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Lafora disease proteins laforin and malin negatively regulate the HIPK2-p53 cell death pathway

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ABSTRACT

Lafora disease (LD) is an autosomal recessive, progressive, and fatal form of a neurodegenerative disorder characterized by the presence of Lafora polyglucosan bodies. LD is caused by defects in either the laforin protein phosphatase or the malin E3 ubiquitin ligase. Laforin and malin were shown play key roles in proteolytic processes, unfolded stress response, and glycogen metabolism. Therefore, the LD proteins laforin and malin are thought to function as pro-survival factors and their loss thus could result in neurodegeneration. To understand the molecular pathway leading to the cell death in LD, in the present study, we investigated the possible role of LD proteins in the p53-mediated cell death pathway. We show that loss of laforin or malin results in the increased level and activity of p53, both in cellular and animal models of LD, and that this is primarily due to the increased levels of Hipk2, a proapoptotic activator of p53. Overexpression of laforin or malin confers protection against Hipk2-mediated cell death by targeting the Hipk2 to the cytoplasmic compartment. Taken together, our study strengthens the notion that laforin and malin are pro-survival factors, and that the activation of Hipk2-p53 cell death pathway might underlie neurodegeneration in LD.

1. Introduction

The progressive myoclonus epilepsy of Lafora, or the Lafora disease (LD), is characterized by the presence of glycogen-rich inclusions in the neurons and other affected tissues [1,2]. The symptoms of LD include the disease defining myoclonic epilepsy, ataxia, dementia and cognitive defects [1,2]. Being an autosomal recessive disorder, LD is caused by mutations in both copies of either the *EPM2A* gene coding for the protein phosphatase laforin [3,4] or the *NHLRC1* gene coding for an E3 ubiquitin ligase named malin [5,6]. Studies have revealed that laforin and malin are recruited to aggresomes upon proteasomal impairment, and as a functional complex, promote the degradation of cytotoxic misfolded proteins [7,8]. Loss of laforin or malin renders cells susceptible for ER stress. In addition to ER stress, laforin and malin as a complex with CHIP (C-terminus hsp70 interacting protein) activate heat-shock factor-1 (HSF1), and protects cells from heat stress-mediated cell death [9]. Since LD is associated with neuronal cell death, laforin and malin are thought to play a pro-survival role in neuronal growth and maintenance [1,10].

The homeodomain-interacting protein kinase-2 (HIPK2) is known to function as a tumor suppressor and as a stress kinase [11]. HIPK2 is a very unstable protein that resides in the nucleus during normal physiological conditions, and its basal level is regulated by the E3 ubiquitin ligases, Siah-1 and Wsb-1 [12,13]. Upon DNA damage, HIPK2 is known to get stabilized, activated, and promote cell death via the activation of p53 pathway [14]; HIPK2 phosphorylates p53 at serine 46, leading to the activation of the latter [14,15]. The level and the activity of HIPK2 are regulated by the E3 ubiquitin-protein ligase MDM2 [16]; conversely, HIPK2 regulates the level and/or activity of MDM2 [17,18] and the transcriptional co-repressor CtBP [19], suggesting a complex interplay between pro- and anti-apoptotic factors. Intriguingly, the subcellular localization of HIPK2 is known to play an important role in its activity and stability [20,21]; cytoplasmic HIPK2 may be a negative marker for p53-dependent pro-apoptotic activity. Since CHIP is known to modulate the interaction between p53 and HIPK2 and the eventual activation of p53 [22,23], and since CHIP requires malin and laforin

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for its cellular activity [9], in the present study, we tested the possible role of laforin and malin in the Hipk2-p53 cell death pathway.

2. Materials and methods

2.1. Reagents and antibodies

The following antibodies were used for the experiments: anti-GFP (Roche India), anti-Myc (Roche India), anti-γ-tubulin, anti-FLAG, anti-V5, anti-HA (all from Sigma–Aldrich India Pvt. Ltd), anti-HIPK2 (Aviva Systems Biology, USA), anti-p53, anti-S46p53, anti-GRP75 (Santa Cruz Biotechnology, Inc), and anti-histone H4 (acetyl K8) (Abcam, USA). The secondary antibodies were obtained from Jackson ImmunoResearch Inc, USA. All chemical were purchased from Sigma–Aldrich India Pvt. Ltd. unless otherwise stated.

2.2. Expression constructs

The expression vectors coding Myc-, FLAG- or GFP-tagged wild-type or the mutant form of laforin or malin were described previously [7–9]. The shRNA knock-down constructs for the silencing the *Epm2a*, *Nhlrc1* or *CHIP* gene were validated and reported in our previous studies [9] (Supplementary Fig. S1). Expression construct coding for V5/myc-tagged CHIP was generously provided by Dr Nihar R. Jana (NBRC, India). Construct coding for the wild-type HIPK2 was provided by Dr. Thomas G. Hofmann (German Cancer Research Institute, Germany). The p53 reporter construct coding for firefly luciferase was obtained from Addgene (Plasmid id: 16442, PG13-luc containing wild-type p53 binding sites). Renilla luciferase encoding plasmid pGL4.74 was generously gifted by Dr. Amitabha Bandyopadhyay (IIT Kanpur, India).

2.3. Cell culture, treatment and transfection

COS-7 and Neuro2A cells were grown at 37 °C (5% CO_2) in Dulbecco's modified Eagle's medium (Sigma–Aldrich India Pvt Ltd) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and100 mg/ml streptomycin. Transfection was performed using Turbofect transfection reagent (Thermo Fisher Scientific India), according to the manufacturer's protocol. Cells were harvested 36–48 h post-transfection as recommended by the manufacturer.

2.4. Immunocytochemistry and cell counting

Cells grown on gelatin-coated sterile glass coverslips were transfected and processed for immunofluorescence microscopy as described [7–9]. Fluorescence images were captured using a fluorescence microscope (Axiovision) with 40× objective lens with ApoTome module (Carl Zeiss). At least 300 transfected cells were counted for each set of experiments. Each experiment was repeated at least three times and scoring was done in a blinded manner.

2.5. Dual-luciferase assay

Cells were transfected with luciferase reporter plasmid containing the p53 responsive element, p53-Luc (plasmid Id 16442) along with the shRNA construct for the knock-down of laforin, malin or CHIP, or a non-silencing construct. As an internal control for transfection efficiency, the cells were co-transfected with vector (pGL4.74) which codes for the renilla luciferase. At 36 h post-transfection, the cells were lysed in Passive Lysis Buffer (Promega Corporation, India), and the luciferase activity was measured with the dual-luciferase assay kit (Promega Corporation, India). The relative light units per second (RLU/s) were normalized to the Renilla luciferase activity and expressed as fold difference of control. All experiments were performed in triplicate.

2.6. Subcellular fractionation

Trypsinized cells were washed once with 1 × PBS, centrifuged, and the fractionation was performed using nuclear/cytosol fractionation kit (BioVision, USA) according to manufacturer's instructions. The relative ratio of cytoplasmic to nuclear fraction was maintained, and the fractions were resolved on a SDS-PAGE and immunoblotted.

2.7. Immunoblotting

Protein samples were resolved on an 8 or 10% SDS–PAGE and were transferred onto a nitrocellulose membrane (MDI, India). After blocking with 5% non-fat dry milk powder at 37 °C for one hour, the membranes were processed for immunodetection of antigen using a chemiluminescence detection kit (SuperSignal West PICO, Thermo Scientific India) as described [9]. Signal intensities on the digital images of immunoblots were quantified using NIH image software (ImageJ).

2.8. RNA extraction and real-time PCR

Total RNA was isolated using Trizol reagent (Banglore Genei) and the total RNA was converted into cDNA by using muMLV reverse transcriptase (Thermo Scientific India) using random hexamers as primers. The resulting cDNA was then used as a template to assess the relative mRNA levels of target genes using SsoFast EvaGreen supermix as per the recommendation of the manufacturer (Bio-Rad Laboratories India). Primers used for amplifying the targets genes are given in Supplementary Material Table S1.

2.9. MTT assay

Transfected cells were incubated with 0.5 mg/ml MTT (thiazolyl blue tetrazolium bromide) for 4–5 h. After removal of the medium, the cells were incubated with DMSO (100%) for 20 min to dissolve formazon crystals. The change in optical density was recorded through spectrophotometer at λ_{570nm} against background reading at λ_{650nm}.

2.10. Laforin- and malin-deficient animals

Laforin- or malin-deficient mice were genotyped and tissue lysate of the knockout mice and their littermates were processed for immunoblotting. For genotyping details, see Supplementary Fig. S2B. The study protocol was approved by the animal ethics committee of the institute.

2.11. Statistical analysis

The average of the each experiment was calculated and plotted. Standard deviations for the observed values were calculated, and statistical significance was analyzed with two tailed unpaired t-test ($P < 0.05$) using the GraphPad software.

3. Results

3.1. Loss of laforin or malin results in the increased levels and activity of p53

CHIP is one the critical regulators of the level and activity p53 [22]. Since laforin and malin are known to function together with

CHIP in heat-shock response [9] and since neuronal cell death is associated with LD [10], we wanted to check if loss of laforin and malin would also alter the level and/or activity of p53. There was a significant increase in the total level of p53 when laforin or malin was partially knocked down in COS-7 or Neuro2A cells, suggesting that laforin-malin complex regulates the cellular level of p53 (Fig. 1A and Supplementary Fig. S2A). Partial knockdown of CHIP also showed similar effect (Fig. 1A; Supplementary Fig. S2A), as reported earlier [22]. The efficiency of individual knock-down construct was confirmed by RT-PCR (see Supplementary Fig. S1). To further check whether the increased level of p53 correlates with an increase in its active form, we looked at the level of S46 phosphorylated of p53 using a specific antibody. Phosphorylation of S46 residue activates p53 for its apoptotic function [16]. As shown in Fig. 1A (lower panel), partial loss of laforin, malin or CHIP led to a significant increase in the phospho-S46-p53. An increase in the p53 level was also noted in the brain lysates from the laforin- or malin-deficient 3-month-old mice (Fig. 1B). We have also measured the p53 activity using a luciferase reporter construct. As shown in Fig. 1C (and Supplementary Fig. S2C), partial loss of laforin, malin or CHIP led to a significant increase in the p53 reporter activity, both in Neuro2A and COS7 cells. We also selected four endogenous target genes of p53 and looked at their transcript level. These include Bak1, Bax, and Puma [24–27]. We have also tested the expression level of p53 since its expression is known to be autoregulated [28]. As shown in Fig. 1D, the transcript level of all three targets was significantly higher upon partial knock-down of malin or laforin. Similar pattern was also noted for p53, in line with earlier finding of an auto activation loop for p53. Taken together ours results clearly indicate that loss of LD proteins activate the p53-mediated cell death pathway, similar to the loss of CHIP as reported earlier [22,23].

3.2. LD proteins regulate the levels and subcellular localization of Hipk2

The homeodomain-interacting protein kinase-2 (HIPK2) is a critical regulator of p53, and is known to activate p53 by phosphorylating its S46 residue and eventual stabilization [15,16]. Since loss of laforin or malin results in the activation of p53 via S46 phosphorylation, we next wanted to check whether Hipk2 level is altered in the laforin or malin-deficient cells. As shown in Fig. 2A, transient knock-down of laforin, malin or CHIP led to a significant increase in the Hipk2 levels. We next tested whether the increased activity of p53 is indeed via Hipk2. For this we transiently overexpressed Hipk2 either with the wild-type malin, laforin, CHIP or their catalytically inactive mutants (as controls) and looked at the level of phospho-S46-p53 – the active form p53. As shown in Fig. 2A, the level of phospho-S46-p53 was at reduced levels when the wild-type form of malin, laforin or CHIP was overexpressed and such a difference was not observed when their catalytically inactive mutant forms found in LD families [1,7–9] were co-expressed with Hipk2. We have also carried out luciferase-based reporter assay to measure the Hipk2-mediated

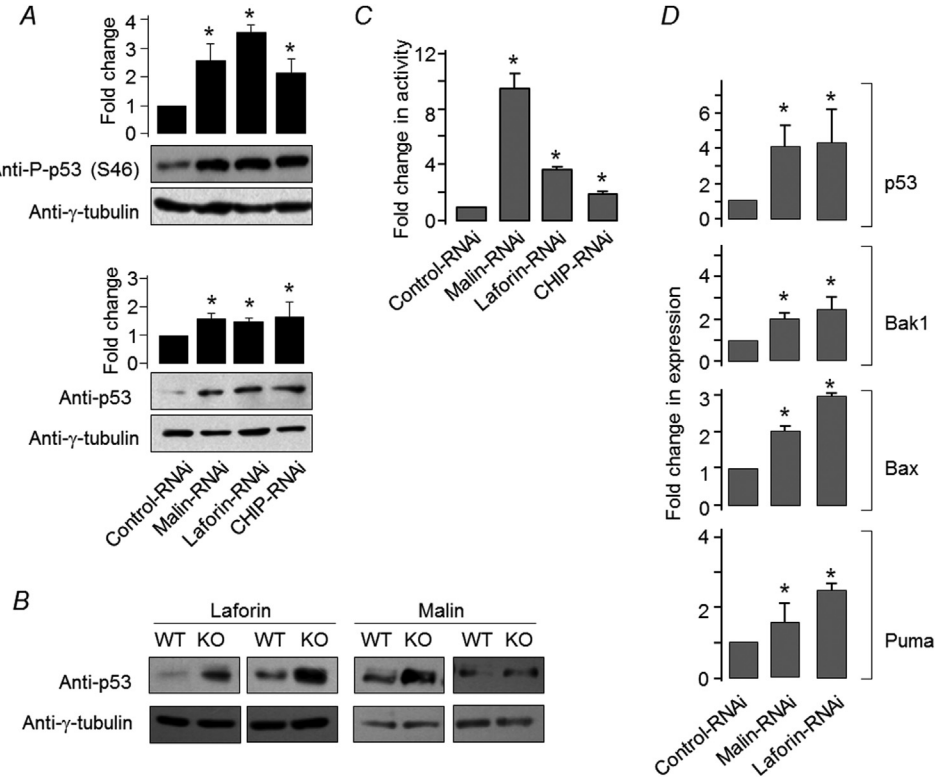

Fig. 1. Loss of laforin or malin results in the increased levels and activity of p53 (A) (A) COS7 cells transiently transfected with the knock-down construct for laforin, malin or CHIP were processed for probing the total level of p53 (bottom) or its S46-phospho form (top) using specific antibodies. The bar diagram shown above display the fold changes in p53/phospho-p53 signal as compared to γ-tubulin as measured by densitometric analysis and normalized to control set (N = 3; *, $p < 0.05$). (B) Immunoblot showing the signal intensity for the total and phosphor form of p53 in brain tissues from a pair of wild-type (WT) or laforin or malin-deficient mice (KO), as indicated. (C) Bar diagram showing fold change in the p53 reporter activity; COS7 cells were co-transfected with the p53 promoter reporter construct "p53-luc" driving the firefly luciferase, the pGL4.74 construct driving renilla luciferase under TK promoter, and the knock construct for malin, laforin, CHIP or a non-silencing construct. The luciferase activity for each set was measured as ratio of firefly to renilla and was normalized with activity of no heat-shock control set (taken as 1 fold) and plotted as fold change (N = 3; *, $p < 0.05$). (D) Fold change in the transcript level of p53 target genes, Bak1, Bax, Puma and p53, as indicated. RNA extracted from Neuro2A cells transiently transfected with knockdown construct for laforin, malin or a non-silencing construct was used for the real-time PCR analysis. Value of the control set (non-silencing construct) was taken as 1 (N = 3; *, $p < 0.05$).

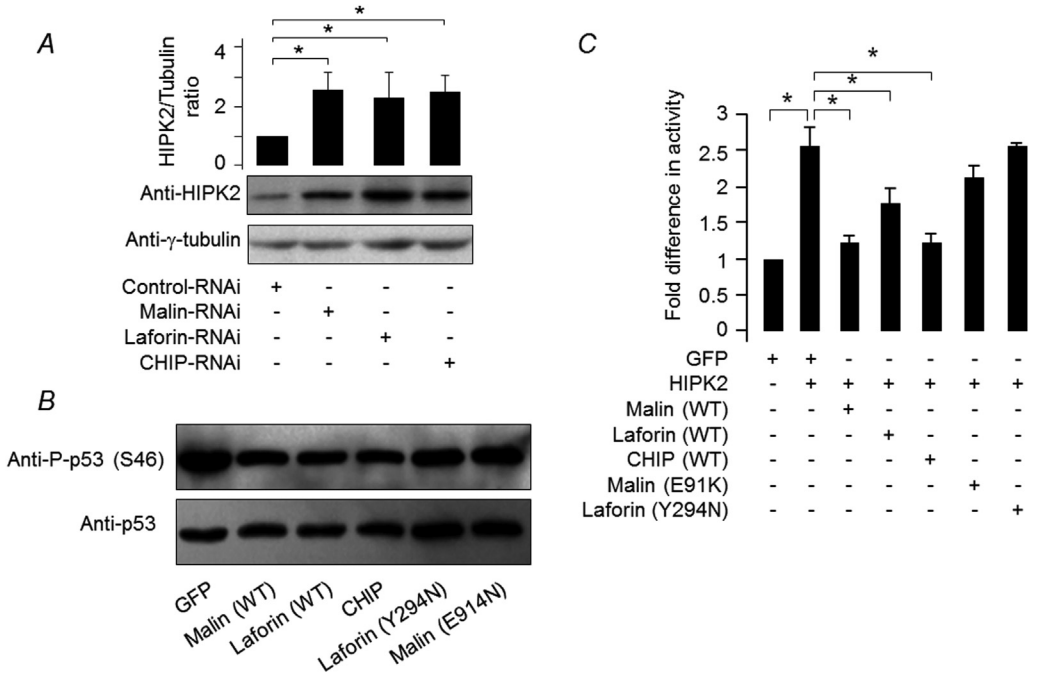

Fig. 2. Laforin and malin regulate the level and activity of Hipk2: (A) Immuoblot showing the level of Hipk2 in COS7 cells transiently transfected with knock-down construct for laforin, malin or CHIP (or control vector). The bar diagram above shows the fold changes in signal intensities, based on densitometric analysis normalized to control set (N = 3; *, $p < 0.05$). (B) FLAG-tagged HIPK2 was transiently expressed in COS7 cells along with wild-type (WT) forms of laforin, malin or CHIP, or with the mutant form of laforin or malin (E91K) or with GFP and the level of total and S46 phospho-p53 was measured by immunoblotting. (C) Dual-luciferase activity – as elaborated in Fig. 1C, for HIPK2 mediated activation of p53 reporter in COS7 cells. HIPK2 and the wild-type (WT) or mutant forms of laforin, malin and CHIP were transiently expressed and the p53 reported activity was measured. The activity in cells expressing GFP was considered as 1 (control), the other values were normalized to this control.

p53 activation. As shown in Fig. 2C (and Supplementary Fig. S3A), Hipk2 showed lower levels of p53-mediated reporter activity when co-expressed with the wild-type forms of malin, laforin and CHIP, as compared with when co-expressed with GFP or with the catalytically inactive mutant forms of laforin and malin Thus LD proteins appear to negatively regulate the Hipk2-mediated activation of p53.

The pro-apoptotic function of Hipk2 is also known to be associated with its nuclear localization [20,21]. Therefore we wanted to test whether laforin and malin would alter the subcellular localization of Hipk2. For this we transiently expressed FLAG-tagged Hipk2 with laforin, malin or CHIP, and scored for its nuclear localization. Co-expression of laforin, malin or CHIP, but not GFP, significantly increased the number of cells with cytoplasmic localization of Hipk2 (Fig. 3A,B; Supplementary Figs. S4 and S5). The disease associated, catalytically inactive laforin or malin did not alter the subcellular localization of Hipk2 (Fig. 3B), suggesting that the ubiquitin ligase activity of malin and the phosphatase activity of laforin are essential for the cytoplasmic translocation of Hipk2. The cytoplasmic translocation of Hipk2 resulting from the overexpression of laforin, malin or CHIP was validated by immunoblotting in the nuclear and cytoplasmic fractions of the transfected cells – both for the endogenous Hipk2 (Fig. 3C) and the transiently expressed Hipk2 (Fig. 3D). Overexpression of Hipk2 is known to induce death in cell lines [17]. Since the nuclear localization of Hipk2 is associated with its pro-apoptotic function, and loss of laforin or malin results in the increased activity of p53, we next tested whether co-expression of laforin, malin or CHIP would confer protection against the Hipk2-induced cell death. As shown in Fig. 3E (and Supplementary Fig. S3B), cells that co-expressed malin, laforin or CHIP along with Hipk2 survived better as compared to cells that co-expressed GFP with Hipk2, suggesting laforin, malin and CHIP confer protection against Hipk2 mediated cell death. The absence of any such protection from the disease associated, catalytically inactive mutants of laforin and malin suggest that the ligase activity of malin and phosphatase activity of laforin are required for the protection (Fig. 3E and Supplementary Fig. S3B).

4. Discussion

We and others have previously reported a role for Lafora disease proteins laforin and malin in cellular stress response [9,29,30], proteolytic processes [7,8,31], glucose metabolism [32–34] and RNA metabolism [35]. In this report, we attempt to propose a link for the LD proteins in the HIPK2-p53-mediated cell death pathway. The pro-apoptotic function of p53 is regulated by multiple factors and HIPK2 is the one that activate p53 via post-translation modification leading to the conformational stability and activity of the latter [14,15]. Consistent with this model, the cytoplasmic HIPK2 was not able to activate p53, suggesting the presence of other factors that regulate subcellular localization of HIPK2 and its activity [20,35]. CHIP is one of such factors and is known to modulate the HIPK2–p53 interaction in stress-dependent manner [23]. We demonstrate here that the LD proteins are two other modulators. Similar to CHIP, the LD proteins laforin and malin were able to regulate the cytoplasmic localization of HIPK2 and confer protection against HIPK2-mediated cell death, both in neuronal and non-neuronal cells. Our observation that partial depletion of malin, laforin or CHIP, but not their overexpression, led to a difference in the level of HIPK2 suggests an indirect role for LD proteins in HIPK2 regulations. Intriguingly zyxin, ATM kinase and Src kinase were reported to regulate HIPK2 level by modulating its proteasomal degradation [12,36,37]. It would be of interest to check the possible cross-talk between DNA damage and heat shock response pathways, and to explore if LD proteins are involved in DNA damage

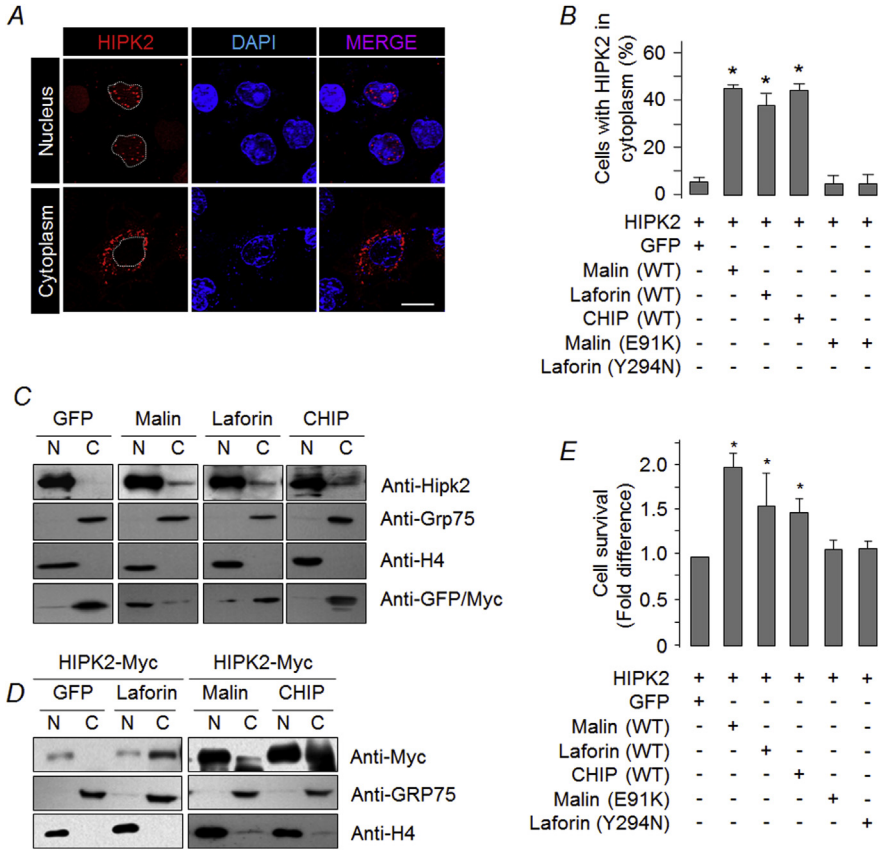

Fig. 3. Laforin- and malin-dependent subcellular localization of HIPK2: (A) Representative images showing the nuclear and cytoplasmic localization of transiently expressed HIPK2 in COS7 cells. The nucleus was stained with DAPI. Dotted lines in HIPK2 panel named HIPK2 indicate the nuclear boundary (for more images, see Supplementary Figs. S4 and S5). (B) Bar diagram showing percent transfected cells with cytoplasmic localization of HIPK2 when expressed with GFP, malin, laforin or CHIP, as indicated (N = 3; *, $p < 0.05$). (C) Immunoblots showing the presence of endogenous HIPK2 in the nuclear or cytoplasmic fraction (identified as "N" or "C" respectively) of COS7 cells transiently expressing GFP, laforin, malin or CHIP as indicated. The blots were also probes with anti-GRP75 and anti-histone (H4) antibodies to show purity of the nuclear and cytoplasmic fractions. Anti-GFP antibody detected the overexpressed GFP, GFP-tagged laforin and malin, while anti-Myc detected the CHIP. (D) Same as C except that transiently transfected with HIPK2 was probed with the Myc-tag antibody. (E) MTT assay to measure cell survival in COS7 cells transiently overexpressing HIPK2 along with GFP, wild-type (WT) laforin, wild-type malin, wild-type CHIP, laforin mutant Y294N or malin mutant E91K, as indicated. Cell survival for the sample set expressing GFP was considered as 1 and the fold difference in the survival for other groups was accordingly plotted (N = 3; *, $p < 0.05$).

signaling networks and whether HIPK2 would be involved in some of the LD pathology.

Being an activator of p53, it is expected that an increase in HIPK2 level/activity would increase the p53 level and its activity [14–16]. Consistent with this model, we found that loss of malin or laforin led to an increase in the p53 levels – both in cellular and animal models. The increased p53 level also correlated with its increased transcriptional activity, suggesting that LD proteins are anti-apoptotic in nature and that the cell death observed in laforin- or malin-deficient mice could be due to the activation of p53. Consistent with this view, the transcript levels of four pro-apoptotic factors were at higher levels in cells partially knockdown for laforin or malin. Taken together, our study suggests that activation of HIPK2-p53 cell death pathway might underlie some of the cell death phenotype seen in case of Lafora disease.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.018.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.018.

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