Malin regulates Wnt signalling pathway through degradation of dishevelled 2

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Running title: Malin degrades Dvl2

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Capsule:
Background: Mutations in malin cause approximately 40% cases of Lafora disease, however the underlying mechanism of disease pathogenesis is poorly understood.
Results: Malin interacts with and promotes degradation of disheveled 2 leading to down-regulation of β-catenin-mediated transcriptional activity.
Conclusion: Malin negatively regulates Wnt signaling.
Significance: Aberrant Wnt signaling pathway might be implicated in Lafora disease pathogenesis.

Using yeast-two hybrid screening followed by co-immunoprecipitation assay we have found that Lafora disease ubiquitin ligase malin interacts with dishevelled2, a key mediator of Wnt signalling pathway. Overexpression of malin enhances the degradation of dishevelled2 and inhibits Wnt signalling, which is evident from the down-regulation of β-catenin target genes and decrease in β-catenin-mediated transcriptional activity. Partial knockdown of malin significantly increases the level of dishevelled2 and up-regulates Wnt signalling. Several malin mutants are found to be ineffective in degrading dishevelled2 and regulating the Wnt pathway. We have also found that malin enhances K48 and K63-linked ubiquitination of dishevelled2 that could lead to its degradation through both proteasome and autophagy. Altogether, our results indicate that malin regulates Wnt signalling pathway through the degradation of dishevelled2 and suggest possible deregulation of Wnt signalling in Lafora disease.

Introduction

Lafora disease (LD) is the most severe form of progressive myoclonus epilepsy and inherited as an autosomal recessive manner. Severe myoclonic seizures, dementia, ataxia, muscle wasting and respiratory failure typically characterize the disease (1-5). Onset of the disease is usually in teen age and patient dies within 10 years of first seizure. One of the common pathological hallmarks of LD is the intracellular accumulation polyglucosan inclusions (commonly known as Lafora bodies) in many organs including brain, liver, heart, skeletal muscle and skin (2,3,6). LD is caused by mutations in either EPM2A or EPM2B genes (7,8). EPM2A gene encodes for a dual specificity phosphatase protein called laforin, while EPM2B gene encodes malin, an E3 ubiquitin ligase having RING finger domain (7-10).

Several recent studies have demonstrated that malin in association with laforin regulates the turnover of a variety of proteins mainly those which are involved in glycogen metabolism like glycogen synthase,
glycogen debranching enzyme, protein targeting to glycogen and neuronatin (9,11-16). These findings could potentially explain the formation of Lafora bodies in LD but how mutations in either malin or laforin cause disease pathogenesis and the involvement of Lafora bodies in the disease progression is poorly understood. There are reports suggesting that the formation of Lafora bodies and neuronal loss in LD might be two independent consequences (17,18). This is further supported by some recent studies indicating the role of either laforin or malin in cell survival pathways (19,20). Emerging evidence now pointing towards the defect in intracellular protein degradation pathways in LD pathogenesis (21-24). Since malin is an ubiquitin ligase, its loss of function could results in improper clearance and accumulation of its target substrates leading to the pathogenesis of LD. Therefore, identification of novel cellular substrates of malin could provide further insight in understanding the disease pathogenesis.

Dishevelled (Dvl) is a central mediator of Wnt signalling pathway, which is involved in development, tissue self-renewal and tumorigenesis (25-29). It is a cytoplasmic phosphoprotein and exits as three isoforms in mammals (Dv1-1 to 3). Dvl has three highly conserved domains: an amino terminal DIX domain, a central PDZ domain and a carboxyl terminal DEP domain (29). In canonical Wnt pathway, Dvl regulates Wnt/β-catenin mediated target gene expression by preventing the constitutive degradation of cytosolic β-catenin through ubiquitin-proteasome system (25,26,29). The binding of Wnt ligand to its membrane receptor frizzled causes polymerisation and assembly of Dvl in cell membrane. This provides a platform for axin and glycogen synthase kinase (GSK)-3β, along with adenomatous polyposis coli protein and casin kinase-1, to relocate to the membrane leading to disassembly of the β-catenin destruction complex. β-catenin then translocates to the nucleus and form complexes with transcription factors of lymphoid enhancer-binding factor/T-cell factor family and mediates transcription of Wnt target genes (25-27). Dvl also plays an important role in non-cannonical and calcium-dependent Wnt pathways (29).

The activity of Dvl is dynamically modulated by phosphorylation and ubiquitin-mediated degradation (29,30). Several ubiquitin ligases are implicated in the selective ubiquitination and degradation of Dvl (31-33). Here we demonstrate for the first time that malin interacts with Dvl2 and promotes its degradation through both proteasome and autophagy. Malin also negatively regulates Wnt signalling pathway.

**Experimental Procedures**

**Expression plasmids, antibodies and other chemicals**—The construction of full-length and delRING malin in pcDNA3.1 vector having V5 and His tags and source of C26S malin were described elsewhere (34). Dvl2-FLAG construct was a kind gift from Dr Mariann Bienz (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). TOPflash luciferase vector (measures T-cell factor-dependent transcriptional activity) was kind gift from Dr. Randall T. Moon (University of Washington, Seattle) and mutant ubiquitin constructs were kind gift from Dr. Lim Kah Leong (National Neuroscience Institute, Singapore).

All cell culture reagents; MG132, rapamycin, bafilomycin A (BFA), and mouse monoclonal anti-FLAG were purchased from Sigma. Lipofectamine® 2000, optiMEM, mouse monoclonal anti-V5 and mouse monoclonal anti-GTPDH and anti-β-catenin, were purchased from Invitrogen. Mouse monoclonal anti-myc, rabbit polyclonal anti-GAPDH and human-specific malin and β-catenin siRNA (a pool of 3 target specific 20-25 nucleotide siRNA) along with control (scrambled sequence) were purchased from Santa Cruz Biotechnologies. Alkaline phosphatase and fluorophore conjugated by guest on October 1, 2017 http://www.jbc.org/ Downloaded from
secondary antibodies were purchased from Vector Laboratories. All other chemicals were purchased from Sigma unless otherwise mentioned.

**Yeast two-hybrid screening**—Wild type human malin was cloned into the pGBK7 plasmid and used as bait to screen a human fetal brain cDNA library (Clontech, Palo Alto, CA) as described earlier (16). Colonies were grown on selection medium lacking amino acids (Leu, Trp and His) and positive clones were screened using the auxotrophic marker genes and the α-galactosidase assay. Plasmids isolated from yeast were transformed in E. coli, amplified and sequenced. The interactions were confirmed by re-transforming the identified pACT-cDNA plasmids together with the bait (pGBK7-malin) into the yeast.

**Cell culture, transfection and RT-PCR analysis**—HEK293 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics penicillin/streptomycin. For routine experiments, cells were plated onto six-well tissue culture plates at sub confluent density. After 24 h of plating, cells were transfected with various plasmids using Lipofectamine®-2000 according to the manufacturer’s instruction (transfection efficiency was about 50-60%) and then cells were processed for co-immunoprecipitation, immunoblotting analysis or immunofluorescence studies. The RT-PCR analysis of malin was described earlier (16).

**Co-immunoprecipitation and immunoblotting experiment**—HEK293 cells were transiently transfected with wild type or mutant malin plasmids in 6-well tissue culture plates. After 36 h, cells were washed with ice cold phosphate-buffered saline (PBS), collected by centrifugation, and lysed on ice for 30 min with Nonident P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonident P-40, and complete protease inhibitor cocktail). Cell lysates were briefly sonicated, centrifuged for 10 min at 15 000 × g at 4ºC and the supernatants were used for immunoprecipitation as described earlier (35). Bound proteins were eluted from the beads with SDS (1×) sample buffer, vortexed, boiled for 6 min, and analyzed by immunoblotting. The total cell lysate and the immunoprecipitated proteins were separated through SDS-polyacrylamide gel electrophoresis and proceeded for immunoblot analysis as previously described (35). Dvl2, β-catenin, cyclin D1, GAPDH and FLAG antibodies were used in 1:1000 dilutions and V5 and HA antibodies were used in 1:5000 dilutions.

**Immunofluorescence techniques**—HEK293 cells grown in chamber slides were transiently transfected with appropriate plasmids. Thirty-six hours post transfection, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed 4-5 times, and then blocked with 4% non-fat dried milk in PBS for 2 h. Cells were then incubated with primary antibody overnight at 4ºC. After multiple washings with PBS, cells were incubated with appropriate fluorescent-labelled secondary antibody (1:500 dilutions) for 1 h, washed several times, and then mounted. Samples were observed in a fluorescence microscope (Apotome, Zeiss, Germany), and digital images were assembled using Adobe Photoshop. The anti-Dvl2, anti-V5 and anti-myc were used in 1:1000 dilutions and Anti-HA was used in 1:250 dilutions.

**Luciferase reporter assay**—HEK293 cells were transfected onto 6-well tissue cultured plates with a mixture of TOPflash reporter and PRL-SV40 (express Renilla luciferase) plasmids along with either various malin or Dvl2 constructs as indicated in respective figure legends. Thirty-six hours after transfection, cells were harvested and subjected to dual luciferase assay according to the manufacturer’s instruction. PRL-SV40 used for co-transfection to normalize the data (ratio of Firefly to Renilla values) and transfected at very low concentration (500-fold lower than TOPflash luciferase plasmid). Data was represented as relative luciferase activity.

**In vitro ubiquitination assay**—The in vitro ubiquitination assay was performed as described earlier (36). Dvl2-FLAG protein was immunoprecipitated with FLAG antibody from the HEK293 cell transfected with Dvl2-FLAG.
The immunoprecipitated protein was washed several times and then used for in vitro ubiquitination assay. Briefly, equal amounts of immunoprecipitated Dvl2-FLAG was incubated in a reaction volume of 50 ml of 50 mM of Tris-HCl, pH 7.4 containing 50 ng of E1, 500 ng of E2 (UBCH5c), 2 mg of wild type or delRING malin, 6 mg of ubiquitin, 1 mM of DTT, 2 mM of MgCl2 and 4 mM of ATP. The incubation was carried out at 30°C for 2 h. The reaction was terminated by the addition of SDS-sample buffer, boiled and separated through 10% SDS-polyacrylamide gel electrophoresis. Blot was probed with ubiquitin antibody.

Statistical analysis—Statistical analysis was performed out using SigmaStat software. Values are presented as mean ± SD. Inter group comparisons were analyzed by two-tailed Student’s t test. P<0.05 was considered statistically significant.

Results

Malin interacts with Dvl2—In order to identify the novel interacting partner of malin, we employed yeast two-hybrid screening of human fetal brain cDNA library using wild type malin as bait. DNA sequencing analysis revealed few of the clones as Dvl2 (Fig.1A). Interaction between Dvl2 and malin was further confirmed by co-immunoprecipitation assay. HEK293 cells were transiently transfected with malin or its RING domain deleted mutant (delRING) and then cell lysates were processed for co-immunoprecipitation using Dvl2 antibody. Blots were detected with Dvl2 and V5 antibodies (that detects full-length and delRING malin). We found that both wild type and delRING malin interacts with Dvl2 (Fig.1B). Figure 1C depicts the domain structure of wild type malin and Dvl2 along with the position of various mutants that were used in the experiment. The C26S mutant of malin is detected in subsets of LD patients and it is functionally inactive. The delRING deletion mutant is also functionally inactive as RING domain necessary for ubiquitin ligase function. We next performed double immunofluorescence staining of malin and Dvl2 to study their co-localization. HEK293 cells were transiently transfected with plasmids encoding wild type and mutant malin along with Dvl2 and 36 h later, cells were subjected to immunofluorescence staining. As shown in Figure 2, ectopic expression of Dvl2 formed cytoplasmic puncta that was demonstrated earlier (37,38). Interestingly, wild type malin as well as two of its mutants (delRING and C26S mutant) were co-localized with cytoplasmic puncta of Dvl2. These finding further suggests that wild type malin or its two mutants (delRING and C26S) also associates with Dvl2. It is also important to note that wild type main as well as its mutant showed both cytoplasmic and nuclear distribution that has been shown earlier ((34). Because of the unavailability of good malin antibody, we were unable to check the endogenous co-localization of malin with Dvl2. However, we studied the co-localization of very low levels of over expressed malin with endogenous Dvl2 in HEK293 cells. The endogenous Dvl2 showed diffuse cytoplasmic staining with few punctate structures where malin was co-localized. We have also noticed decrease in the numbers of cytoplasmic puncta of Dvl2 in malin over expressed cells (Supplementary Fig.1).

Malin promotes degradation of Dvl2 and negatively regulates Wnt signalling pathway—Since malin interacts and co-localizes with Dvl2, we further investigated the effect of malin on Dvl2 degradation and regulation of Wnt signalling pathway. HEK293 cells were transfected with different concentrations of plasmid encoding malin and 36 h later, cells were collected and subjected to immunoblot analysis using antibodies against Dvl2 and other markers of Wnt pathway. As shown in Figure 3A and B, the steady-state level of endogenous Dvl2 was decreased upon over expression of malin in a concentration dependent manner. The endogenous level of β-catenin and its two downstream target genes (c-myc and cyclin D1) were also significantly reduced. Malin, however, did not have any direct effect on β-catenin, because there was no interaction between these two proteins (Supplementary Fig.2). Malin also degraded...
ectopically expressed Dvl2 (Fig. 3C and D). We further studied the effect of malin on Wnt/β-catenin-mediated transcriptional activity using TOPflash reporter gene assay. HEK293 and HeLa cells were transfected with different concentrations of malin plasmid along with a mixture of TOPflash reporter and PRL-SV40 plasmids and 36 h of post transfection, cells were collected and subjected to dual luciferase reporter assay. In one set of experiment, cells were treated with Wnt3a to stimulate the Wnt signalling pathway. Figure 4A and B showed that malin significantly reduced the TOPflash reporter activity in a concentration dependent way in both uninduced and induced HEK293 and HeLa cells. Figure 4C and D demonstrated the suppressive effect of malin on TOPflash reporter activity when HEK293 or HeLa cells were treated with different concentrations of Wnt3a. To further confirm our findings, we performed siRNA-mediated knockdown of malin and then studied the level of Dvl2 and TOPflash activity under unstimulated and stimulated conditions. Partial knockdown of malin significantly increased the level of Dvl2 and β-catenin and concomitantly stimulated (significantly over control) the Wnt signalling as evident from the increase in TOPflash reporter activity (Fig. 5). Partial knockdown of β-catenin prevented the Wnt3a-induced TOPflash activity (Fig. 5C). We also studied the effect of two malin mutants (delRING and C26S) on Dvl2 degradation and TOPflash reporter activity and observed that none of the mutants had any significant effect on Dvl2 degradation and TOPflash reporter activity (Fig. 6).

Malin mediates degradation of Dvl2 via proteasome and autophagy—Dvl2 has been shown to be degraded through both proteasome and autophagy (30). To explore the pathway by which malin degrades Dvl2, we first checked the effect of proteasome inhibitor MG132 on Dvl2 level and found that proteasome inhibition partially recovered the malin-induced down-regulation of Dvl2 (Fig. 7A and B). Since Dvl2 also degraded through autophagy, we further checked the effect of autophagy inducers on Dvl2 degradation.

HEK293 cells were transfected with plasmids encoding malin along with Dvl2 and then cells were starved by serum deprivation to induce autophagy. In some experiments, cells were treated with autophagy inhibitor BFA before collecting the cell. Serum starvation decreased the level of Dvl2 and the treatment of BFA partially recovered its level (Fig. 7C and D). Serum starvation also augmented malin-induced decrease in the level of Dvl2, which can be rescued by BFA (Fig. 7C and D). The malin-induced degradation of Dvl2 was also induced upon treatment of autophagy inducer, rapamycin (Supplementary Fig. 3).

Ubiquitination of Dvl2 by malin—To study the effect of malin on Dvl2 ubiquitination, we performed both in vivo and in vitro ubiquitination assay. In in vivo ubiquitination assay, HEK293 cells were transfected with plasmids encoding malin and two of its mutant along with Dvl2 and ubiquitin-HA. Cells were collected and subjected to co-immunoprecipitation using FLAG antibody (to pull down Dvl2) and blots were probed with FLAG and HA antibodies. Figure 8A showed that over expression of malin induced the ubiquitination of Dvl2. The delRING and C26S malin had no effect on Dvl2 ubiquitination. Malin (but not its mutant) also induced ubiquitination of Dvl2 in in vitro ubiquitination assay (Fig. 8B). It was shown that K48-linked ubiquitination targets a substrate for proteasomal degradation, while K63-linked ubiquitination for autophagy (39-41). Since malin promotes degradation of Dvl2 through both proteasome and autophagy, we checked the involvement of malin in K48 and K63-linked ubiquitination of Dvl2. Figure 8C showed that malin induced K48 and K63-linked ubiquitination of Dvl2 in in vivo ubiquitination assay. Lysine-less ubiquitin (K0) was used as control, which was not attached to Dvl2. Double Immunofluorescence staining confirmed that Dvl2 puncta co-localized with both K48 and K63 ubiquitin suggesting further that Dvl2 is ubiquitinated by both K48 and K63-linked ubiquitin chain (Fig. 8D).
Discussion

Here we provide evidence that malin promotes degradation of Dvl2 and thereby negatively regulates Wnt signalling pathway. First, we have shown that malin interacts with Dvl2 in yeast two-hybrid system followed by co-immunoprecipitation assay. Over expression of malin promotes the degradation of Dvl2 and consequently inhibits Wnt signalling. Partial knockdown of malin increases the steady-state level of Dvl2 and stimulates Wnt pathway. Catalytically inactive malin mutants are ineffective in degrading Dvl2 and regulating Wnt signalling. We have also demonstrated that malin promotes K48 and K63-linked ubiquitination of Dvl2, which could leads its degradation through both proteasome and autophagy.

Dvl is a key component of Wnt signalling and transmit Wnt signals from membrane receptors to downstream effectors (25,26,28,29). Current model of canonical Wnt pathway suggests that in the absence of Wnt signals, β-catenin is phosphorylated and degraded by ubiquitin proteasome system. The phosphorylation of β-catenin occurs as part of multi-protein complex that includes GSK-3β, axin, casin kinase-1 and adenomatous polyposis coli. Wnt ligand initiates binding of Dvl to frizzled and axin to LDL-receptor-related protein-5/6 that results in disassembly of the β-catenin destruction complex and consequently accumulation of β-catenin in the nucleus. In the nucleus, β-catenin form complex with T-cell factor/lymphoid-enhancer bindings factor family of transcription factors and activates the transcription of target genes like c-myc, cyclin D1 etc. Ectopic expression of Dvl also causes activation of canonical Wnt signalling pathway (32). Our findings indicate that malin could be one of the important regulators of canonical as well as non-cannonical and calcium-dependent Wnt signalling by promoting the degradation of Dvl2.

Several ubiquitin ligases have been demonstrated to promote the ubiquitination and proteasome-mediated degradation of Dvl including NEDL1, Kelch-like-12-Cullin-3, and prickle-1 (31-33). Over expression of these ligases negatively regulates the Wnt pathway. A recent report demonstrates the degradation of Dvl2 through autophagy under metabolic stress and suggests that autophagy could potentially regulate Wnt signalling pathway (30). Our findings show that malin promote the degradation Dvl2 through both proteasome and autophagic degradation pathways. Malin also promotes both K48 and K63-linked ubiquitination of Dvl2 indicating further that malin uses both proteasomal and autophagic pathways for the clearance of Dvl2. Increasing evidence now suggest that K48-linked ubiquitination targets the substrate for proteasomal degradation while K63-linked ubiquitination for autophagy (39-41).

Interestingly, laforin (a protein phosphatase), another protein implicated in LD has been shown to down-regulate the Wnt signalling pathway by activating GSK-3β (20). Inactivation of laforin results in increased Wnt signalling and tumorigenesis (20). Laforin is an important interacting partner of malin and these two proteins have been suggested to regulate some common physiological pathways, because, LD patients with mutations in either malin or laforin are phenotypically indistinguishable (5,42). In fact, laforin-malin complex have been shown to regulate the level and activity of many key enzymes that are involved in glycogen metabolism (11-15). These findings could provide mechanistic basis in the formation of Lafora bodies in each and every LD patients. Our present findings along with other (20) suggests Wnt signalling pathway could be another common pathway that is regulated by both malin and laforin (Fig.9).

In addition to its well known involvement in several developmental processes including synaptic differentiation, the Wnt signalling pathway also been implicated in regulating synaptic plasticity and neurogenesis in adult (28,43). Loss of function of either malin or laforin could potentially increase Wnt signalling in developing or adult brain leading to abnormal synaptic
differentiation, synaptic plasticity or other abnormalities. The teen-age onset of the disease also indicate possible defect in developmental program in LD. At present there is no evidence of altered Wnt signalling in LD. Interestingly, mutation in prickle-1, another ubiquitin ligase involved in the down-regulation of Wnt signalling through degradation of Dvl, also causes autosomal recessive progressive myoclonus epilepsy-ataxia syndrome (33,44). These findings together with ours suggest possible role of altered Wnt signalling in progressive myoclonus epilepsy including LD. Further studies in malin and laforin knockout mice could provide insight in understanding the role of altered Wnt signalling in LD pathogenesis.

Laforin has also been implicated in the regulation of autophagy and laforin knockout mice exhibits defect in autophagic degradation pathway (23,24). Our findings of malin dependent K63-linked ubiquitination and autophagic degradation of Dvl2 suggests laforin-malin complex might be involved in the autophagic degradation of not only Dvl2 but also other autophagic substrates.

In conclusion, our studies identified Dvl2 as a new substrate of malin. Malin negatively regulates Wnt signalling through degradation of Dvl2. Our findings suggest a possible involvement of altered Wnt signalling in LD pathogenesis.

References
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Figure legends

Fig.1. Malin interacts with Dvl2. A) Identification of Dvl2 as an interacting partner of malin in yeast two-hybrid screening. The wild type malin was used as a bait to screen human fetal cDNA library as described in the Materials and methods. Identified Dvl2 clone (in GAL4-BD vector) was re-transformed into yeast cells along with malin or lamin C (negative control) and grown on plates lacking amino acids Leu, Trp and His in culture media. In positive control, yeast cells were transformed with pGADT7-RecT (express SV40 large T-antigen as a GAL4-AD fusion) along with pGBK T7-53 (express p53 as a GAL4-BD fusion). B) Co-immunoprecipitation of Dvl2 with malin. HEK293 cells were transiently transfected with plasmids encoding LacZ, wild type and delRING malin (all having V5 tag) and 48 h later cells were collected, lysates were made and subjected to co-immunoprecipitation using Dvl2 antibody. Blots were probed with antibodies against Dvl2 and V5 (to detect LacZ, wild type and delRING malin). WCL indicates whole cell lysate. C) Domain structure of malin and Dvl2 including position of specific mutations.

Fig.2. Malin and its mutants co-localize with Dvl2 vesicles. HEK293 cells were transfected with plasmids encoding wild type malin or its two mutants along with Dvl2-FLAG (250 ng of each plasmid/well of 2-well chamber slide). Thirty-six hours later, cells were processed for double immunofluorescence staining using antibodies against Dvl2/V5 (to detect wild type and delRING malin) and Dvl2/myc (to detect C26S). FITC-conjugated secondary antibody was used to recognize wild type or mutant malins and Texas Red-conjugated secondary antibody was used to label Dvl2. Nuclei were counterstained with DAPI. Scale bar; 20 μm.

Fig.3. Malin decreases Dvl2 level and downstream targets of Wnt/β-catenin pathway in the HEK293 cell. A) Cells were transfected with different concentrations of wild type malin and 48 h of post transfection, cell lysates were made and subjected to immunoblot analysis using antibodies against Dvl2, β-catenin, c-myc, cyclin D1, GAPDH and V5 (to detect malin). B) Band intensities of the blot shown above were quantified using NIH image analysis software, normalized with GAPDH and expressed as percent change. Values are mean ± SD of three independent experiments. *p<0.05 in comparison with control. C) Cells were transfected with different concentrations of plasmid encoding malin along with fixed amounts of Dvl2 for 48 h. Collected cells were then subjected to immunoblot analysis using FLAG, GAPDH and V5 antibodies. D) Quantitation of the Dvl2 blot shown in C. Band intensities were normalized with GAPDH and expressed as percent change. *p<0.01 in comparison with control.

Fig.4. Malin inhibits Wnt signalling pathway. A, B) Malin inhibits β-catenin-dependent luciferase activity (TOPflash activity) in a concentration dependent manner. HEK293 (A) or HeLa (B) cells were plated onto 6-well tissue cultured plate and on the following day cells were transfected with increasing concentrations of plasmid encoding malin along with a mixture of TOPflash (500 ng) and Renilla luciferase (1 ng) vectors. Twenty-four hours of post transfection, cells were left untreated or treated with Wnt3a (100 ng/ml) for another 24 h. Cells were then collected and subjected to dual luciferase assay. Values are mean ± SD of three independent experiments each performed in triplicate. *p<0.01 in comparison with respective control group. C, D) HEK293 or HeLa cells were transfected with either empty pcDNA3.1 or malin plasmid (2 μg/well) along with a mixture of TOPflash and Renilla reporter vectors and 24 h of post transfection, cells were treated with different concentrations of Wnt3a for another 24 h. Collected cells were then processed for dual luciferase assay. Values are mean ± SD of two independent experiments each performed triplicate. *p<0.01 in comparison with respective control group.
Fig. 5. Knockdown of malin increases the level of Dvl2 and activates Wnt signalling pathway. A) HEK293 cells were transfected with scrambled siRNA (control) or human malin specific siRNA (each 50 pmol/well of 6-well tissue culture plate) for 48 h. Cells were then collected and either processed for RNA extraction followed by RT-PCR analysis of malin and β-actin or subjected to immunoblot analysis using antibodies against Dvl2, β-catenin and GAPDH. B) Quantitation of the level of Dvl2 and β-catenin in the knock down experiment described above. Values were normalized against GAPDH and expressed as percent change. Values represent the mean ± SD of three independent experiments. *p<0.05 in comparison with respective control group. C) HEK293 cells were transfected with scrambled siRNA (control), β-catenin siRNA or malin siRNA (each 50 pmol/well of 6-well tissue culture plate) along with TOPflash and Renilla luciferase vector for 24 h. Cells were then treated with Wnt3a (200 ng/ml) for another 24 h and collected cells were subjected to dual luciferase assay. Values are mean ± SD of three independent experiments each performed triplicate. *p<0.001 in comparison with control siRNA transfected + Wnt3a treated group.

Fig. 6. Effect of two malin mutants on Dvl2 level and TOPflash activity. A) HEK293 cells were transfected with plasmids encoding wild type or mutants of malin (delRING and C26S) along with Dvl2-FLAG (each plasmid 2 µg/well of 6-well plate). Thirty-six hours later, cells were processed for immunoblot analysis using antibodies against FLAG (to detect Dvl2), V5 (to detect wild type and delRING malin), myc (to detect C26S) and GAPDH. B) Dvl2 blot collected from three independent experiments were quantified using NIH Image analysis software, normalized against GAPDH and expressed as percent change. Values are mean ± SD of three independent experiments. *p<0.001 in comparison with control group. C) HEK293 cells were transiently transfected with plasmid encoding wild type and mutant malins (along with Dvl2-FLAG and mixture of TOPflash and Renilla luciferase vector). Thirty-six hours later, cells were processed for luciferase assay. Values are mean ± SD of three independent experiments each performed triplicate. *p<0.001 in comparison with only Dvl2 transfected group.

Fig. 7. Malin mediates Dvl2 degradation via proteasomal and autophagic pathways. A) HEK293 cells were transfected with Dvl2-FLAG plasmid along with either empty pcDNA3.1 (control) or malin plasmid (each 2 µg/well of 6-well plate) for 36 h. In some experiment, cells were treated with 10 µM of MG132 for 8 h before collecting the cell. Cell lysates were then subjected to immunoblot analysis using FLAG, V5 and GAPDH antibodies. B) Quantitation of the level of Dvl2 in experiment described in A. Values was normalized against GAPDH. Values represent the mean ± SD of three independent experiments. *p<0.01 in comparison with control. C) Cells were transfected with different plasmids as above and then starved with serum deficient media for 6 h before collection. In some experiment, cells were treated with bafilomycin A (BFA) along with serum starvation. Collected cells were then subjected to immunoblot analysis using FLAG to detect Dvl2 and GAPDH antibodies. D) Quantitation of the level of Dvl2 in experiment described in C. Values was normalized against GAPDH. Values represent the mean ± SD of three independent experiments. *p<0.05.

Fig. 8. Malin ubiquitinates Dvl2 in vivo and in vitro. A) HEK293 cells were transiently transfected onto 6-well tissue cultured plate with plasmids encoding wild type malin or its mutants (2 µg/well) along with Dvl2-FLAG (2 µg/well) and ubiquitin-HA (1 µg/well). Forty-eight hours later, cells were collected and processed for co-immunoprecipitation using FLAG antibody and blots were probed with either FLAG or HA antibody. B) Malin induces ubiquitination of Dvl2 in in vitro ubiquitination assay. Equal amounts of immunoprecipitated Dvl2 were incubated with malin, delRING malin along with ubiquitin, E1 and E2 (UBCH5c) as described in Materials and methods. Blot was
detected with ubiquitin antibody. C) Cells were transfected with plasmids encoding Dvl2, wild type malin as in A along with wild type or various mutant forms of ubiquitin-HA (each 1 μg/well) constructs. Forty-eight hours later cells were processed for co-immunoprecipitation using FLAG antibody and blot was detected with HA antibody (to detect ubiquitinated Dvl2). D) HEK293 cells were transfected with Dvl2-FLAG along with K48 or K63-ubiquitin-HA constructs in chamber slides. Thirty-six hours later, cells were processed for double immunofluorescence staining using antibodies against Dvl2/HA (to detect ubiquitin). FITC-conjugated secondary antibody was used to detect ubiquitin and Texas Red-conjugated secondary antibody was used to label Dvl2. Nuclei were counterstained with DAPI. Scale bar; 20 μM.

Fig.9. Schematic representation of the regulation of Wnt pathway by malin and laforin. Laforin is shown to function as a phosphatase for GSK-3β. It stimulates the degradation of β-catenin and down regulates Wnt signalling by activating GSK-3β ((20). Our finding shows that malin down regulates Wnt pathway by promoting degradation of Dvl2. Therefore, loss of function of either malin or laforin could results in aberrant Wnt signalling, which might be one of the possible cause of LD pathogenesis.
Fig. 1

A

Positive control

Malin + Dvl2

Negative control

B

<table>
<thead>
<tr>
<th></th>
<th>WCL</th>
<th>IP: Anti-Dvl2</th>
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<tr>
<td>LacZ</td>
<td>Malin</td>
<td>delRING</td>
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IP: Dvl2
IB: V5

LacZ

Malin

delRING

Dvl2

C

Malin

Dvl2

C26S

RING

NHL

NHL

NHL

NHL

NHL

395

DIX

PDZ

DEP

1 13 93 267 355 414 507 736
Fig. 4

A. HEK293

B. HeLa

C. Relative luciferase activity vs. Wnt 3a (ng/ml)

D. Relative luciferase activity vs. Wnt 3a (ng/ml)
Fig. 5

A

Immunoblot

Control siRNA

Malin siRNA

Dvl2

β catenin

GAPDH

RT-PCR

Malin

β actin

B

Percent Change

Control siRNA

Malin siRNA

Dvl2

β catenin

C

Relative luciferase activity

Wnt3A

Con. siRNA

β cat. siRNA

Malin siRNA

- + - + - + - + - +
Fig. 6

A

Control | Malin | delRING | C26S
--- | --- | --- | ---
Dvl2-FLAG
GAPDH
Malin-V5
delRING-V5
C26S-myc

B

Percent change

Control | Malin | delRING | C26S
--- | --- | --- | ---

C

Relative luciferase activity

Dvl2 | Malin | delRING | C26S
--- | --- | --- | ---
- | - | + | +
+ | + | + | +
- | + | - | -
- | - | - | +
Fig. 9

Ubiquitination and degradation of Malin lead to the degradation of Dvl2, which in turn activates Wnt signalling. Laforin and phosphorylation of GSK3β also contribute to this process.

Dvl2

GSK3β

Wnt signalling

Ubiquitination

Dephosphorylation

and degradation

and activation