Malin and laforin are essential components of a protein complex that protects cells from thermal stress

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Summary

The heat-shock response is a conserved cellular process characterized by the induction of a unique group of proteins known as heat-shock proteins. One of the primary triggers for this response, at least in mammals, is heat-shock factor 1 (HSF1) – a transcription factor that activates the transcription of heat-shock genes and confers protection against stress-induced cell death. In the present study, we investigated the role of the phosphatase laforin and the ubiquitin ligase malin in the HSF1-mediated heat-shock response. Laforin and malin are defective in Lafora disease (LD), a neurodegenerative disorder associated with epileptic seizures. Using cellular models, we demonstrate that these two proteins, as a functional complex with the co-chaperone CHIP, translocate to the nucleus upon heat shock and that all the three members of this complex are required for full protection against heat-shock-induced cell death. We show further that laforin and malin interact with HSF1 and contribute to its activation during stress by an unknown mechanism. HSF1 is also required for the heat-induced nuclear translocation of laforin and malin. This study demonstrates that laforin and malin are key regulators of HSF1 and that defects in the HSF1-mediated stress response pathway might underlie some of the pathological symptoms in LD.

Key words: Stress response, Heat-shock response, Co-chaperone, Ubiquitin ligase, Malin, Laforin, Lafora disease

Introduction

The heat-shock response is a process that is evolutionarily conserved, has a rapid onset, is short-term and is essential for cells to survive under conditions of stress. It is characterized by an increase in the cellular level of molecular chaperones known as heat-shock proteins (HSPs) (Akerfelt et al., 2010). Besides heat shock, a large variety of stressors are known to induce the expression of HSPs. Therefore, the heat-shock response is now considered synonymous to the cellular stress response (Morimoto, 1998). The proximal trigger in all these stresses seems to be damage to the proteome of the cell, and hence HSPs are induced to stabilize partially unfolded proteins (Morimoto, 1998; Morimoto, 2008). The HSPs, especially the inducible forms, are thus protective against stress-induced cell death (Kalmar and Greensmith, 2009; Hartl, 1996). HSPs are also present in non-stressed cells, although at a lower level, to help newly synthesized proteins to fold properly and to target old proteins for degradation. Thus, there appears to be a delicate balance between the demand and the supply of HSPs, and hence HSPs are rapidly induced when the cells are exposed to unfolded protein stress. This is primarily achieved, at the transcriptional level, by heat-shock factor 1 (HSF1), a transcription factor that binds to heat-shock elements present in the promoter region of genes encoding HSPs (Akerfelt et al., 2010). The activation of HSF1 involves its translocation from the cytoplasm to the nucleus, trimerization and hyperphosphorylation (Sarge et al., 1993; Baler et al., 1993; Ali et al., 1998; Zhou et al., 1998; Akerfelt et al., 2010). Emerging evidence suggests that several cellular chaperones and a few co-chaperones play crucial roles in both the activation and attenuation of HSF1 (Abravaya et al., 1992; Baler et al., 1992). For example, Hsp90 forms a complex with the HSF1 monomer but, upon heat shock, its dissociation leads to the trimerization of HSF1 (Sarge et al., 1993; Akerfelt et al., 2010). By contrast, elevated levels of Hsp90 and Hsp70 can prevent the activation of HSF1, thereby providing a negative feedback to the cellular stress response (Shi et al., 1998; Zhou et al., 1998). Besides HSPs, co-chaperones, such as immunophilins, Hip, Hop and the C-terminus of Hsc70-interacting protein (CHIP), among others, are also known to regulate the activation and/or the attenuation of HSF1, suggesting that HSF1 exists as a heterocomplex with a diverse set of regulatory proteins (Carrello et al., 1999; Zou et al., 1998; Bharadwaj et al., 1999; Ballinger et al., 1999).

Lafora disease (LD), a fatal form of neurodegenerative disorder, is caused by mutations in the genes *EPM2A* or *NHLRC1* (reviewed by Ganesh et al., 2006; Singh and Ganesh, 2009). The *EPM2A* gene encodes the protein phosphatase laforin, which harbors a carbohydrate-binding domain (CBD) at its N-terminus and a dual-specificity phosphatase domain (DSPD) at its C-terminus (Singh and Ganesh, 2009). Laforin is a cytoplasmic protein that associates with the endoplasmic reticulum (ER) membrane and polyribosomes (Ganesh et al., 2000). Malin, encoded by *NHLRC1*, is an E3 ubiquitin ligase; it harbors a RING finger domain at its N-terminus and NHL repeat domains at its C-terminus (Singh and Ganesh, 2009). Malin interacts with laforin and promotes its degradation (Gentry et al., 2005). The laforin and malin proteins appear to function in at least three

distinct physiological pathways. First, a role for laforin has been established in glucose metabolism; laforin is a glycogen phosphatase, and the aggregation of hyperphosphorylated glycogen could underlie the formation of the Lafora bodies seen in LD (Tagliabracci et al., 2007). A laforin–malin complex is thought to regulate the cellular levels of glycogen synthase, the R5 regulatory subunit of protein phosphatase-1 (also known as PTG and regulatory subunit 3C) and the glycogen-debranching enzyme by promoting their degradation through the ubiquitin–proteasome system (Cheng et al., 2007; Vilchez et al., 2007; Solaz-Fuster et al., 2008; Worby et al., 2008). Thus, loss of laforin or malin can result in increased glycogen synthesis and Lafora bodies (Vilchez et al., 2007). Second, laforin and malin associate with the ER membrane (Ganesh et al., 2000; Mittal et al., 2007), and loss of laforin results in increased ER stress and decreased proteasomal function (Vernia et al., 2009). Third, malin and laforin are recruited to aggresomes upon proteasomal blockade (Mittal et al., 2007), and the malin–laforin complex, together with Hsp70, ameliorates the toxicity of misfolded proteins (Garyali et al., 2009). The latter observation suggests that laforin and malin can function as co-chaperones, given that they both interact with Hsp70, and the presence of laforin and malin is required for Hsp70 to confer protection against stress induced by misfolded proteins (Garyali et al., 2009). Recently, a regulatory role for CHIP on malin has been suggested (Rao et al., 2010b) but a functional significance for the interaction has yet to be determined. Interestingly, CHIP is known to activate HSF1 and confer protection against heat shock (Dai et al., 2003). Because both CHIP and malin are E3 ubiquitin ligases, and both interact with Hsp70 and promote the clearance of misfolded proteins (Garyali et al., 2009; Jana et al., 2005), we addressed the possible role of LD-causing proteins in the heat-shock response. We show that laforin and malin are essential for the cells to achieve the heat-shock response and that malin activates the HSF1 through an unknown mechanism.

Results

Laforin and malin translocate to nucleus upon heat shock

CHIP, a predominantly cytoplasmic protein, is known to associate with Hsp70 and translocate to the nucleus when the cells are exposed to heat shock (Dai et al., 2003). In a manner similar to CHIP, the LD proteins laforin and malin associate with Hsp70 (Garyali et al., 2009). We therefore checked whether laforin and malin also showed any change in their localization upon exposure to heat shock. For this, CHIP, laforin or malin were transiently expressed in cells that were subsequently exposed to heat shock (42°C for 1 hour) and the subcellular localization of the proteins was evaluated. As shown previously (Dai et al., 2003), CHIP translocated to the nucleus in the majority of cells upon heat shock (Fig. 1A,B; supplementary material Fig. S1A). Laforin and malin also translocated to the nucleus in a substantial proportion of cells that were exposed to heat shock (Fig. 1A,B; supplementary material Fig. S1B,C). Such translocations were seen even when the two proteins were co-expressed (Fig. 1C). This heat-shock-induced translocation of laforin and malin was independent of the tags used for the detection of the transiently expressed protein (GFP or Myc tags; data not shown), the cell lines used for the transfection (Neuro2A, COS-7 or HeLa cells; see supplementary material Fig. S1A–C) or the expression level of the tagged protein (COS-7 cells stably expressing Myc-tagged laforin or malin; data not shown). The heat-shock-induced translocation of laforin and malin was

further confirmed by fractionation followed by immunoblotting. As shown in Fig. 1D, the relative intensity of immunoreactive bands for transfected malin and laforin in the nuclear fraction of the heat-shocked cells showed a substantial increase as compared with that in the control cells. We used the same approach to test whether endogenous malin and laforin would translocate to the nucleus upon exposure heat shock. As shown in Fig. 1E, there was a substantial increase in the immunoreactive bands for laforin and malin in the nuclear fraction of the heat-shocked samples as compared with that in the control cells. The specificity of these antibodies was validated using RNA interference (RNAi)-mediated partial knockdown of the *EPM2A* and *NHLRC1* transcripts (supplementary material Fig. S2A). We used Neuro2A cells for the detection of endogenous laforin and malin because these antibodies work better with these cell lines. The antibodies, however, did not work in immunofluorescence stainings.

A number of disease-associated mutations have been identified in malin and laforin and most of them affect the enzymatic activity of the protein or their interaction with their cellular substrates (reviewed in Singh and Ganesh, 2009). We selected four mutants of laforin (two affecting the CBD and two affecting the DSPD) and four mutants of malin (two affecting the NHL domain, one affecting the RING domain and one located close to RING domain) (supplementary material Fig. S3A) and tested the cellular translocation of these proteins upon exposure to heat shock. The subcellular localization of laforin mutants E28K, Q293L and Y294N was very similar to that of wild-type laforin in both control and heat-shocked cells (supplementary material Fig. S3B). The laforin mutant F88L, however, localized in the nucleus even in control conditions, as described previously (Ganesh et al., 2002), and there was no significant difference in the proportion of cells in which the mutant protein was in the nucleus upon heat shock (supplementary material Fig. S3B). Intriguingly, two of the mutations that affected the malin RING domain (C26S and E91K) prevented the translocation into the nucleus upon heat shock (supplementary material Fig. S3B). The two NHL mutants (W219R and V356fs32), however, behaved very similarly to the wild-type malin when exposed to heat shock (supplementary material Fig. S3B).

Malin is required for the nuclear translocation of laforin and CHIP upon heat shock

Malin and laforin are known to function as a complex (Garyali et al., 2009). We were therefore interested in checking whether malin would require laforin, and vice versa, for its nuclear translocation upon heat shock. As shown in Fig. 2B, the heat-induced nuclear translocation of overexpressed laforin was impaired when malin was knocked down by RNAi. By contrast, overexpressed malin was able to translocate to the nucleus even when laforin was knocked down (Fig. 2C). Next we checked, using a similar overexpression approach, whether malin and/or laforin required CHIP for its nuclear translocation. As shown in Fig. 2B,C, both laforin and malin required CHIP for their nuclear translocation upon heat shock. We also tested whether CHIP required malin and laforin for its heat-induced nuclear translocation. As shown in Fig. 2A, loss of malin or laforin impaired the heat-induced nuclear translocation of CHIP. The efficiency of individual knockdown constructs was validated by immunoblot analysis (supplementary material Fig. S2A).

Malin and laforin are essential for the cell to achieve the heat-shock response

It has been shown previously that, upon exposure to thermal challenge, fibroblasts derived from CHIP-deficient mice exhibit increased apoptosis as compared with those derived from wild-type littermates (Dai et al., 2003). Because laforin and malin showed heat-shock-induced nuclear translocation, and are essential for the nuclear translocation of CHIP, we wanted to check whether loss of endogenous laforin or malin would affect the viability of the cell when exposed to heat shock. Exposure of cells to temperatures ranging from 42 to 45°C is known to activate the heat-shock response without substantially affecting the cell viability (Jolly et al., 1997). We therefore exposed cells to 45°C for 1 hour and assessed the cell viability using two different methods. In the first method, cells were co-transfected with an expression construct for GFP and either a short-hairpin RNA (shRNA) construct targeting the protein of interest or control shRNA, exposed to heat stress and analyzed for cell death by scoring the number of

abnormal nuclei in GFP-positive cells (Fig. 3A). In the second approach, cells were transfected with an shRNA construct, exposed to heat shock and the cell viability was measured using an MTT assay. As shown in Fig. 3B,C, loss of malin or laforin resulted in a significant reduction in cell viability upon exposure to heat shock, and this reduction was comparable to that in cells where CHIP was knocked down. Laforin and malin form a complex with Hsp70, which protects the cells against the misfolded protein stress (Garyali et al., 2009). CHIP is also known to interact with Hsp70 (Ballinger et al., 1999). Therefore, the possibility of laforin, malin, CHIP and Hsp70 acting in the same stress response pathway was explored. For this, cells were transiently transfected with the shRNA construct for the knockdown of endogenous CHIP, laforin or malin together with an overexpression construct for a potential partner (laforin, malin, CHIP or Hsp70) (supplementary material Fig. S4). Intriguingly, none of the overexpressed proteins was able to confer protection against heat-induced cell death in the absence of laforin, malin or CHIP (supplementary material Fig. S4A–C). Taken
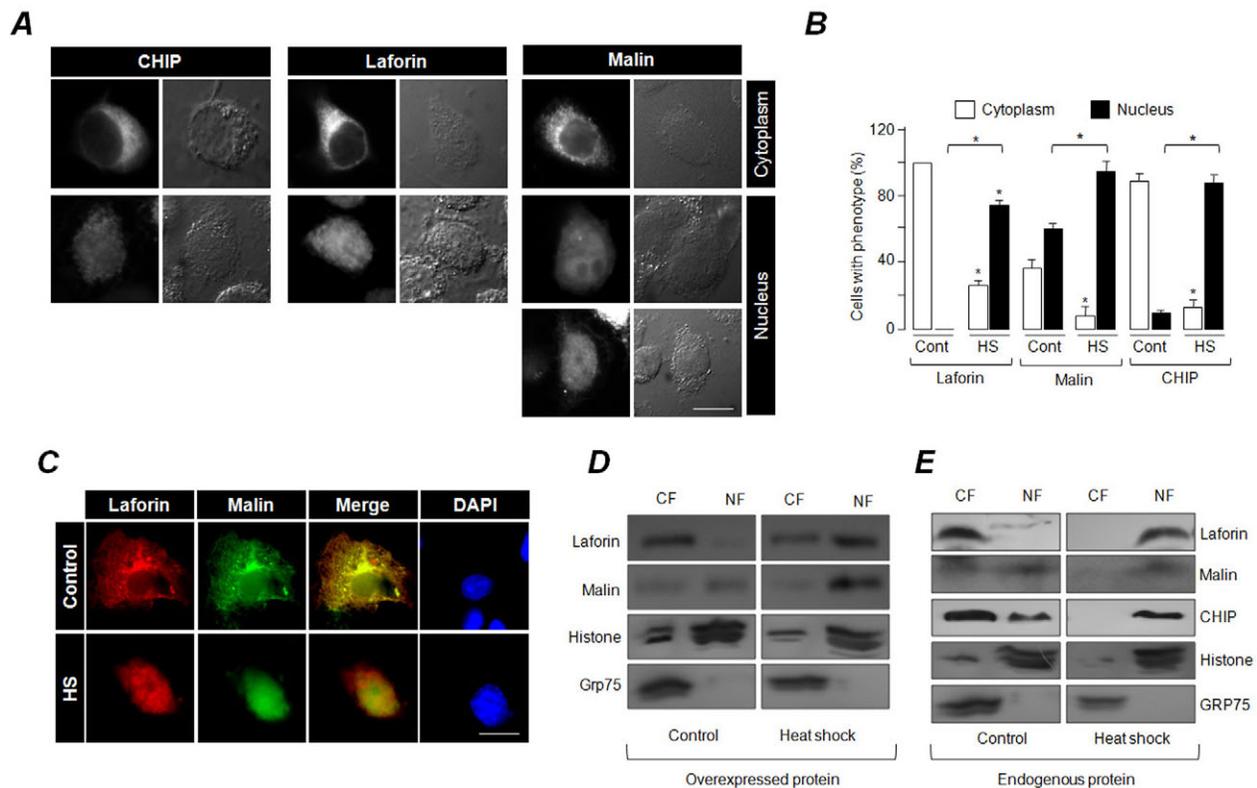

Fig. 1. Malin and laforin, in a manner similar to CHIP, translocate to the nucleus upon heat shock. (A) Representative fluorescence images (left-hand side), along with their phase-contrast images (right-hand side), showing the subcellular localization of CHIP (V5-tagged), laforin (GFP-tagged) or malin (GFP-tagged) when transiently expressed in COS-7 cells, as indicated. The top panel for each protein represents its cytoplasmic localization, and the bottom panels represents its nuclear localization (either exclusively in the nucleus or in both the nucleus and cytoplasm). The bottom panel represents the localization of proteins from the heat-shocked cells. The middle panel for malin represents its nuclear localization, even in unstressed cells. Scale bar: 10 µm. (B) Histogram showing the percentage of cells with cytoplasmic and nuclear localization (as defined as in A) of the indicated protein, when transiently expressed in COS-7 cells. The scoring was performed for cells that were grown at 37°C (Cont) or those were exposed to heat shock (HS) at 42°C for 1 hour. *P<0.005 as determined by Student's t-test (n=3 independent transfections; 300 cells per transfection). (C) COS7 cells transiently overexpressing GFP-tagged malin and Myc-tagged laforin were either exposed to heat shock (HS) or left at 37°C (control) and visualized for the subcellular localization, as indicated. Cells were counterstained with DAPI (blue color) to reveal the nucleus. Scale bar: 10 µm. (D) COS7 cells transiently expressing Myc-tagged laforin or malin were exposed or not exposed to heat shock, and the cytoplasmic fraction (CF) and nuclear fraction (NF) of the cells was immunoblotted with anti-Myc antibody (for laforin and malin), anti-histone antibody (a marker for the nuclear fraction) or anti-Grp75 antibody (a marker for the cytoplasmic fraction), as indicated. (E) Similarly, Neuro2A cells were fractionated to detect the endogenous laforin and malin. Note the enrichment of laforin and malin proteins in the nuclear fraction when the cells were exposed to heat shock, both when transiently overexpressed (D) and for the endogenous protein (E).
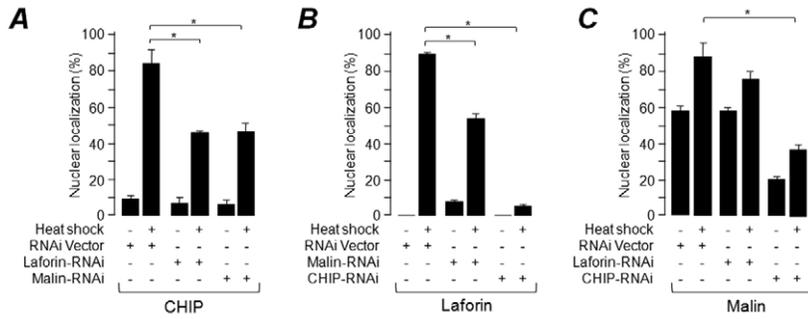
Fig. 2. CHIP requires malin and laforin for its translocation to the nucleus upon heat shock. Histogram showing the percentage of cells with a nuclear localization of CHIP (**A**), laforin (**B**) or malin (**C**) when each protein was transiently expressed in COS-7 cells. The cells were co-transfected with the indicated RNAi construct (shRNAmir) (RNAi Vector, control shRNA construct), exposed or not exposed to heat shock, and the localization pattern was scored. *$P<0.005$ as determined by Student's t-test ($n=3$ independent transfections; 300 cells per transfection).

together, these results suggest that the presence of each one of the three players tested here is crucial for the cell to achieve the heat-shock response. Malin, however, was able to elicit a heat-shock response when overexpressed alone (see below).

Because the present study indicates that CHIP is required for laforin or malin to confer protection against the thermal stress, and vice versa, we next tested whether laforin and malin physically interacted with CHIP. For this, His-tagged CHIP was expressed with laforin or malin and processed for a pull-down assay using Ni-affinity resin. As shown in Fig. 3D,E, CHIP was able to pull-down both laforin and malin. In the control assay, where laforin or malin was expressed in the absence of CHIP, the Ni-resin did not pull-down laforin or malin, demonstrating the specificity of the assay employed (Fig. 3D,E). However, our repeated attempts to pull down the endogenous CHIP by overexpressing malin or laforin were not successful, suggesting that the interaction could be weak and/or the relative cellular levels of these proteins could be crucial for the interaction. We note that a functional interaction between

malin and CHIP was reported recently, but a physical interaction could not be established by immunoprecipitation (Rao et al., 2010b), possibly owing to the differences in the sensitivity of the methods used.

Laforin and malin require HSF1 for their translocation into the nucleus upon heat shock

HSF1 is known to translocate into the nucleus upon heat shock (Sarge et al., 1993) (see also supplementary material Fig. S2B). Because laforin and malin also show a similar property, we were interested in checking whether the nuclear translocation of HSF1 under heat shock is dependent on the presence of laforin and/or malin or, conversely, whether the translocation of laforin and/or malin is dependent on the presence of HSF1. For this, we coexpressed HSF1 with an shRNA construct for malin or laforin, and the cells were either given or not given heat shock. As shown in Fig. 4A, the heat-shock-induced nuclear translocation of HSF1 was not affected by the absence of laforin or malin. Intriguingly,
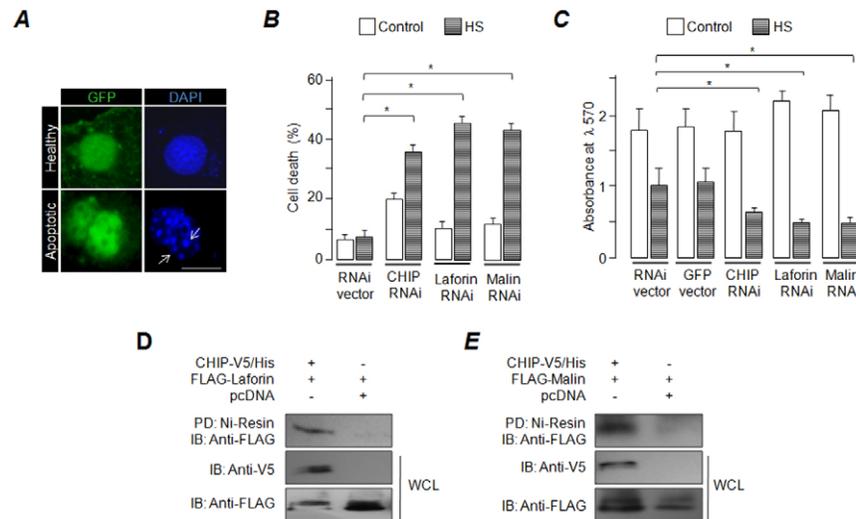

Fig. 3. Malin and laforin are essential for the cell to survive a heat stress, and both proteins interact with CHIP. (**A**) Representative fluorescent images of COS7 cells co-transfected with GFP expression vector (green) and an RNAi construct for laforin, showing healthy and abnormal nucleus as visualized by DAPI staining (blue). Scale bar: 10 μm. (**B**) Histogram showing the percentage of cells with abnormal nuclei in GFP-positive cells, as shown in A, that were transfected with the indicated RNAi construct (RNAi vector, control shRNA construct). The cells were given either a heat shock (HS) or no heat shock (control), as indicated. *$P<0.005$ as determined by Student's t-test ($n=3$ independent transfections; 300 cells per transfection). (**C**) Histogram representing the viability of the cell as measured by an MTT assay. Cells transfected with indicated constructs were processed for the measurement. A reduction in the absorbance level indicate reduced cell viability. *$P<0.005$ as determined by Student's t-test ($n=3$ independent transfections). (**D,E**) Malin and laforin physically interact with CHIP. V5 and His-tagged CHIP was coexpressed with FLAG-tagged laforin (D) or FLAG-tagged malin (E) in COS-7 cells and processed for the pull-down assay using the Ni-affinity resin, as indicated. As a negative control, cells were co-transfected with empty vector (pcDNA) along with laforin or malin. The pulled down products (PD) and whole-cell lysates (WCL) were immunoblotted (IB) with anti-V5 and anti-FLAG antibodies, as indicated.
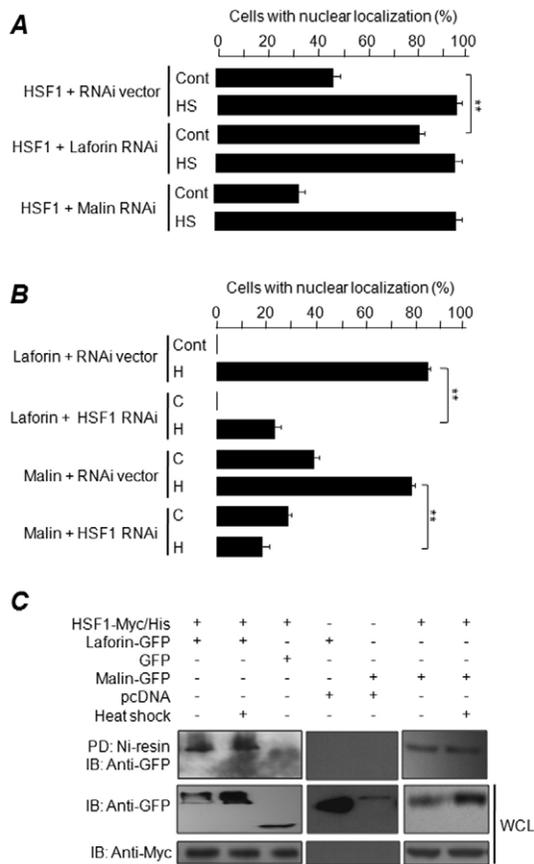
Fig. 4. Malin and laforin require HSF1 for their nuclear translocation under heat shock. Histogram showing the percentage of cells with nuclear localization of HSF1 (**A**), and laforin or malin (**B**), when each protein was transiently expressed in COS-7 cells. The cells were co-transfected with the indicated RNAi (shRNAmir) constructs (RNAi vector, control shRNA construct), maintained at 37°C (C, Cont) or exposed to heat shock (HS) and the localization pattern was scored. *$P<0.005$ as determined by Student's t-test (n=3 independent transfections; 300 cells per transfection). Representative images of the Myc-tagged HSF1 protein, both under control and heat-shock conditions, are shown in supplementary material Fig. S2B. (**C**) Malin and laforin physically interact with HSF1. Myc- and His-tagged HSF1 was coexpressed with GFP-tagged laforin or malin in COS-7 cells and processed for the pull-down assay using the Ni-affinity resin, as indicated. As a negative control, cells were co-transfected with empty vector (pcDNA) along with laforin or malin. Cells that coexpressed HSF1 with GFP were also used as a control. The pulled down products (PD) and whole cell lysates (WCL) were immunoblotted (IB) with anti-Myc and anti-GFP antibodies, as indicated.

loss of laforin led to a significant increase in the number of cells having HSF1 in the nucleus even under control conditions. We next checked whether laforin or malin required HSF1 for their nuclear translocation upon heat shock. As shown in Fig. 4B, the heat-shock-induced nuclear translocation of both laforin and malin was severely impaired when HSF1 was knocked down. Taken together, these results demonstrate that laforin and malin are dependent on HSF1 for their nuclear translocation under the condition of heat shock. We were therefore interested in checking whether laforin and malin physically interacted with HSF1, and whether any such interaction was mediated by heat shock. For this, Myc or His-tagged HSF1 was coexpressed with GFP-tagged laforin

or malin and processed for a Ni-affinity resin pull-down assay. As shown in Fig. 4C, HSF1 was able to pull down both laforin and malin, in both control and heat-shock conditions, suggesting that HSF1 interacts with laforin and malin, and that this interaction occurs during stress, as well as in unstressed conditions.

Malin and laforin are required for the activation of HSF1 under heat shock

Because laforin and malin interact with both HSF1 and CHIP, and given that CHIP is known to activate HSF1 during thermal stress (Dai et al., 2003), we next examined the possible role of laforin and malin in the activation of HSF1. We first tested the expression level of one of the target genes of HSF1, that encoding Hsp70. For this, cells were transiently transfected with the RNAi construct for laforin, malin or CHIP; the RNAi construct for HSF1 was used as a positive control. The transfected cells were either given or not given a heat shock (42°C for 1 hour) and processed for immunoblotting with anti-Hsp70 antibody. As shown in Fig. 5A,B, there was a significant reduction in the level of Hsp70 in cells where malin or laforin was knocked down and cells were exposed to heat shock as compared with that in the control set. A similar effect was seen in cells that were depleted for CHIP or HSF1 (Fig. 5A,B), as demonstrated previously (Qian et al., 2006; McMillan et al., 1998; Zhang et al., 2002). Taken together, these results suggest that the laforin–malin complex regulates the HSF1-mediated induction of Hsp70-encoding gene through some unknown process. We also found that knockdown of malin or CHIP resulted in an increase in the cellular level of Hsp70 when cells are not exposed to heat shock (Fig. 5A,B) (see Discussion). To test whether loss of malin or laforin enhanced the transcription of the Hsp70-encoding gene when cells were not under stress, we used a reporter gene assay [pHSE-luc construct (Feng et al., 2006)] to assess the Hsp70 promoter activity. As shown in Fig. 5C, knockdown of malin, laforin or CHIP did not alter the activity of reporter construct, whereas knockdown of HSF1 (the known activator of Hsp70 transcription) reduced its activity to nearly 50% of that in the control cells. Using a semi-quantitative reverse transcription (RT)-PCR assay we also found that the loss of laforin or malin did not alter the levels of Hsp70-encoding transcripts in cells that were not exposed to heat shock (supplementary material Fig. S5). Next, we examined the effect of loss of laforin or malin on the *Hsp70* promoter activity when cells were under heat shock. As shown in Fig. 5C, and as reported previously for CHIP and HSF1 (Dai et al., 2003), knockdown of laforin or malin significantly reduced the *Hsp70* promoter activity when the cells were exposed to heat shock, suggesting that HSF1 requires laforin and malin for its activation under heat-shock conditions. To confirm these findings, we examined another target of HSF1, the satellite III locus at 9q12, in HeLa cells. In human cell lines, HSF1 is known to activate the transcription of satellite III repeats by binding to the 9q12 locus and forming what is known as nuclear stress bodies (nSBs) (Jolly et al., 2004). The formation of nSBs, which are induced by a variety of stressors including heat shock, is dependent on the activation of HSF1 (Jolly et al., 2004; Sengupta et al., 2009). We therefore evaluated whether HSF1 would require malin, laforin or CHIP for the induction of nSBs. As shown in Fig. 5D,E, nearly 85% of HeLa cells that were co-transfected with a GFP expression construct along with a control RNAi vector showed HSF1-positive nSBs, as reported previously (Jolly et al., 2004; Sengupta et al., 2009). However, there was a significant reduction in the number of transfected cells that were positive for nSBs upon knockdown
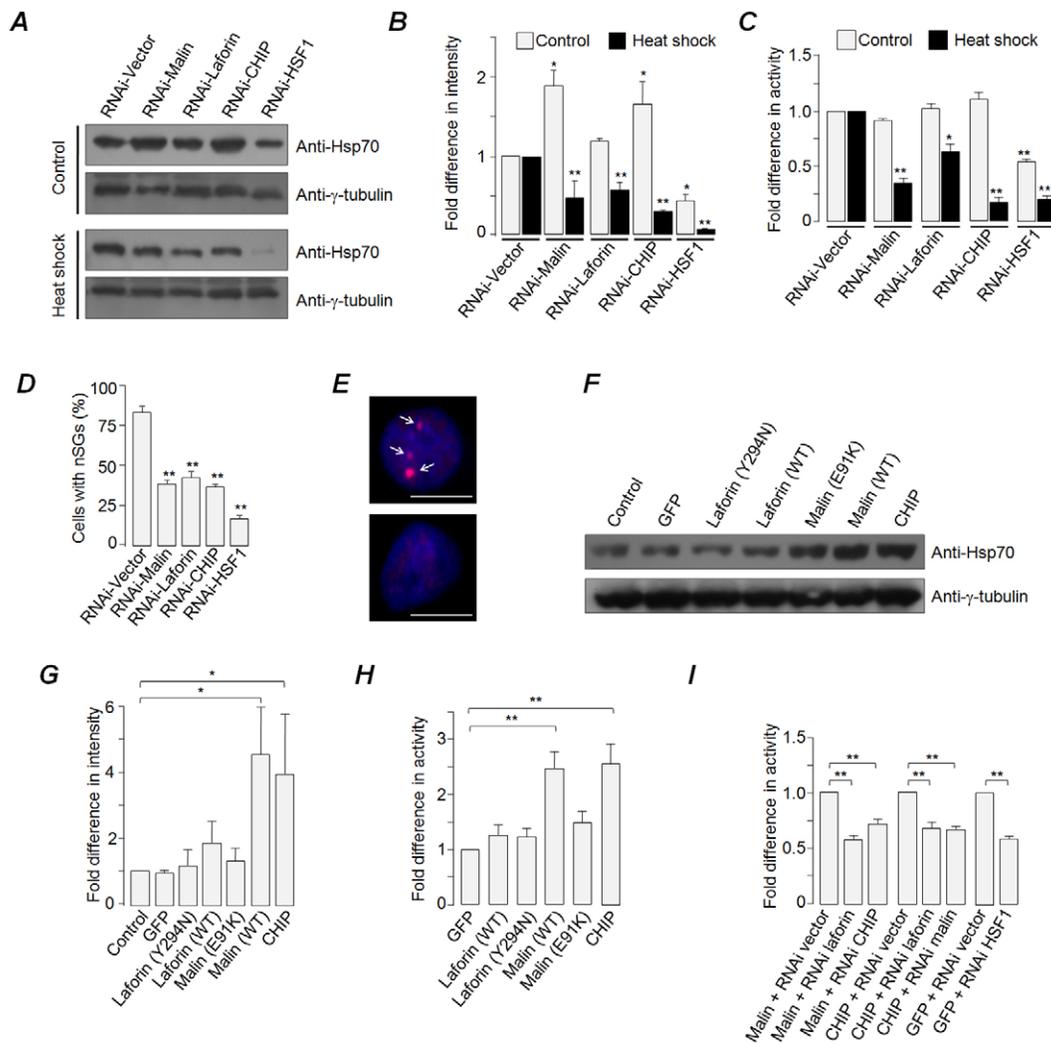
Fig. 5. Malin and laforin are required for the activation of HSF1. (A) Lysates of the Neuro2A cells, transfected with RNAi constructs (RNAi-Vector, control shRNA construct) and exposed or not exposed to heat shock as indicated, were processed for immunoblotting with the anti-Hsp70 antibody. Probing the same blot for γ-tubulin served as a loading control. **(B)** The relative intensity of signals detected for Hsp70 in the immunoblot shown in A was estimated with densitometry scanning, normalized for the intensity of γ-tubulin and plotted. The ratio for the Hsp70 and tubulin in the RNAi-vector lane was considered as 1, and the relative difference for rest of samples was calculated and plotted. *$P<0.05$; **$P<0.005$ as determined by Student's t-tests (n=3 independent experiments). **(C)** Neuro2A cells were co-transfected with the heat-shock-responsive pHSE-luc reporter construct encoding firefly luciferase along with a construct that encodes *Renilla* luciferase (internal control for transfection efficiency), and an RNAi knockdown construct as indicated. Control refers to cells that were not exposed to heat shock. The cells were given or not given heat shock, harvested and subjected to a dual luciferase assay. The ratio between the activity of firefly luciferase and *Renilla* luciferase in the RNAi-vector sample was considered as 1, and the relative difference for rest of samples was calculated. The difference in the activity, as compared to the control set (RNAi-vector), was tested for statistical significance. *$P<0.05$; **$P<0.005$ as determined by Student's t-tests (n=3 independent experiments). **(D)** Histogram showing the proportion of transfected cells (HeLa) exhibiting HSF1-positive nuclear stress bodies (nSBs) when transiently transfected with the constructs as indicated. A difference in the proportion of cells having nSBs, as compared with that in the control set (RNAi-vector), was calculated for statistical significance. *$P<0.05$; **$P<0.005$ as determined by Student's t-tests (n=3 independent transfections for each construct; 300 cells scored per transfection). **(E)** Representative images of the DAPI-stained nuclei showing the presence (top panel) and the absence (bottom panel) of nSBs (magenta dots; identified by arrows) in HeLa cells that were transfected with a RNAi empty vector (top) or a malin-RNAi construct (bottom) and exposed to heat shock. Scale bar: 10 μm. **(F)** Neuro2A cells were transiently transfected with expression constructs as indicated and the lysates were processed for immunoblotting using an anti-Hsp70 antibody (to see the difference in the level of Hsp70 protein) and an anti-γ-tubulin antibody (as a loading control). Control, untransfected cells. **(G)** The relative intensity of Hsp70 signals in the immunoblot shown in F were estimated with densitometry scanning, normalized for the intensity of γ-tubulin and plotted. The ratio for the Hsp70 and tubulin in the RNAi-vector lane was considered as 1, and the relative difference for rest of samples was calculated and plotted. *$P<0.05$ as determined by Student's t-test (n=3 independent experiments). **(H)** Neuro2A cells were co-transfected with the pHSE-luc reporter construct encoding firefly luciferase, a construct that encodes *Renilla* luciferase and an expression construct as indicated and subjected to a dual luciferase assay as described for C. A difference in the activity, as compared to the control set (GFP), was calculated for statistical significance. **$P<0.005$ as determined by Student's t-test (n=3 independent transfections). **(I)** Similarly, the pHSE-luc reporter construct (with *Renilla* luciferase construct as an internal control) was coexpressed with malin or CHIP, and one RNAi construct as indicated. A control set, in which GFP and an RNAi construct for HSF1 or an empty vector, was also used. The ratio between the activity of firefly luciferase and *Renilla* luciferase in the sample that expressed malin, CHIP or GFP with the empty RNAi vector was considered as 1, and the relative difference for rest of samples was calculated. A difference in the activity, as compared with that in the control set (RNAi-vector), was calculated for statistical significance. **$P<0.005$ as determined by Student's t-test (n=3 independent transfections).

of laforin or malin (Fig. 5D,E). Similar observations were made when CHIP or HSF1 was knocked down (Fig. 5D). Knockdown of laforin, malin or CHIP in cells that were not exposed to heat shock did not show nSBs (data not shown) or any difference in the level of Hsp70-encoding transcripts (supplementary material Fig. S5). These results demonstrate that HSF1 requires malin, laforin and CHIP for the activation of its target genes under the condition of heat shock.

The activation of HSF1 involves its heat-shock-induced hyperphosphorylation and its conversion into a DNA-binding homotrimer complex (Sorger et al., 1987; Sorger and Pelham, 1988; Baler et al., 1993; Sarge et al., 1993). We therefore evaluated the hyperphosphorylation status of HSF1 after transfecting cells with the knockdown constructs for laforin, malin or CHIP, or with a empty vector knockdown construct as a control, and the cells were then either exposed or not exposed to heat shock (42°C for 1 hour). An equal quantity of protein from each set was subjected to electrophoresis and immunoblotted with an anti-HSF1 antibody. As shown in supplementary material Fig. S6A, and as reported previously (Sorger and Pelham, 1988), a higher-molecular-mass hyperphosphorylated form of HSF1 was observed in all samples that were given a heat shock, suggesting that the presence of laforin or malin is not required for the heat-induced hyperphosphorylation of HSF1. Similarly, knockdown of CHIP did not substantially affect the hyperphosphorylation status of HSF1 under heat-shock conditions (supplementary material Fig. S6A). We next checked, by treating the cells with the chemical cross-linker disuccinimidyl suberate (DSS) and immunoblotting, whether laforin and malin are required for the stress-induced homotrimerization of HSF1, which gives the form capable of binding to DNA (Chen and Parker, 2002). For this, we transiently transfected cells with the knockdown construct for laforin, malin or CHIP, exposed the cells to heat shock and DSS and then processed the cells for immunoblotting. As shown in supplementary material Fig. S6B, knockdown of laforin, malin or CHIP did not substantially alter the trimerization of HSF1 when the cells were exposed to heat shock. Besides hyperphosphorylation and trimerization, the activation of HSF1 also involves other modifications, such as sumoylation and dephosphorylation (Hong et al., 2001; Hilgarth et al., 2003). Thus, the laforin–malin–CHIP complex might regulate HSF1 through one of such modifications.

Malin overexpression induces Hsp70 by activation of HSF1

Having established that laforin and malin are required for the activation of HSF1 upon heat shock, we next explored whether overexpression of laforin or malin activated HSF1 even when the cells were not exposed to heat shock – a property previously demonstrated for CHIP (Dai et al., 2003). For this, we transiently overexpressed laforin, malin or CHIP and examined the cellular level of Hsp70. We also overexpressed a laforin mutant (Y294N) and a malin mutant (E91K) (see supplementary material Fig. S3A). These mutations are associated with the LD phenotype, and hence we wanted to test whether they represent loss-of-function mutations. As shown in Fig. 5F,G, overexpression of wild-type malin resulted in the elevated levels of Hsp70, and this was comparable with that upon overexpression of CHIP. By contrast, overexpression of laforin, its Y294N mutant or the malin mutant E91K did not significantly alter the expression level of Hsp70 as compared with cells that overexpressed GFP or untransfected cells (Fig. 5F,G). Because overexpression of malin resulted in elevated Hsp70, we

also checked whether overexpression of malin (or laforin) activates HSF1 through its hyperphosphorylation. As shown in supplementary material Fig. S6C, overexpression of malin, laforin or CHIP did not result in the higher-molecular-mass hyperphosphorylated HSF1 being observed in the immunoblot. Similarly, overexpression of laforin or malin did not enhance the trimerization of HSF1, although overexpression of CHIP did result in an increase in the trimerized form of HSF1 (supplementary material Fig. S6D), as reported previously (Dai et al., 2003). Thus, malin – when overexpressed – appears to activate HSF1 through a process independent of hyperphosphorylation and trimerization. To confirm further that the observed difference in the cellular level of Hsp70 is mediated through HSF1-dependent transcription of Hsp70-encoding gene, we used a reporter gene assay to assess the *Hsp70* promoter activity. Malin overexpression resulted in an increase in the *Hsp70* promoter activity and the reporter activity was comparable to that of CHIP (Fig. 5H). Overexpression of the malin mutant E91K, or laforin or its mutant Y294N, did not cause such an increase in the promoter activity when compared with that in cells overexpressing GFP (Fig. 5H). Taken together, our results suggest that malin, but not laforin, is able to regulate HSF1 activity though an unknown process when the cells are not under stress.

Having established that overexpression of malin or CHIP is able to activate HSF1, we next checked whether laforin is required for malin or CHIP to activate HSF1. For this, we overexpressed malin or CHIP, along with the reporter gene construct, in the presence of the knockdown construct for laforin and measured the luciferase activity. Control experiments in which the laforin-knockdown construct was replaced with an empty vector were processed in parallel. As shown in Fig. 5I, loss of laforin significantly decreased the malin- or CHIP-mediated *Hsp70* promoter activity, suggesting that laforin is required for malin and CHIP to activate HSF1 when overexpressed. Similarly, we also checked whether malin required CHIP, and vice versa, for the *Hsp70* promoter activity when overexpressed. As shown in Fig. 5I, *Hsp70* promoter activity was significantly reduced when malin was overexpressed with a knockdown vector for CHIP or when CHIP was overexpressed with a knockdown vector for malin. Taken together, these results suggest that all three proteins (laforin, malin and CHIP) are crucial for the activation of HSF1.

Discussion

Here, we have shown that malin and laforin might function as co-activators of HSF1 to achieve the cellular heat-shock response and that their functions are similar, but not redundant, to that of CHIP. First, both laforin and malin translocate to the nucleus upon heat shock, and this property was hampered in some of LD-associated mutants. We have demonstrated this property for malin and laforin in more than one cell line and for both overexpressed and endogenous proteins. Thus, the heat-induced translocation of malin and laforin appears to be an important cellular and/or physiological function of these proteins. The suggestion is strengthened by the observation that, under identical conditions, CHIP exhibited a very similar property, as reported in a previous study (Dai et al., 2003). Thus, the malin–laforin complex appears to be a crucial regulator of HSF1 function.

Our cell survival assays demonstrate that both laforin and malin are essential for the cell to achieve the heat-shock response and protect the cell against thermal stress. In this regard, it is intriguing that laforin was unable to translocate to the nucleus in the absence of malin although laforin is essential for the cell to induce the heat-

shock response. It is possible that, among the two proteins, malin is the primary 'sensor' of heat shock and requires laforin for its function only in the nucleus. We demonstrate further that the co-chaperone CHIP is another component of this complex. CHIP is known to function as a protein complex and its cellular partners include Hsp70/Hsc70 (Ballinger et al., 1999), Hsp90 (Connell et al., 2001), BAG-2 (Arndt et al., 2005) and the E3 ubiquitin ligase parkin (Imai et al., 2002). A recent study demonstrates that CHIP functions redundantly with parkin (Morishima et al., 2008), suggesting that not all components of the functional complex are essential for its function. What we have demonstrated here, using a knockdown approach, is that all the three members of the malin–laforin–CHIP complex are required for the cell to achieve the heat-shock response. Our observations that laforin and malin are promoting the translocation of CHIP to the nucleus upon heat shock, and that CHIP failed to provide complete protection against the heat shock in the absence of malin or laforin, suggest that the LD-associated proteins malin and laforin probably act at an early step(s) of the CHIP-mediated stress response pathway.

Our studies demonstrate that laforin and malin interact with HSF1 and are required for HSF1 to activate the transcription of its target genes. The overexpression or the knockdown of malin and/or laforin does not seem to affect the nuclear localization, hyperphosphorylation or the trimerization property of HSF1. Thus laforin and malin are likely to function in an as yet unknown step of the HSF1 activation process. Given that laforin and malin interact with CHIP, and these three proteins appear to function as a non-redundant functional complex, laforin and malin probably represent new co-activators, with a role identical to that proposed for CHIP in regulating HSF1 (Dai et al., 2003). In this regard, it is interesting that both laforin and malin interact with HSF1 even when the cells are not under stress. Thus, it is probable that laforin and malin also regulate inactive HSF1. Conversely, HSF1 might regulate some of the functions of laforin–malin complex in the heat-shock response given that laforin and malin were unable to translocate to nucleus in the absence of HSF1. Clearly, the functional interdependence between HSF1 and the laforin–malin complex in the heat-shock response needs to be studied further.

It is intriguing that although the loss of laforin or malin under heat-shock conditions resulted in the reduction of the Hsp70 level, their loss in the control conditions led to an increase in the cellular levels of Hsp70. One possible explanation for this observation is that CHIP, laforin and malin work as a complex, and loss of either laforin or malin affects the ubiquitin ligase activity of CHIP. As a result, CHIP would not be able to promote the degradation of Hsp70 – one of its established substrates (Qian et al., 2006) – when laforin or malin is absent and hence there is an increase in the Hsp70 level. We have not tested whether malin or laforin regulate the ubiquitin ligase activity of CHIP or measured the half-life of Hsp70. Nonetheless, our semi-quantitative RT-PCR assay and the *Hsp70* promoter reporter assay suggest that loss of laforin or malin does not alter the level of Hsp70-encoding transcripts when the cells are not under stress; therefore, the increased level of Hsp70 protein is probably due to its inefficient clearance from the cellular pool. In this regard, it is interesting that the loss of laforin or malin results in reduced proteasomal activity (Vernia et al., 2009; Rao et al., 2010a). Taken together, the results from the present study suggest that loss of any one of the components of the malin–laforin–CHIP complex affects the ability of the cell to achieve the stress response and might also result in the accumulation of unwanted proteins. Indeed the loss of the laforin–malin complex

results in ER stress and the accumulation of misfolded and abnormal proteins (Vernia et al., 2009; Liu et al., 2009; Garyali et al., 2009). Our finding that the induction of nSBs is dependent on the presence of all three members of the laforin–malin–CHIP complex is intriguing. The HSF1-induced nSBs are thought to regulate chromatin remodeling, transcription and splicing processes under the condition of heat stress (Jolly and Lakhotia, 2006; Biamonti and Vourc'h, 2010). Given the indispensible role of HSF1 in the stress response pathway, and its functional conservation (Liu et al., 1997), the present set of findings underscore the crucial role of laforin–malin complex in transcriptional and post-transcriptional regulation under conditions of physiological stress. Thus, defects in the HSF1-mediated stress response pathway might underlie some of the pathological symptoms in LD.

Materials and Methods
Cell culture and transfection
COS-7, HeLa and Neuro2A cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum, 100 units per ml penicillin and 100 mg/ml streptomycin. PolyFect transfection reagent (Qiagen) was used for the transfection according to the manufacturer's protocol.

Expression constructs and antibodies
The expression vectors encoding Myc-, FLAG- or GFP-tagged wild-type or mutant forms of laforin and malin are described in our previous studies (Mittal et al., 2007; Garyali et al., 2009; Puri et al., 2009). For the HSF1 expression construct, the coding sequence of HSF1 was amplified from cDNA derived from HeLa cells and cloned into pcDNA3 vector in-frame next to a Myc-epitope coding region. The RNAi constructs (shRNAmir) for laforin, malin, CHIP and HSF1 were purchased from Open Biosystem, USA (Expression Arrest microRNA-adapted shRNA libraries) and were validated for their efficiency of knockdown (supplementary material Fig. S2A). The expression constructs for V5-tagged CHIP was provided by Nihar R. Jana (National Brain Research Center, Manesar, India), the Hsp70 expression construct was provided by Harm H. Kampinga (University Medical Center Groningen, Groningen, Netherlands) and the reporter construct for Hsp70 promoter (pHSE-Luc) was provided by Karl Riabowol (University of Calgary, Calgary, Canada). The following antibodies were used for the experiments. From Roche India: anti-GFP and anti-myc antibodies. From Sigma-Aldrich: anti-γ-tubulin, anti-FLAG, and anti-V5 antibodies. From Cell Signaling Technology: anti-Hsp70 (detects both constitute and inducible forms of Hsp70), anti-HSF1 and anti-CHIP antibodies. From Santa Cruz Biotechnology: anti-GRP75 antibody. From Abcam: anti-histone H4 (acetylated K8) antibody. From NeuroMab: anti-malin antibody. The anti-laforin antibody was reported in Puri et al. (Puri et al., 2009). Secondary antibodies were obtained from Jackson Immuno Research.

Immunocytochemistry, RNA in situ hybridization and cell viability assays
Mammalian cells grown on gelatine-coated sterile glass coverslips were processed for immunofluorescence microscopy, as reported previously (Ganesh et al., 2000), or for RNA in situ hybridization, as reported previously (Sengupta et al., 2009). For cell viability assays, transfected cells (GFP positive) were scored for abnormal nuclei, as reported previously (Garyali et al., 2009) (Fig. 3A). Cell counting was done in a blinded manner. For the MTT assay, transfected cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 36 hours after transfection for 6 hours at 37°C. The metabolic product of MTT was dissolved in DMSO and measured at 570 nm using a spectrophotometer.

Immunoblotting
Protein samples were resolved on using SDS–PAGE (10% gels), transferred onto a nitrocellulose membrane and the immunoreactive proteins were detected using a chemiluminescent detection system, as described previously (Garyali et al., 2009). The signal intensity of the immunoblot was quantified using the NIH image software (ImageJ).

Subcellular fractionation
The nuclear and cytoplasmic fractions were collected using the nuclear/cytosol fractionation kit (BioVision), as recommended by the manufacturer, and the fractions were then subjected to immunoblotting.

Pull-down experiments
To establish the physical interaction between malin or laforin and the target protein (HSF1 and CHIP), a expression construct that encoded His-tagged malin, laforin, CHIP or HSF1 was coexpressed with a construct that encoded the desired protein. Then cell lysates were incubated with Ni-affinity resin (Sigma-Aldrich) for 4–6 hours at 4°C and processed for pull-down assays, as reported previously (Gariyali et

al., 2009). Pull-down products were detected by immunoblotting using specific antibodies.

DSS cross-linking

Transfected cells that had expressed desired proteins were either given heat shock at 42°C for 1 hour or left untreated. Cells were then treated with 5 mM DSS (Pierce) for 1 hour at 4°C and then the activity was quenched with 25 mM Tris-HCl (pH 7.5), as recommended by the manufacturer. Cells were washed briefly in ice-cold PBS, lysed and the proteins were subjected to immunoblotting.

Dual luciferase reporter assay

The Neuro2A cells were transfected with the luciferase reporter plasmid containing the heat-shock response element (pHSE-Luc) (Feng et al., 2006) (a gift from Karl Riabowol), along with an expression construct coding *Renilla* luciferase gene driven by the CMV promoter, and an expression construct that encoded wild-type or a mutant form of laforin, malin, CHIP or HSF1 or their knockdown construct (shRNA), as desired. At 36 hours post transfection, cells were given either a heat shock at 42°C for 1 hourr or left untreated. The cells were allowed to recover at 37°C for 1 hour and then processed for luciferase activity with a luminometer, as recommended by the manufacturer (Promega). The relative light units per second were normalized to the *Renilla* luciferase activity and expressed as a fold change compared with control.

Statistical analysis

Means±s.d. were calculated and plotted for every experiment, and statistical significance (taken at $P<0.05$) was tested with unpaired Student's *t*-tests using the GraphPad software.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/13/2277/DC1

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