoblotting as described previously (Dubey and Ganesh 2008). As shown in figure 1B and C, while laforin showed a higher molecular weight dimeric band as reported earlier (Dubey and Ganesh 2008), no such additional high molecular weight band was visible for malin, suggesting that malin might not form dimers at detectable level though it physically interacts with itself.

Since malin is an E3 ubiquitin ligase, and it interacts with itself, the possibility that malin–malin interaction would promote its own degradation via an auto-ubiquitination process was next explored. For this, wild-type or its catalytic

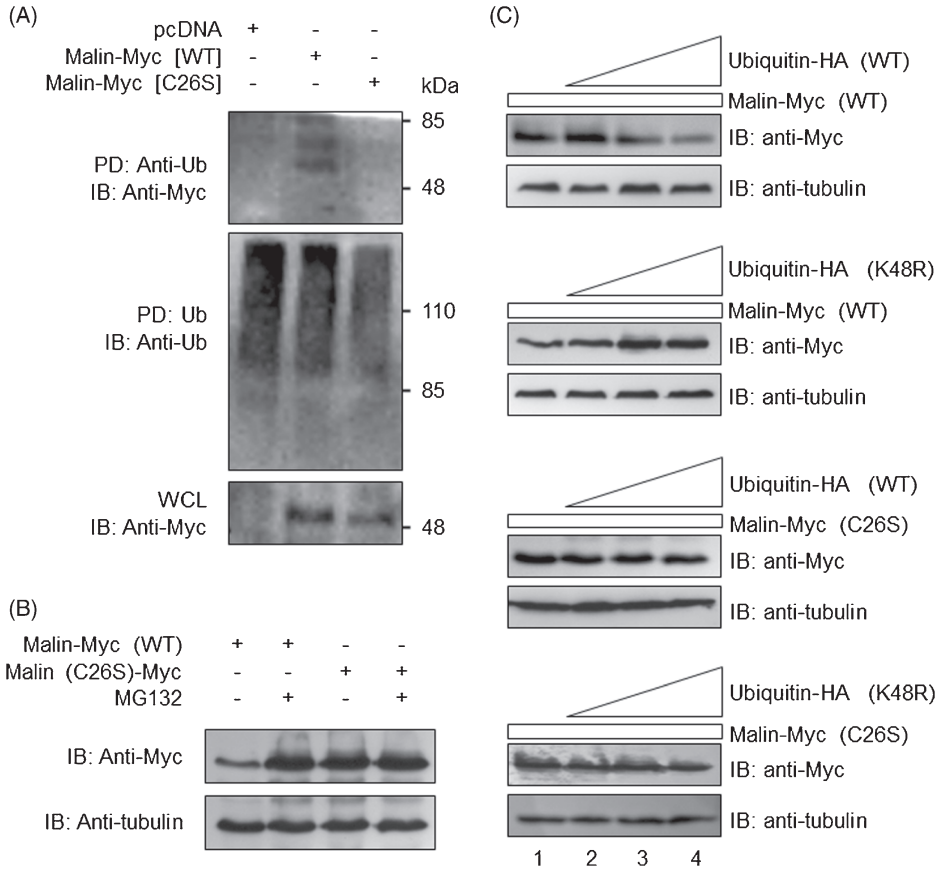

Figure 2. Malin promotes its own degradation: (**A**) The wild-type (WT) or the catalytically inactive (C26S) mutant malin was overexpressed in COS-7 cells and processed for pull-down assay using anti-ubiquitin antibody. The pull-down products (PD) and whole cell lysates (WCL) were resolved and immunoblotted (IB) with indicated antibodies (**B**) Myc/His-tagged malin or Myc/His-tagged C26S malin was transiently expressed in COS-7 cells either in the presence or absence of MG132 as indicated. Equal amount of whole cell lysate for each combination was resolved in SDS-PAGE and immunoblotted with anti-Myc antibody or anti-γ-tubulin antibody (as loading control) to show the difference in the cellular levels of malin. (**C**) Transfections were done in COS-7 cells in 24-well plate for Myc-tagged wild-type malin or the Myc-tagged C26S mutant malin (300 ng/well) with increasing proportion of an expression construct (0, 100, 200, or 400 ng/well in lanes 1 to 4, respectively) for the HA-tagged wild-type ubiquitin (WT) or its mutant (K48R) as indicated. Equal amount of whole cell lysate from each well was resolved in SDS-PAGE and immunoblotted with anti-Myc antibody or anti-γ-tubulin antibody (as loading control) to show the difference in the cellular levels of the proteins.

inactive mutant (RING finger mutant C26S) (Gentry *et al.* 2005; Garyali *et al.* 2009) was expressed in COS-7 cells, the cells were treated with MG132 to prevent proteasomal degradation of malin, and the poly-ubiquitinated proteins were pulled-down using the ubiquitin enrichment kit. The pulled-down product and the whole cell lysate were probed with anti-Myc antibody to detect the overexpressed malin. As shown in figure 2A, overexpression of wild-type malin, but not its mutant version, led to the detection of higher molecular weight, ubiquitinated species in the ubiquitin-enriched fraction. Probing the same blot with anti-ubiquitin antibody revealed the efficiency of pull-down, and the presence ubiquitinated proteins in all three sets. Taken together, these results suggest that the transient expression of malin led to its poly-ubiquitination. Further, to establish that poly-ubiquitination of malin leads to its degradation, the wild-type or its catalytically inactive mutant form (C26S) was overexpressed in the absence or presence of a proteasomal blocker (MG132) and their cellular levels measured by immunoblot. As shown in figure 2B, there was a significant decrease in the cellular level of wild-type malin when compared to the mutant malin or the one that were treated with MG132. Thus, poly-ubiquitination of malin might promote its degradation. The ubiquitination of malin is most likely to be done by the auto-ubiquitination process because the catalytically inactive malin (C26S) did not show any difference

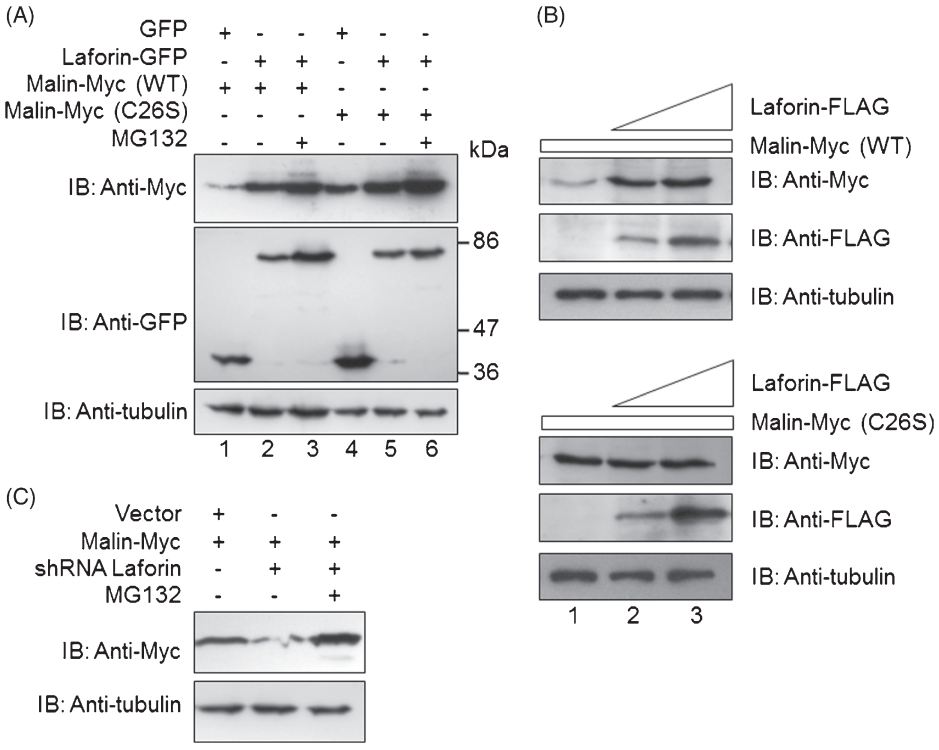

Figure 3. Laforin regulates cellular levels of malin: (**A**) Myc-tagged wild-type malin or its mutant (C26S) was co-expressed with GFP-tagged laforin or GFP in the presence or absence of MG132 in COS-7 cells, as indicated. Equal amount of the whole cell lysate for each combination was resolved in SDS-PAGE and immunoblotted with anti-Myc antibody for detecting malin level, anti-GFP antibody for detecting laforin level or with anti-γ-tubulin as loading control to show the difference in the cellular levels of malin. The fold change in the malin level was calculated by considering the signal intensity of wild-type or the mutant malin (in lane 1 and 4 respectively) as '1', and normalizing the values in lanes 2 and 3 (or 5 and 6) to the loading control, tubulin. The values are in lane 1 to 6 are: 1, 3, 2.8, 1, 1, and 2.4. (**B**) Transfections were done in COS-7 cells for expression constructs that code for Myc-tagged wild-type (WT) malin or its mutant (C26S) (400 ng/well in a 24-well plate) with increasing proportion of FLAG-tagged laforin (0, 200, or 400 ng/well in lanes 1 to 3, respectively). For each combination, the total amount of DNA used was adjusted with an empty vector (pcDNA). Equal amount of whole cell lysate from each well was resolved in SDS-PAGE and immunoblotted with anti-Myc, anti-FLAG or anti-γ-tubulin antibody (as loading control) to show the difference in the level of the protein. (**C**) Myc/His-tagged malin was co-expressed with the shRNA knockdown construct for laforin in the presence or absence of MG132 in Neuro2a cells. Equal amount of whole cell lysate from each well was resolved in SDS-PAGE and immunoblotted with anti-Myc or anti-γ-tubulin antibody (as loading control) to show the difference in the cellular levels of the malin protein. The efficiency of knockdown construct was demonstrated in our previous studies (Garyali *et al.* 2009; Sengupta *et al.* 2011).

in the cellular level when treated with the proteasomal blocker (figure 2B). To further strengthen this suggestion, the wild-type or the mutant (C26S) form of malin was expressed along with increasing concentrations of either the wild-type or the mutant (K48R) form of ubiquitin and checked for its cellular levels by the immunoblot analysis. As shown in figure 2C, the wild-type malin levels tend to decrease in a ubiquitin dose-dependent manner, and such an effect was not observed when the mutant form of malin was co-expressed with the wild-type ubiquitin or when the K48R ubiquitin mutant was co-expressed with the wild-type malin. Taken together, these results demonstrate that overexpression of malin promotes its own degradation via ubiquitination.

3.2 *Laforin regulates the cellular level of malin by presenting itself as a substrate*

Since laforin is an established substrate of malin (Gentry *et al.* 2005) and since malin promotes its own degradation, we speculated that laforin could prevent malin's auto-degradation by presenting itself as a substrate. To test this possibility, wild-type malin was co-expressed with GFP-tagged laforin or with GFP and the level of malin was evaluated by immunoblotting (figure 3A). Similarly, the catalytic mutant of malin (C26S) was co-expressed with laforin as a control. An identical set but treated with the proteasomal inhibitor MG132 was also used as control (figure 3A). The cellular level of wild-type malin was found

to increase several folds when it was co-expressed with laforin (compare lane 1 with lane 2 in figure 3A). However the fold difference observed for the catalytic malin mutant upon co-expression with laforin was much lower as compared to that of the wild-type malin (compare difference in signal intensity between lanes 4 and 5 with lanes 1 and 2 in figure 3A), suggesting that the overexpressed laforin likely increases the cellular level of wild-type malin by presenting itself as a substrate and thus partially preventing the autodegradation of malin. We further show that the wild-type malin level increase in a laforin dose-dependent manner but such an effect was not observed when laforin was co-expressed with the catalytic inactive malin mutant (C26S) (figure 3B). Since Neuro2A cell is known to express laforin endogenously (Garyali *et al.* 2009; Sengupta *et al.* 2011), the possibility that depletion of endogenous laforin would further decrease the cellular levels of overexpressed malin was next explored. For this, malin was overexpressed and the endogenous laforin was knocked down with a shRNA construct in the presence or absence of MG132 and an immunoblot was carried out. As shown in figure 3C, there was a significant reduction in the cellular level of malin when laforin was knocked down but not when treated with MG132.

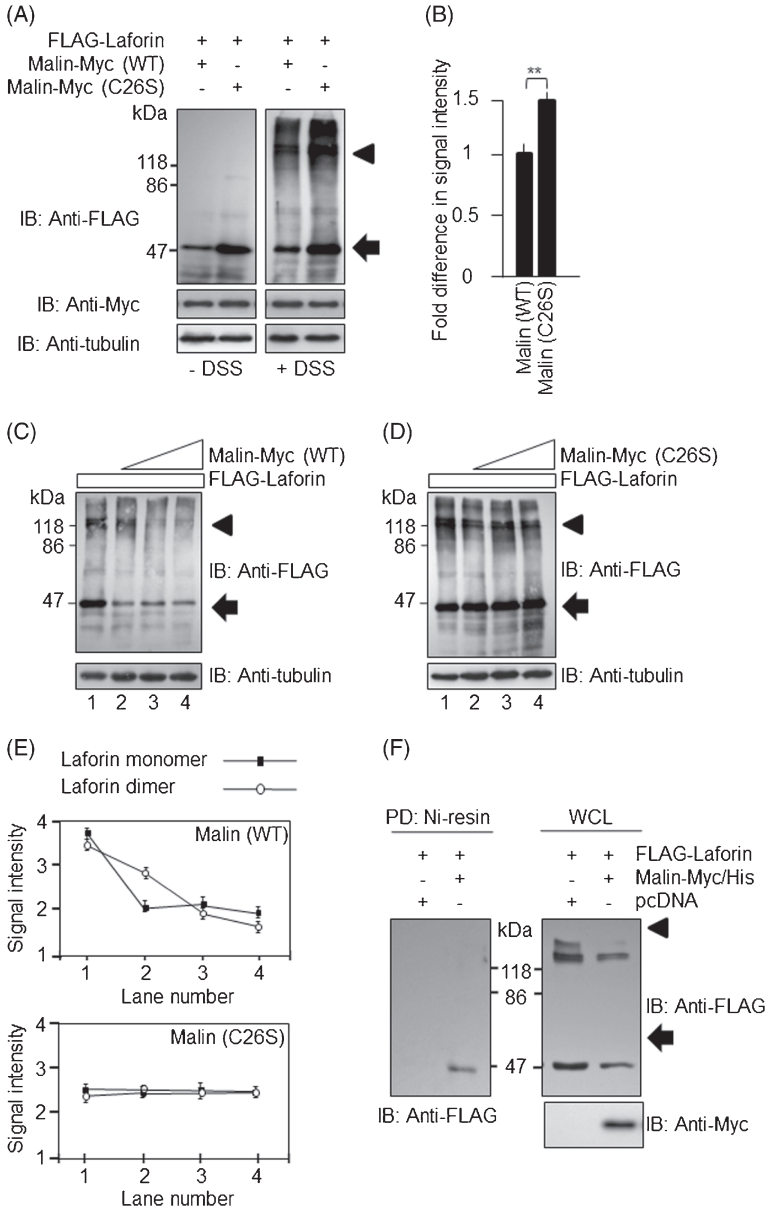

Figure 4. Malin prefers laforin monomer as its substrate. (**A**) FLAG-tagged wild-type laforin was co-expressed with Myc/His-tagged wild-type malin or malin mutant C26S in COS-7 cells that are treated (+) or not treated (−) with cross linker DSS as indicated. The total cell lysates were resolved on SDS PAGE and immunoblotted with anti-FLAG, anti-Myc or anti γ-tubulin antibody. The monomeric and dimeric forms of laforin are identified by an arrow and arrowhead, respectively. (**B**) Bar diagram indicates the relative signal intensity of the monomeric band of laforin as compared to its dimeric band when co-expressed with wild-type malin or the C26S malin mutant, as indicated. The values represent the mean of two independent transfections (+SD) and the signal intensity in the digital images was measured using the Quantity One Discovery Series software of Bio-Rad Laboratories. The *P*-value less than 0.005, calculated by a paired *t*-test, is denoted over the bar by double asterisks (**). (**C, D**) Transfections were done in COS-7 cells in 24-well plate for FLAG-laforin (300 ng/well) and increasing proportion of constructs (0, 100, 200, or 400 ng/well in lanes 1 to 4, respectively) for Myc-tagged wild-type malin (**C**) or its C26S mutant (**D**) and treated with cross linker, DSS. Equal amount of whole cell lysate from each well was resolved in SDS-PAGE and immunoblotted with anti-FLAG or anti-γ-tubulin antibody (loading control). The monomeric and dimeric forms of laforin are identified by an arrow and arrowhead, respectively. (**E**) The line graphs indicating the relative intensity of the band representing the monomeric and the dimeric forms of laforin in the presence of increasing concentration of wild-type malin (WT) or the malin mutant (C26S) construct (for the images shown in **C** and **D**), as indicated. The values represent the mean of two independent experiments (+SD) and the signal intensity in the digital images was measured using the Quantity One Discovery Series software of Bio-Rad Laboratories. (**F**) FLAG-tagged wild-type laforin and Myc/His-tagged wild-type malin were transiently expressed in COS-7 cells separately. The FLAG-laforin expressing cells were treated with the cross linker, DSS. The lysates of cross-linked laforin and untreated malin were mixed and incubated for 2 h at 4°C, and then the Ni-affinity resin was added and the pull down was done as indicated. Lysates from cells that had expressed only the FLAG-laforin was used as control. The pulled-down products (PD) and whole cell lysates (WCL) were immunoblotted (IB) and probed with anti-FLAG or anti-Myc antibody. The monomeric and dimeric forms of laforin are identified by an arrow and arrowhead, respectively.

3.3 *Malin interacts with the phosphatase inactive laforin monomer*

Laforin is known to exist both as a monomer and as dimer. We and others have shown that while the dimeric form is active as a phosphatase (Liu *et al.* 2006; Dubey and Ganesh 2008) but only the monomeric form binds to glycogen (Dubey and Ganesh 2008) suggesting that the two forms of laforin could have distinct functional roles. To explore whether malin show equal preference to the two forms (monomer/dimer) of laforin or whether it prefers one over the other, we co-expressed the wild-type or the mutant form (C26S) of malin with laforin, either treated or not treated the cells with the chemical cross-linker DSS, the cell lysates were resolved in SDS-PAGE and immunoblotted with an antibody to detect the two forms of laforin (figure 4A). The intensity of the signal both for monomeric and dimeric forms of laforin was observed to be lower when co-expressed with wild-type malin as compared with the set wherein the malin mutant was co-expressed (figure 4A). However, the relative intensity of monomeric form of laforin was found to be about 0.5 fold higher when expressed with the mutant malin as compared with the set that co-expressed the wild-type malin (figure 4A and B). This could perhaps mean that both the forms of laforin (monomer/dimer) are in dynamic equilibrium, and that malin prefers the monomeric laforin as a substrate. To strengthen this point further, laforin was co-expressed with increasing concentration of wild-type or the mutant form of malin, treated with DSS, and the cell lysates were processed immunoblotting to detect the monomeric and dimeric forms of laforin. As shown in figure 4C and E, increasing concentration of malin led to a sharp fall in the level of laforin monomer while its dimeric form showed relatively a gradual decrease in the signal intensity. No such difference however was noted when the C26S malin mutant was co-expressed (figure 4D and E). To further establish that the observed drop in the level of laforin dimer is a consequence of loss of its monomeric form in the cellular pool, the wild-type laforin (FLAG-tagged) and wild-type malin (Myc/His-tagged) were overexpressed separately, treated the FLAG-laforin expressing cells with the cross linker (DSS), and then mixed the cell lysates and incubated at 4°C. Subsequently, the lysates were mixed with Ni-resin and malin was pulled down using its His-tag and immunoblotted for laforin with anti-FLAG antibody. As shown in figure 4F, while both forms of laforin were detected in the whole cell lysate, only the monomeric form of laforin was detected in pulled-down product, suggesting that malin's interaction with laforin is restricted to its inactive monomeric form.

4. Discussion

E3 ubiquitin ligases are known to form functional dimers. For example, Cullin3 interacts with itself and form dimeric complex that is active as an E3 ligase (Nikolay *et al.* 2004; Tang *et al.* 2007; Wimuttisuk and Singer 2007; Linke *et al.* 2008). Similarly, E3 ligases are also known to interact with themselves and promote their degradation via auto-ubiquitination (Yang and Li 2000; Zhang *et al.* 2000; Steller 2008). In the present study, while malin was found to interact with itself but this interaction did not appear to

result in its dimerization. The interaction could, however, promote its ubiquitin-mediated auto-degradation because treatment of cells with the proteasome blocker (MG132), or co-expression of the ubiquitin mutant K48R which prevents poly-ubiquitination, led to an increase in the cellular level of wild-type malin. This suggestion is further strengthened by the observation that the wild-type malin is poly-ubiquitinated when overexpressed. Absence of any such changes in the cellular level of the catalytic mutant form of malin suggests that the degradation is indeed through auto-ubiquitination and is not because of an E3 ligase other than malin expressing in the COS-7 cells. These observations, together with the studies with MG132 or the shRNA-mediated knockdown, suggest that the observed changes in the wild-type malin are not due to its overexpression, difference in the transfection efficiency, epitope or the expression vector used. The present set of observations parallel the findings on parkin – an E3 ubiquitin ligase involved in Parkinson's disease (Kitada *et al.* 1998; Imai *et al.* 2000). In this regard, it is of interest to note both malin and parkin are recruited to aggresome when proteasome is blocked (Ardley *et al.* 2003; Mittal *et al.* 2007), and are involved in the clearance of misfolded proteins by associating with Hsp70 (Imai *et al.* 2000; Garyali *et al.* 2009).

Why malin should auto-ubiquitinate and trigger its own degradation? While we are yet to establish a physiological significance to this finding, it is tempting to speculate that this auto-ubiquitination property could be a critical mechanism by which the cellular levels of malin might be regulated. For example, an increase in the level of malin's substrate in the cellular milieu could result in an increase in the level of malin. We did find that co-expression of laforin, an established malin substrate, led an increase in the level of wild-type malin, suggesting that malin is able to regulate its cellular levels via auto-degradation depending on the substrate availability. In this regard it is interesting to note that the cellular level of parkin is regulated by its ubiquitin-like domain which promotes it auto-degradation (Finney *et al.* 2003). Thus, similar to parkin, malin is known to display preference for its substrate (Dubey and Ganesh 2008), associates with HSP70 and help in clearing the misfolded proteins (Garyali *et al.* 2009) and show increased levels of expression under endoplasmic reticulum stress (Vernia *et al.* 2009). Thus, the auto-ubiquitination property may help malin in bringing-down its cellular level to the 'normal state' when the activity of malin is no longer required or when its substrate level goes down in the cell. Taken together our results suggest that the auto-degradation property of malin regulate its cellular of level and that laforin might increase the level of malin by presenting itself as a substrate.

Laforin is known to exist both as a monomer and as dimer. We and others have shown that while the dimeric form is active as a phosphatase (Liu *et al.* 2006; Dubey and Ganesh 2008) but only the monomeric phosphatase-inactive form binds to glycogen (Dubey and Ganesh 2008) suggesting that the two forms of laforin could have distinct functional roles. The present set observations suggest that malin could be one of the regulatory players that selectively degrade the monomeric phosphatase-inactive form of laforin. It is yet to be established whether laforin bind to its substrate as a monomer or as a dimer. It is possible that both forms of laforin may compete for the substrate but only the dimer can catalyse the dephosphorylation. It is likely that the loss of malin or its activity in the LD condition might tilt the monomer/dimer ratio towards the monomeric form such that the cellular level of inactive monomeric laforin might increase and therefore the substrate may remain phosphorylated (or relatively more phosphorylated) even when the *EPM2A* gene is not mutated. This may explain why phospho-glycogen – an established substrate of laforin – remains hyper-phosphorylated in malin-deficient tissues though the level of laforin is very high in that condition (Turnbull *et al.* 2010). An alternate possibility could be that the cellular signals that convert the active (dimeric) form into an inactive form (monomeric) of laforin would also increase in the cellular levels of malin by preventing its auto-degradation by promoting laforin–malin interaction. Thus, loss of laforin in LD conditions, as shown in our knockdown studies, might lead to a significant reduction in the cellular level of malin even when the *NHLRC1* gene is not mutated. This assumption, that laforin and malin regulating each other's activity and level, could offer novel insight into the possible functional interdependence of these proteins and also might help us in understanding the molecular basis of locus heterogeneity in LD.

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