Conclusion
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Nearly a hundred years after the first report of a LD patient, the pathophysiology of the disease is still being elucidated (Lafora and Glueck, 1911). Since, LD is characterized by pathognomonic LBs and neurodegeneration, a causal association between them was initially sought (Minassian, 2001). But, the apparent dissociation between LBs and neurodegeneration is slowly beginning to emerge (Ganesh et al., 2002c; Machado-Salas et al., 2012). Moreover, LBs contain 6-28% protein depending on the tissues studied and their functional relevance to the disease evolution has hardly been addressed (Yokoi et al., 1968). This study for the first time investigates the role of protein aggregation and subsequent sequestration on to the LBs in the pathogenesis of LD.

Nearly 50% of the genetically confirmed LD cases have mutations in NHLRC1 encoding malin, which is an E3 ubiquitin ligase, a critical component of ubiquitin proteasome system (Gentry et al., 2005). Geographical location can further influence the proportion of LD case burden secondary to mutations in malin (27-73%) (Gomez-Abad et al., 2005; Franceschetti et al., 2006; Lohi et al., 2006; Singh et al., 2006). These mutations span the entire coding domain of malin and can bring about structural, molecular and functional changes in wild type malin (Table.2). In this study, the tertiary structures of individual domains of malin (RING and NHL) were studied by computer-assisted structural modeling and effects of LD-associated missense mutations were evaluated (Part-I; Fig.1). Structural analysis showed that approximately 84% of LD-associated missense mutations of malin can cause alterations of stability. These include destabilization or stabilization of tertiary structures of individual domains of malin. Functionally, destabilization can lead to aggregate formation while as stabilization can limit much needed flexibility for substrate interaction in the NHL domain of malin. In addition, structurally neutral mutations of malin can lead to loss of protein-protein interaction interfaces and altered solvent accessibility (Part-I; Fig.2 and 3). Some of the mutations predicted to alter the stability of malin in this study were reported to preserve the interaction with laforin but intriguingly rendered malin dysfunctional. Taken together, these results point towards a possibility that stability of malin is a pre-requisite for its function. In addition, primary structure
analysis of malin suggested that it contained aggregation hot-spots, β-aggregation-prone areas and amino acid stretches of high hydrophobicity.

*In vitro* experiments from this study suggested that wild type malin is aggregate-prone, misfolded, degraded by proteasome and forms higher frequency of aggresomes upon proteasome inhibition (*Part-I; Fig.5, 6 and 7*). Mutations of malin increase its aggregation propensity even in the nuclear environment where wild type malin is relatively stable (*Part-I; Fig.6 and Part-II; Fig.5*). These results along with structural analyses, reiterate that malin is a protein with intrinsic conformational instability. This is also exemplified by the fact that, malin undergoes substrate-induced stabilization in the presence of laforin and malin-laforin function together as a complex to regulate glycogen metabolism and cellular levels of misfolded proteins (Vilchez et al., 2007; Garyali et al., 2009; Zeng et al., 2012). Hence, recruitment of malin to MTOC under conditions of proteolytic stress might simply reflect inability of the cell to digest the misfolded malin (*Part-I; Fig.7*). However, in the presence of laforin, a functional malin-laforin complex might act as a protein quality control complex (Mittal et al., 2007).

Cells possess protein-triage mechanisms (protein quality control machinery) to handle the misfolded proteins and take crucial decisions such as, whether to refold it or to degrade the protein via UPS (*Part-I; Fig.16B*) (Gottesman et al., 1997; Marques et al., 2006). Molecular chaperones like Hsc70/Hsp70 and E3 ligases like CHIP and E6-AP constitute few of the critical molecules involved in protein-triage mechanisms (Connell et al., 2001; Lee and Tsai, 2005; Bukau et al., 2006; Mishra et al., 2009). This study confirmed the regulation of malin stability by chaperones and cellular quality control ligases. Surprisingly, co-chaperone CHIP was found to stabilize malin there-by delaying its proteasome-mediated degradation (*Part-I; Fig.11 and Fig.15A*). This action of CHIP might be attributed to its ability to negatively regulate Hsp70 activity since no direct interaction between malin and CHIP was detected (*Part-I; Fig.13, 14, 15 and 16*) (Ballinger et al., 1999). On the other hand, malin mutants were not only stabilized by CHIP but also showed increased nuclear aggregation in the presence of CHIP (*Part-I; Fig.12*). These results suggest the role of altered protein-triage decisions in stability and
degradation of malin mutants that might underlie some of the mechanisms of LD pathogenesis (Wu et al., 1994; Gregersen et al., 2003).

Previously, malin was reported to be auto-ubiquitinated in a RING domain dependent manner (Gentry et al., 2005). In this study, full length wild type malin, delRING malin and catalytically inactive mutant malin (C26S), were found to be targeted to UPS for degradation at varying rates (Part-I; Fig.8). This suggests that there might be additional mechanisms (possibly involving other E3 ligases) responsible for UPS mediated degradation of malin and its mutants.

Human and mouse LBs were reported to be labeled by anti-ubiquitin antibodies (Acharya et al., 1993; Lohi et al., 2005a; Garcia-Cabrero et al., 2012). The presence of ubiquitin on LBs suggests that they might contain misfolded proteins targeted for proteolytic degradation. However, the exact nature of proteins accumulated on the LBs has seldom been looked into. This study could show the co-localization of aggresomes of malin and its mutants with the proteasome components and chaperone machinery (Part-I; Fig.9). These in vitro observations have been confirmed in the LD patient skin and brain biopsy samples, wherein sequestration of UPS components and chaperone machinery on to the LBs was observed (Part-II; Fig.1, 2 and 3). Interestingly, few of the LD-associated aggregate-prone malin mutants (L126P and delF216_D233) were also seen to be associated with the LBs (Part-II; Fig.6). This suggests that LB biogenesis is an aggresome like process similar to what was hypothesized for Lewy bodies found in Parkinson’s disease (Olanow et al., 2004). Thus, the sequestration of UPS components and chaperone machinery on to the LBs is selective and regardless of the underlying genetic mutation (laforin, malin or third locus) (Part-II; Fig.1 and 4). This is further reconciled from animal models of LD where-in, LBs were found to be ubiquitinated irrespective of the underlying genetic modifications (LKO and MKO), reiterating that it is a generic phenomenon unlike as described before (Lohi et al., 2005a; Garcia-Cabrero et al., 2012).

Sequestration of misfolded proteins to an inclusion body has been described for many neurodegenerative diseases including polyglutamine disorders, Parkinson’s disease and myotonic dystrophy (Miller et al., 2000; Suhr et al., 2001; Vilotti et al., 2012). Sequestrations on to the inclusion bodies are reported to be cytoprotective
(Taylor et al., 2003; Tanaka et al., 2004; Saini et al., 2010). However, prolonged sequestration can produce massive proteinaceous inclusions that a cell might find difficult to clear forcing it to rely on compensatory proteolytic pathways such as autophagy (Lamark et al., 2009; Chen et al., 2011; Matsumoto et al., 2011). Compensatory activation of autophagy secondary to UPS impairment can be explained by the fact that they share common substrates and regulatory proteins (HDAC6, p62 and Alfy) (Rideout et al., 2004; Simonsen et al., 2004; Bjorkoy et al., 2005; Pandey et al., 2007; Kim et al., 2011; Sharma et al., 2012). In this study, malin mutants were found to have increased aggregation propensity as compared to wild type malin. They were also found to be proteasome substrates with varying rates of degradation. Recent discovery of autophagy defects in malin and laforin knock out mouse models suggests that misfolded malin mutants including those that are highly aggregate-prone (e.g. delF216_D233) have to depend heavily on UPS for their degradation (Nedelsky et al., 2008; Korolchuk et al., 2009; Aguado et al., 2010; Criado et al., 2012; Puri et al., 2012). On the other hand, defects in endosomal and autophago-lysosomal pathways can be secondary to UPS dysfunction itself, thus forming a vicious cycle leading to proteolytic stress (Strous and Govers, 1999; Dennissen et al., 2012).

UPS is mechanistically related to cellular levels of short-lived regulatory proteins and protein aggregation can cause impairment of this system (Bence et al., 2001; Glickman and Ciechanover, 2002; Cardinale et al., 2003; Tank and True, 2009). In vitro experiments in this study found that overexpression of aggregate-prone LD-associated mutants, led not only to a decreased proteasome activity secondary to sequestration of UPS components on to the aggresomes but also altered the global ubiquitination profile in transfected cells (Part-II; Fig.9C and 9D). In addition, d1EGFP- a novel reporter substrate of UPS with half-life of one hour, was found to specifically accumulate in only mutant malin transfected cells suggesting mutant malin induced proteasome impairment (Part-II; Fig.9A and B). Malin-laforin complex was also shown to suppress cellular toxicity caused by misfolded proteins, by subjecting them to proteasome-mediated degradation (Garyali et al., 2009). Given the causal association between protein aggregation and UPS impairment, dysfunctional malin-laforin complex secondary to malin or laforin mutations is expected to affect proteasome activity (Bence et al., 2001).
Intriguingly, decreased proteasome activity has been found in the liver of laforin knockout mice without any pronounced changes in the brain (Vernia et al., 2009a). However, since UPS function in the central nervous system is compartmentalized at sub-cellular levels (neuronal nucleus, axonal boutons and dendritic spines), a detailed study of these specialized compartments needs to be undertaken; preferably using a transgenic model system overexpressing LD-associated mutations of malin or laforin (Tai and Schuman, 2008). Moreover, transcriptional dysregulation has been reported in LKO mouse brain, as early as six months of age (Ganesh et al., 2005). This observation becomes crucial since, it is the UPS, which mainly regulates the short lived transcription factors in cells (Ciechanover, 2012; Kravtsova-Ivantsiv and Ciechanover, 2012). Thus, chronic proteolytic stress in the form of accumulation of misfolded proteins can lead to UPS dysfunction, which might underlie some of the pathogenic mechanisms in the LD. Since, malin is an E3 ligase and malin-laforin complex are critical components of UPS, this study proposes that UPS dysfunction in LD can be both cause as well as a consequence of evolution of the disease.

Recently, the critical role of UPS in the regulation of cell death pathways has been elucidated (Bernassola et al., 2010). Novel therapies to induce cancer cell death by targeting UPS are therefore being pursued (Nawrocki et al., 2006; Dalla Via et al., 2012). In line to this, overexpression of LD-associated mutants of malin, have been shown in this study to induce caspase-3 dependent apoptotic cell death in vitro suggesting a toxic-gain-of-function by mutants of malin (Part-I and Part-II; Fig.10). Interestingly, overexpression of deletion and missense mutations of laforin were also observed to cause apoptotic cell death (Liu et al., 2009a). Toxic-gain-of-function has been suggested to be involved in pathogenesis of many neurodegenerative disorders including Alzheimer’s disease, Parkinson’s disease and Huntington’s disease (Winklhofer et al., 2008). However, toxic-gain-of-function conferred by mutations cannot satisfactorily explain symptom free heterozygous individuals in diseases with an autosomal-recessive inheritance pattern like LD. But, several possible explanations could account for this apparent paradox. Firstly, the perpetual aggregation of LD-associated proteins might be seen specifically in homozygous individuals whereas heterozygotes might be able to overcome the overall cellular proteolytic stress using partially functional malin-
laforin complex. In addition, long term follow-up studies of heterozygous parents of LD patients are highly lacking, which can give an insight about possible gene dosage effect (Glaser et al., 1994). Surprisingly, heterozygous littermates of LKO and MKO models were also reported to be symptom free (Ganesh et al., 2002c; Valles-Ortega et al., 2011; Garcia-Cabrero et al., 2012). This could relate to the intrinsic limitations of the knockout mouse model itself that is unable to replicate the tremendous aggregate load seen in LD patients. This notion is supported by the fact that LD patients die within 10 years of the disease onset in contrast to the homozygous LD mouse models that have a normal life span (Ganesh et al., 2002c; Valles-Ortega et al., 2011). It is intriguing to note that few of the LD-associated missense mutations (D146N and H187P) of NHL domain do display a differential effect not only on structural stability of malin but also on phenotypic outcomes of the LD (Baykan et al., 2005; Traore et al., 2009). These results suggest a possibility of aggregation, sequestration and subsequent UPS dysfunction being modifiers of LD severity.

Malin-laforin complex is also reported to perform many cytoprotective functions such as clearance of polyglucosan aggregates, resisting endoplasmic reticulum (ER) stress and conferring protection against thermal stress (Vernia et al., 2009a; Couarch et al., 2011; Sengupta et al., 2011; Zeng et al., 2012). Concurrently, in vitro loss of laforin or malin has been shown to predispose neuronal and non-neuronal cells to ER stress and subsequently to cell death via apoptosis (Vernia et al., 2009a; Zeng et al., 2012). These findings are further supported by the observations of upregulated ER and oxidative stress markers in the LD patient brain samples (Vernia et al., 2009a). Taken together, this study proposes that in homozygous LD patients, toxic-gain-of-function coupled with concurrent loss of cytoprotective function might underlie some of the mechanisms of disease pathogenesis. On the contrary, in individuals heterozygous for malin or laforin mutations, partial cytoprotective functions conferred by normal NHLRC1 and EPM2A alleles might be sufficient to suppress a clinically recognizable disease phenotype. Clearly, more work is needed to understand possible gene dosage effect in the LD.
Despite the ubiquitous expression of malin seen in human cortex (Part-II; Fig. 7 and 8), recent reports of selective neuronal loss (i.e., PV positive and GAD67 positive neurons) in MKO mouse models are intriguing, since PV knockout mice share few of the behavioural and electrophysiological abnormalities with MKO and LKO mice (Ganesh et al., 2002c; Gregory et al., 2008; Valles-Ortega et al., 2011; Criado et al., 2012; Garcia-Cabrero et al., 2012). These abnormalities include locomotor hyperactivity, increased frequency of stereotyped behaviour, reduced threshold for acoustic startle response, no preference for new objects in novel object recognition task and enhanced hippocampal long term potentiation (Gregory et al., 2008; Shamir et al., 2012). This suggests that selective neuronal loss might underlie some of the disease-related pathogenic mechanisms of LD. However, the mechanistic details of selective neuronal loss are still elusive.

Finally, this study has also discovered that LBs display aggresome-like characteristics with sequestration of misfolded proteins, UPS components and chaperone. In addition, other studies done in LD patient, LD dog and LD mouse models have suggested sequestration of GSK3β, EPM2AIP1 (laforin interacting protein of unknown function), GRP78 (an ER-associated chaperone), p62 (associated with autophagy signalling), Rab5 and Rab7 (early and late endosomal markers respectively) on to the LBs (Chan et al., 2004b; Lohi et al., 2005a; Puri et al., 2012). The presence of ubiquitin, Hsc70, GRP78 and p62 on the LBs suggests an active interference by the cell to degrade the misfolded proteins accumulated on the LBs (Lamark et al., 2009; Wang et al., 2009; Matsumoto et al., 2011; Matsumura et al., 2011; Ciechanover, 2012). In this context, this study has explored the role of Hsp70 chaperone in countering the aggregation and toxic-gain-of-function by mutations of malin. Overexpression of the Hsp70 in mutant malin expressing cells not only decreased its aggregation but also attenuated the mutant-malin induced cell death. This suggests that chaperones, such as Hsp70/Hsc70 can be a suitable therapeutic target to stabilize aggregation-prone malin mutants and to attenuate some of the disease-associated pathogenic mechanisms, such as neurodegeneration. Recently, overexpression of a neuronal chaperone HSJ1a (DNSJB2a) in the Huntington’s disease mouse model (R6/2) was shown to suppress aggregation of mutant huntingtin by chaperone-mediated modifications of high molecular weight complexes (Labbadia et al., 2012). Interestingly, HSJ1a has
been shown to regulate functional specificity of Hsp70 chaperone machinery (Kampinga and Craig, 2010). This study thus provides for the first time a novel therapeutic target and suggests exploration of chaperone-mediated neuroprotection for LD.

In summary, the role of mutant malin aggregation and its subsequent sequestration along with UPS components and chaperone machinery on to the LBs was explored and a possibility of sequestration induced UPS dysfunction has been proposed as one of the mechanisms of LD pathogenesis (Part-II; Fig.12). In addition, intrinsic conformational instability of wild type malin and increased aggregation propensity of malin mutants were noted. Here, the role of protein-triage machinery, including molecular chaperones, such as Hsp70/Hsc70 was explored and found to play a key role in both wild type and mutant malin stabilization. Moreover, as a proof-of-principle, Hsp70 was shown to attenuate the toxic-gain-of-functions by malin mutants in vitro, thus rescuing cells from apoptotic death.