Activation of the Unfolded Protein Response and Proteostasis Disturbance in Parkinsonism-Dementia of Guam

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Abstract
Guam parkinsonism-dementia (G-PD) is a progressive and fatal neurodegenerative disorder among the native inhabitants of the Mariana Islands that manifests clinically with parkinsonism as well as dementia. Neuropathologically, G-PD is characterized by abundant neurofibrillary tangles composed of hyperphosphorylated tau, marked deposition of transactive response DNA-binding protein 43 kDa (TDP-43), and neuronal loss. The mechanisms that underlie neurodegeneration in G-PD are poorly understood. Here, we report that the unfolded protein response (UPR) is activated in G-PD brains. Specifically, we show that the endoplasmic reticulum (ER) chaperone binding immunoglobulin protein/glucose-regulated protein 78 kDa and phosphorylated (activated) ER stress sensor protein kinase RNA-like ER kinase accumulate in G-PD brains. Furthermore, proteinaceous aggregates in G-PD brains are found to contain several proteins related to the ubiquitin-proteasome system (UPS) and the autophagy pathway, two major mechanisms for intracellular protein degradation. In particular, a mutant ubiquitin (UBB^{+1}), whose presence is a marker for UPS dysfunction, is shown to accumulate in G-PD brains. We demonstrate that UBB^{+1} is a potent modifier of TDP-43 aggregation and cytotoxicity in vitro. Overall, these data suggest that UPR activation and intracellular proteolytic pathways are intimately connected with the accumulation of aggregated proteins in G-PD.

Key Words: Autophagy, Endoplasmic reticulum stress, Guam parkinsonism-dementia, Mutant ubiquitin, Tauopathy, TDP-43, Ubiquitin-proteasome system, Unfolded protein response.

INTRODUCTION
The island of Guam is one of several (former) high-incidence foci of amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia (PD) in the western Pacific (1–4). Other foci have been identified in the Kii peninsula of Japan (5) and in southern West New Guinea (Papua) (6, 7). Guam parkinsonism-dementia (G-PD), afflicting the native Chamorro people of Guam and the Mariana Islands, is clinically characterized by progressive mental deterioration in combination with extrapyramidal signs. At the neuropathological level, G-PD is characterized by abundant neurofibrillary tangles (NFTs) composed of hyperphosphorylated microtubule-associated protein tau, cytoplasmic deposition of transactive response DNA-binding protein 43 kDa (TDP-43), and neuronal loss (8). NFTs present in G-PD have biochemical and ultrastructural properties similar to those present in Alzheimer disease (AD) brains. Tau- and TDP-43-positive astrocytic and oligodendroglial inclusions can also be found, but senile plaques are virtually absent in most G-PD cases (8). Although several causes have been suggested for ALS and PD on Guam, such as genetic factors, infections, trauma and early-life stress, minerals and metals in soils and drinking water, toxins in cy-cad seeds, and other environmental toxins, the etiology remains unclear. Incidences of both ALS and PD have declined dramatically on Guam, coincident with increased accul-turation and westernization (8–10). Hence, it is plausible that environmental factors play an important role in their causation. Epidemiological studies indicate that increased risk for ALS and PD is acquired early in life or after long-term residence in the high-incidence areas (11, 12). Understanding the cause(s) and pathogenic mechanisms underlying these disor-ders is of great interest, as this could lead to new insights into...
more common neurodegenerative diseases throughout the world.

The accumulation and aggregation of misfolded amyloid-like proteins is a common feature of many neurodegenerative diseases (13, 14). While it is clear that these aggregating proteins are harmful to cells, the precise molecular mechanisms of aggregate toxicity remain largely unknown. A potential mechanism involves the interaction of aggregating proteins with essential cellular molecules, resulting in deregulation of their normal functions. Misfolded and toxic proteins could disrupt neuron-specific functions, such as axonal transport and maintenance of synaptic integrity. In addition, multiple pathological proteins may interact with each other and cause additive effects on disease progression (15). Cellular defense mechanisms (proteostasis mechanisms) exist to clear misfolded proteins from cells, but these can fail, leading to the detrimental accumulation of aberrant proteins (16). For example, accumulation of abnormal proteins in the endoplasmic reticulum (ER) triggers a cellular stress response called the unfolded protein response (UPR), which can protect cells against toxic protein buildup (17–19). Binding of the ER chaperone binding immunoglobulin protein/glycose-regulated protein 78 kDa (BiP/GRP78) to unfolded or misfolded proteins in the lumen of the ER results in dissociation of BiP/GRP78 from ER stress sensors located in the ER membrane. These ER stress sensors, inositol-requiring protein 1α (IRE1α), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6), subsequently signal to the cytosol and nucleus to adjust proteostasis capacity. The primary goal of the UPR is to re-establish homeostasis in the ER by inhibiting general protein translation and modulating expression of UPR target genes that alleviate ER stress (e.g. through regulating protein folding, redox status, ER-associated degradation [ERAD], autophagy, amino acid metabolism, and lipid biosynthesis). However, severe or sustained ER stress and UPR activation can contribute to neurodegeneration (17–19).

In this study, we investigate whether the UPR is activated in G-PD brains. For this purpose, expression of BiP/GRP78 and phosphorylated (activated) PERK (pPERK) is assessed by immunohistochemistry on postmortem brain tissue (hippocampal sections) from G-PD patients and controls.

In addition, we previously reported the accumulation of a mutant ubiquitin protein, ubiquitin-B+1 (UBB+1), in G-PD patient brains (20). UBB+1 is a marker for dysfunction of the ubiquitin-proteasome system (UPS), the major pathway for regulated degradation of intracellular proteins, and has been associated with multiple neurodegenerative disorders, including AD and other tauopathies (21–23). The mutant protein, carrying a 19-amino acid C-terminal extension generated by a transcriptional mutation event, likely contributes to an environment that favors the accumulation of abnormal proteins through inhibiting ubiquitin-dependent proteolysis (24). Transgenic expression of UBB+1 in mice results in behavioral deficits that are compatible with neurodegenerative disease (25, 26). Interestingly, cells undergoing ER stress fail to efficiently clear UBB+1 (27). Here, we explore whether UBB+1 can be detected in additional G-PD cases. We also investigate whether UBB+1 can modify the aggregation and cytotoxicity of TDP-43 in vitro. Critical components of the UPS and the autophagy-lysosome system, the latter representing another important mechanism for protein degradation in cells, are visualized in G-PD brain sections to determine if they are present in disease-associated aggregate structures.

**MATERIALS AND METHODS**

**Ethics Approval**

This study was approved by the Ethics Committee of Shinshu University (No. 3397; Kiyomitsu Oyanagi). Controls in this study were participants at the US National Institutes of Health, National Institute of Neurological Disorders and Stroke (NINDS) Research Center on Guam. Autopsy tissues from controls in this study were part of an approved NIH protocol (NINDS 83-N-34; PI: Ralph M. Garruto). Written permissions to conduct autopsies were secured by the NINDS Research Center on Guam and fixed and frozen tissues sent to the NIH and subsequently to Binghamton University.

**Immunohistochemistry on Human Postmortem Brain Tissue**

Information on cases used in this study is listed in Table 1. In summary, 8 G-PD cases (mean age 58.8 years) and 3 Guamanian controls (mean age 47.7 years) were included for immunohistochemical analysis. For BiP/GRP78 intensity comparisons in Figure 1, 2 non-Guamanian non-demented controls (NC1, NC2) were used.

Formalin-fixed, paraffin-embedded (FFPE) hippocampal sections (6-μm thick) were immunostained for various antigens using standard procedures. In brief, sections were deparaffinized in xylene and rehydrated in a descending alcohol series, after which they were treated with formic acid for 1 hour followed by washes in tap water for 30 minutes. Next, sections were incubated with primary antibodies (Table 2) for 1 hour at room temperature (RT) followed by an overnight incubation at 4°C. All primary antibodies were diluted in Super Mix (SuMi; TBS pH 7.6 with 0.5% Triton X-100 and 0.25% gelatin). Subsequently, sections were washed in TBS and incubated with biotinylated horse-anti-mouse or horse-anti-rabbit antibodies (1:400) (Vector Laboratories, Burlingame, CA) for 1 hour at RT. Next, sections were rinsed in TBS and incubated with avidin-biotin-peroxidase complex (1:800) (Elite ABC kit; Vector Laboratories) for 1 hour at RT. After final washes in TBS, sections were reacted with 0.5 mg/mL 3,3′-diaminobenzidine (Merck, Darmstadt, Germany) containing 0.2% nickel ammonium sulfate and 0.01% hydrogen peroxide (H2O2; Merck) in TBS. Reactions were stopped in distilled water. Stained sections were dehydrated in an ascending alcohol series, cleared in xylene, and coverslipped with Entellan (Merck).

Postmortem microscopic examinations were performed blinded to clinical diagnosis by a single investigator (B.M.V.). Immunohistochemical staining was scored semiquantitatively (−: negative; +, mild; ++, moderate; ++++, strong). Average scores from 3 independent examinations of complete hippocampal sections were used. Slides were imaged using a Zeiss Axio Imager M2 microscope equipped with an AxioCam ICc 3 camera.
**Yeast Experiments**

**Yeast Strains and Growth Conditions**

Yeast strains used in this study were on a BY4741 genetic background. Wild-type (WT) and ∆ubi4 strains were obtained from Dharmacon/Horizon Discovery (Cambridge, UK). The deletion strains ∆ubr2 and ∆ubr2/Δrpm4 were obtained from (28). Yeast strains were grown in YPD, or in synthetic complete (SC) media as previously described in (29). SC media contained 2% carbon sources, either glucose (SCD) or galactose (SCGal). Carbon sources and agar for YPD and SC media, and amino acids and supplements for SC media were obtained from Serva Electrophoresis (Heidelberg, Germany) or Carl Roth (Karlsruhe, Germany). Yeast extract and bacterial peptone for YPD medium were obtained from Formedium (Norfolk, UK). Plasmid transformation and maintenance were done by growth in selective SC media. For SC media were obtained from Sigma-Aldrich (Taunton, MA), and yeast nitrogen base for SC media was bought from Formedium (Norfolk, UK). Plasmid transformation and maintenance were done by growth in selective SC media, using the auxotrophic markers of the yeast strains. Gene expression was under the control of constitutive (MET25), inducible (galactose-inducible [GAL10]), or doxycycline-repressible [TetOff] promoters.

**Measurement of TDP-43 Aggregation**

The influence of UBB\textsuperscript{+1} on TDP-43 aggregation was measured in the indicated wild-type and deletion strains by determining the number of TDP-43-EGFP foci per cell in the presence or absence of UBB\textsuperscript{+1}. Yeast cells were co-transformed with TDP-43 (pUG35-TDP-43-EGFP, MET25 promoter, URA3, CEN, [30]) and UBB\textsuperscript{+1} (pESCnFLAG-UBB\textsuperscript{+1}, GAL10 promoter, HIS3, 2μ, [31]) expression constructs or vector controls (pUG35-EGFP, MET25 promoter, URA3, CEN; pESCnFLAG, GAL10 promoter, HIS3, 2μ). Transformed clones were grown overnight in 96-well plates with 250 rpm at 30°C in selective SCD-URA/HIS media. Under these conditions, TDP-43-EGFP formed aggregate-like EGFP foci under nontoxic levels, and UBB\textsuperscript{+1} expression was repressed. The cell densities of the overnight cultures were measured (OD\textsubscript{600}), the cultures were diluted in fresh media to an OD\textsubscript{600} of 0.2, and were incubated with 250 rpm at 30°C for 4 hours. After centrifugation, the supernatants were discarded, cell pellets were resuspended in expression media (SCGal-URA/HIS), and incubated overnight with 250 rpm at 30°C. The number of aggregate-like TDP-43-EGFP foci was determined in at least 50 cells per condition and experiment using epifluorescence microscopy. Epifluorescence microscopy was performed using an AxioPlan 2 microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with a Plan Neofluar 100×1.30 Ph3 oil objective and an Evolution VF Mono Cooled monochrome camera (Intas Science Imaging Instruments, Göttingen, Germany) with QCapture Pro 6 software (QImaging, Surrey, BC, Canada).

**Spot Dilution Assays for Measuring Cytotoxicity**

The influence of UBB\textsuperscript{+1} on TDP-43-triggered cytotoxicity was measured by determining growth deficits on agar plates upon UBB\textsuperscript{+1} and/or TDP-43 expression. Yeast clones co-transformed with TDP-43 (pCM190-TDP-43-wt, TetOff promoter, URA3, 2μ, [30]) and UBB\textsuperscript{+1} (pESCnFLAG-UBB\textsuperscript{+1}, GAL10 promoter, HIS3, 2μ, [31]) expression constructs or vector controls (pCM190, TetOff promoter, URA3, 2μ, pESCnFLAG, GAL10 promoter, HIS3, 2μ) were grown overnight in 96-well plates with 250 rpm at 30°C in selective SCD-URA/HIS media under non-inducing conditions (with 2 μg/mL doxycycline, Sigma-Aldrich). Cultures were normalized to an optical density (OD\textsubscript{600}) of 0.2 in ddH\textsubscript{2}O (1st dilution), serially diluted (1:10) in 5 steps in ddH\textsubscript{2}O (2nd–6th dilution). Diluted cultures were spotted onto solid nutrient-containing media inducing (SCGal-URA/HIS with low levels of doxycycline, i.e. 0.25 μg/mL) or repressing...
RESULTS

TDP-43 Pathology in G-PD

First, immunostainings were carried out to determine whether cytoplasmic deposition of the DNA- and RNA-binding protein TDP-43, a neuropathological feature of G-PD (32, 33), could be observed in FFPE hippocampal sections from the subjects. Some studies have suggested that NFTs may be a background phenomenon in Guamanians (34, 35). Therefore, we focused on the presence of TDP-43 in these experiments. TDP-43 pathology was expected to distinguish controls from disease subjects better than tau pathology. Indeed, all G-PD sections showed cytoplasmic TDP-43 inclusions (Fig. 1A, B), whereas TDP-43 aggregates were not found in brains of non-neurological Chamorro controls (Table 3). None of the controls displayed prominent NFTs (as determined by anti-MC1 and anti-CP13 staining; data not shown). In sum, TDP-43 pathology is consistently observed in G-PD.

The UPR Is Activated in G-PD

Immunohistochemistry was performed on postmortem brain tissue to evaluate expression of the UPR markers BiP/GRP78 and pPERK. Immunostainings for the ER chaperone BiP/GRP78 revealed prominent accumulation in G-PD cases, as compared to (non-Guamanian) Braak 0 and Braak II control subjects (Fig. 1C–H). BiP/GRP78 is an abundant protein that binds to transducers of ER stress (IRE1α, ATF6, and PERK) and acts as a sentinel for alterations in ER homeostasis. Activation of the UPR results in transcriptional induction of genes involved in the ER-localized stress response, including BiP/GRP78 itself. Thus, the finding of accumulated BiP/GRP78 in G-PD brains indicated ER dyshomeostasis. In contrast, no BiP/GRP78 reactivity was observed in Chamorro controls (Table 3).

Accumulation of pPERK was also detected in G-PD sections (Table 3). pPERK is the activated form of PERK and reflects activation of the UPR pathway. Specifically, pPERK was present in tangle-like and granular structures (Fig. 1I–L). Granular structures may be indicative of granulovacuolar degeneration (36), which has been previously observed through electron microscopic examination of hippocampal tissue from cases with G-PD (37). pPERK accumulation was particularly frequent in the cornu ammonis 1 (CA1) region of the hippocampus in G-PD cases. No staining for pPERK was seen in controls (Table 3). These results show that the UPR is activated in G-PD patient brains, but not in Guam controls.

Deposition of UBB+1 and UPS Components in G-PD

UBB+1 is an aberrant ubiquitin molecule that is generated by a transcriptional mutation event (Fig. 2A). The mutant protein lacks a C-terminal glycine residue that is necessary to ubiquitylate other proteins, but can still be ubiquitylated itself. UBB+1 does not meet the prerequisites for efficient UPS proteolysis and is itself an inhibitor of the UPS (38). ER stress conditions promote UBB+1 accumulation in vitro, indicating...
crosstalk between the ER and the UPS (Supplementary Data Fig. S1). UBB-1 has been found to accumulate in several neurodegenerative disorders, including tauopathies (21, 22) and polyglutamine (polyQ) repeat diseases (39), and has been used as a marker for UPS dysfunction in protein misfolding disorders (23).

To determine whether UBB-1 accumulates in G-PD, we performed immunostainings using UBB-1-specific antibodies.

![Diagram showing the accumulation of UBB-1 in Guam parkinsonism-dementia (G-PD) brains](https://example.com/diagram.png)

**FIGURE 2.** Ubiquitin-B-1 (UBB-1) accumulates in Guam parkinsonism-dementia (G-PD) brains. (A) UBB-1 is a frameshift mutant of ubiquitin that is generated by “molecular misreading,” a form of transcriptional mutagenesis. Dinucleotide deletions (e.g. ΔGU) at repeat motifs (e.g. GAGAG) generate abnormal transcripts that are not detected by nonsense-mediated decay (because they lack a downstream intron). The resulting abnormal transcripts can be translated into aberrant proteins. (B) UBB-1 contains an abnormal C-terminal domain that can be recognized by specific antibodies. However, UBB-1 can be truncated by deubiquitylating enzymes (DUBs), that is ubiquitin C-terminal hydrolase-L3 (UCH-L3), destroying the epitope. Inhibition of these DUBs, for example by oxidative stress conditions, prevents this truncation, preserving the epitope. Immunohistochemistry reveals multiple UBB-1-positive structures in G-PD brains (C, D). UBB-1 shows a granular and tangle-like pattern of distribution in the cytoplasm. In addition, the ubiquitin-proteasome system components Rpt3/S6b (E, F) and UCH-L1 (G, H) are found in G-PD aggregate structures. Representative pictures are shown. Arrowheads indicate different immunoreactive structures. Scale bars: C, E, G = 200 μm; D, F, H = 50 μm.
(Fig. 2B). UBB$^{+1}$ was found to be present in hippocampal sections from all G-PD cases (Fig. 2C, D), but in none of the controls (Table 3). These results are in accordance with previous findings (20). Interestingly, UBB$^{+1}$ was also detected in glial cells, that is astrocytes, in G-PD brains (20). It should be noted that UBB$^{+1}$ staining in cases 1–6 has been described in a previous study (20).

We also performed immunostainings to visualize specific components of the UPS in G-PD brains. Accumulation of the 19S ATPase subunit Rpt3/S6b (also known as PSMC4) (Fig. 2E, F) and the deubiquitylating enzyme (DUB) ubiquitin C-terminal hydrolase-L1 (UCH-L1) (Fig. 2G, H) was observed in G-PD aggregate structures. Deposits containing these proteins were absent in control subjects (Table 3). These findings corroborate previous results on the accumulation of particular UPS components in G-PD (20).

**UBB$^{+1}$ Modifies TDP-43 Aggregation and Cytotoxicity In Vitro**

Intracellular accumulation of UBB$^{+1}$ and formation of intracytoplasmic TDP-43 inclusions are observed in G-PD brains (Figs. 1 and 2). It is of interest to know whether these pathologies are independent from each other or whether they

### TABLE 2. Overview of Primary Antibodies Used for Immunohistochemical Stainings on Human Sections

| Antibody | Species | Dilution | Immunogen/Epitope | Source |
|----------|---------|----------|-------------------|--------|
| MC1      | Mouse   | 1:200    | Disease-specific conformational modification of tau (epitope within aa 312–322) | Dr P. Davies, Albert Einstein College of Medicine |
| CP13     | Mouse   | 1:200    | Tau phosphorylated at Ser202 | Dr P. Davies, Maastricht University |
| TDP-43   | Mouse   | 1:1000   | Recombinant full-length human TARDBP (epitope at aa 205–222) | Abnova, Taipei, Taiwan |
| Ubi2a    | Rabbit  | 1:400    | Mutant ubiquitin extended C-terminal domain | Dr F.W. van Leeuwen, Maastricht University |
| Rpt3     | Rabbit  | 1:400    | Recombinant protein from the C-terminus of *Manduca sexta* proteasome 26S (19S regulator complex) (Rpt3/S6b subunit) | Biomol, Plymouth Meeting, PA |
| UCH-L1   | Rabbit  | 1:500    | Recombinant full-length human PGP9.5 | Biomol |
| BiP (GRP78) | Rabbit | 1:200    | Synthetic peptide conjugated to KLH derived from within residues 600 to the C-terminus of mouse GRP78 (BiP) | Abcam, Cambridge, MA |
| pPERK    | Rabbit  | 1:400    | Protein kinase RNA-like ER kinase phosphorylated at Thr981 | Santa Cruz Biotechnology, Santa Cruz, CA |
| ATG8     | Rabbit  | 1:500    | Recombinant full-length human LC3-I | Dr A. Iwata, Stanford University |
| ATG12    | Rabbit  | 1:500    | N-terminus of human ATG12 | Dr A. Iwata |
| p62      | Rabbit  | 1:400    | Synthetic peptide corresponding to aa 387–436 of human p62 (UBA domain) | Biomol |

### TABLE 3. Overview of Results Obtained From Immunohistochemical Stainings on Human Sections

| Subject | TDP-43 | BiP/GRP78 | pPERK | UBB$^{+1}$ | UCH-L1 | Rpt3 | ATG8 | ATG12 | p62* |
|---------|--------|-----------|-------|-----------|--------|------|------|-------|------|
| 1       | +      | +         | ++    | ++        | ++     | +    | ++   | +     | +    |
| 2       | ++     | +         | ++++  | +++       | ++++   | ++   | +++  | ++    | ++   |
| 3       | +      | +++       | ++    | ++        | N/A    | +++  | +++  | ++    | +    |
| 4       | +++    | +         | +++   | +++       | ++++   | +++  | +++  | ++    | +    |
| 5       | +++    | +         | ++    | ++        | ++     | +    | +    | +     | +    |
| 6†      | +      | +++       | ++++  | +         | N/A    | +    | +    | +     | +    |
| 7       | ++     | +         | ++    | ++        | ++     | +    | +    | +     | +    |
| 8       | +      | +         | ++    | ++        | ++     | +    | +    | +     | +    |
| C1      | -      | -         | -     | -         | -      | -    | -    | -     | -    |
| C2      | -      | -         | -     | -         | -      | -    | -    | -     | -    |
| C3‡     | -      | -         | -     | -         | -      | -    | -    | -     | -    |

Formalin-fixed, paraffin-embedded hippocampal sections were used for this study. Subjects 1–6 correspond to subjects 1–6 described in (20).

*No p62 reactivity was observed in pyramidal neurons in the cornu ammonis 1 (CA1) region of all evaluated sections.

†Sections from subject 6 did not contain CA1 region.

‡A minor reactivity for neurofibrillary tangles (MC1, CP13) was seen in subject C3.

Semiquantitative scores: −, negative; +, mild; ++, moderate; ++++, strong; N/A, not available.
can influence each other. To determine the effects of UBB$^{+1}$ expression on the aggregation and cytotoxicity of (other) disease-associated proteins, we performed in vitro assays in a yeast TDP-43 proteinopathy model, in which the aggregation and cytotoxicity of TDP-43 can be analyzed in an easy and reliable fashion (30). TDP-43 aggregation could be followed by analyzing the formation of TDP-43-EGFP foci in yeast cells, whereas the cytotoxicity of TDP-43 could be measured by its influence on the growth of yeast cells on agar plates using a spot dilution assay. Analyses were performed in wild-type yeast cells, as well as in yeast cells with elevated UPS capacities ($\Delta ubr2$). In the latter strain, the E3 ubiquitin ligase Ubr2, which is required for the degradation of the major UPS transcriptional activator Rpn4 (40), is missing, resulting in high Rpn4 levels and consequently elevated UPS capacity.

The aggregation assay revealed that UBB$^{+1}$ does not affect TDP-43 aggregation in wild-type yeast cells (Fig. 3A, B). However, in $\Delta ubr2$ cells, UBB$^{+1}$ significantly decreased
TDP-43 aggregation. This effect seemed to depend, at least in part, on Rpn4 (the master transcription factor for inducing UPS components in yeast and a substrate of Ubr2 [40]), because the anti-aggregation effect was abolished in a Δabr2/Δrpn4 strain. These data suggest that UBB⁺¹ triggers a transcriptional response at the level of the UPS, counteracting TDP-43 accumulation and aggregation. Interestingly, UBB⁺¹ moderately increased the aggregation of TDP-43 in ubiquitin-deficient yeast cells (Supplementary Data Fig. S2).

Based on the aggregation data, it appeared that UBB⁺¹ accumulation could play a protective role in TDP-43-expressing cells. In agreement with this assumption, growth experiments (spot dilution assays) indicated that the cytotoxicity of TDP-43 was relieved upon UBB⁺¹ expression in both wild-type and Δabr2 cells (Fig. 3C). These data suggest that UBB⁺¹ accumulation in affected cells of G-PD patients is a potentially protective mechanism to reduce the accumulation and toxicity of TDP-43 aggregates.

Key Autophagy Proteins Are Deposited in G-PD

Immunohistochemical expression of the autophagy-related proteins ATG8 (LC3), ATG12, and ubiquitin-binding protein p62 was checked in hippocampal brain sections. ATG8 and ATG12, components of 2 ubiquitin-like conjugation systems, are involved in vesicle expansion and completion in the autophagic process (41–43). p62 (also known as SQSTM1) is an adapter for various signaling pathways as well as a receptor for selective autophagy (44). All of these markers were found to be present in disease-associated aggregates in G-PD cases, but not in non-neurological controls (Fig. 4A–F; Table 3). Thus, disease-associated proteostasis disturbance was not limited to impairment of the UPS, but also involved the autophagy pathway. Strikingly, p62 was not found in any pyramidal cells in the CA1 regions of the evaluated hippocampi. Absence of p62 in particular cells of the CA1 region might be related to selective autophagy induction and differential vulnerability of this specific region in G-PD.

DISCUSSION

Altered proteostasis is a salient feature of neurodegenerative diseases, including G-PD. In this study we showed that the UPR, an adaptive signaling cascade that counteracts proteostasis dysfunction at the level of the ER, is activated in G-PD brains. In addition, immunohistochemical experiments revealed deposition of multiple factors related to the main intracellular protein degradation pathways, that is the UPS and the autophagic-lysosomal pathway, in G-PD brain tissue. Abnormal components of the UPS present in G-PD brains were shown to modify the aggregation and cytotoxicity of disease-associated proteins. Our observations suggest that disrupted proteolytic systems and proteotoxic stress pathways contribute to G-PD pathology.

Cytoplasmic Accumulation of TDP-43 in G-PD

We started with evaluating TDP-43 pathology in G-PD tissue sections. G-PD is generally classified as a tangle forming disorder, but several reports indicate that NFTs are merely a background feature in Guamanians (34, 35). Therefore, we focused on the presence of cytoplasmic TDP-43 inclusions as a criterion for distinguishing controls from disease subjects in this study. Previous work has demonstrated pronounced TDP-43 pathology in G-PD brains (32, 33).

In keeping with previous findings, cytoplasmic TDP-43 deposits were observed in all evaluated G-PD cases. In contrast, no TDP-43 cytoplasmic aggregates were found to be present in Guamanian controls in our experiments. The significance of the previous reports on frequent NFTs in Guamanian controls is unclear but it could indicate a pre-disease state in the subjects, possibly due to shared genetic predisposition or exposure to an environmental trigger. No prominent NFTs were detected in control cases in this work. However, it should be noted that the number of control cases included in this study was rather small. Also, the mean age of controls (47.7 years) was considerably lower than the mean age of G-PD cases (58.8 years). An intriguing possibility is that previously reported tau pathology in controls actually represents primary age-related tauopathy (45). It would be interesting to explore the frequency of neuropathology in a larger group of Chamorro controls in future studies to see if there is a link with proteostasis mechanisms. It will also be important to examine other (non-hippocampal) brain regions and to establish the progression pattern of brain lesions in G-PD in detail. For example, cases were not screened for limbic-predominant
Physiological

ER stress induction
• e.g., misfolded proteins, oxidative stress, environmental insults
• ER homeostasis restored

Pathology

Unresolved ER stress
• Age-related impairments?
• Accumulation of demanding substrates?
• Prolonged UPR activation
• Insufficient autophagy

ER stress
• UPR †
• Autophagy †

Protein degradation by ERAD-UPS

Activation of the UPR in G-PD

To determine whether the UPR is activated in G-PD, we performed immunohistochemistry for UPR markers on G-PD brain tissue. Specifically, we demonstrated that the ER chaperone BiP/GRP78 and phosphorylated, that is activated, PERK accumulate in G-PD hippocampal sections. These differences were not seen in Guam control subjects.

Under normal conditions, BiP/GRP78 is bound to ER stress sensors, such as PERK, which are located in the ER membrane. According to the classic view of UPR activation, binding of BiP/GRP78 to unfolded or misfolded proteins present in the lumen of the ER results in dissociation of BiP/GRP78 from these membrane-associated sensors, resulting in their activation (alternatively, direct binding of unfolded proteins to these sensors may activate the UPR) (18). The main goal of the UPR is to restore ER homeostasis by inhibiting general protein translation and by modifying expression of UPR target genes, for example genes that encode proteins involved in protein folding and quality control. The PERK arm of the UPR specifically inhibits protein synthesis through phosphorylation of eukaryotic translation initiation factor 2α, which in turn reduces the biosynthetic burden of the secretory pathway. However, prolonged UPR activation can contribute to cellular demise through proapoptotic signaling (17–19). pPERK was found to be present in tangle-like and granular structures in the G-PD brain sections. This staining pattern is intriguing, because UPR activation has been shown to be closely associated with an early stage of neurofibrillary degeneration in disease (47, 48). A possible mechanism is that cytoplasmic tau aggregates inhibit processes like the ERAD-proteasome pathway, preventing the normal degradation of target proteins, stimulating ER stress (Fig. 5) (49). TDP-43 pathology has also been linked to ER stress (50). The pPERK-immunoreactive granules that were seen may be indicative of granulovacuolar bodies, which have been previously observed in G-PD brain tissue (37). It has been suggested that these granulovacuolar inclusions are a special type of autophagic vacuole or late-stage endo-lysosomal compartment, but it is unclear whether they reflect a cellular defense mechanism or if their formation inevitably leads to impairment of important cellular functions (36). Modulating the UPR may have therapeutic value in a range of neurodegenerative diseases (17–19).

Accumulation of UBB⁺¹ and UPS Components in G-PD

Ubiquitylated structures can be frequently observed in G-PD brain slices (51), and impairment of the UPS has been implicated in the pathogenesis of many neurodegenerative diseases (52–54). In addition, the UPS could be a promising therapeutic target to prevent neurodegeneration (53, 55).

It was previously shown that a mutant ubiquitin, UBB⁺¹, accumulates in G-PD brains (20). UBB⁺¹ is a frameshift mutant of ubiquitin that has been found to accumulate in a variety of disorders, including tauopathies (21, 22) and polyQ repeat expansion disorders (39). Aberrant ubiquitin is a dose-dependent inhibitor of the UPS and likely contributes to...
an environment that favors accumulation of abnormal proteins (24, 38). Transgenic expression of UBB\(^+\) in mice results in neurological deficits (25, 26) and modifies other pathology in vivo (e.g. by partial ERAD inhibition) (56, 57).

In this study, we extended the findings on UBB\(^+\) deposition to additional G-PD cases. UBB\(^+\) was present in a granular and tangle-like pattern in G-PD hippocampal neurons. Compellingly, UBB\(^+\) was also detected in astrocytes (20). Comparable UBB\(^+\)-reactive glial inclusions have been reported in progressive supranuclear palsy, a disease that exhibits some similarities to G-PD (23). In addition, we replicated previous findings on the accumulation of specific UPS constituents in G-PD brain, that is the ATPase subunit Rpt3/S6b and the DUB UCH-L1 (20). Accumulation of these UPS components has also been observed in other neurodegenerative diseases (58, 59).

The presence of accumulated UBB\(^+\) and UPS components in G-PD brain suggests that UPS dysfunction could play a role in the pathogenesis of the disease (Fig. 5). A crucial role for the UPS in neurodegenerative disease is supported by the finding that mutations in genes encoding components of the UPS can cause or predispose for neurodegeneration (52, 53). The UPS is a major regulator of tau proteolysis (60) and activation of the UPS has been shown to delay tau aggregation and neurodegeneration in vitro and in vivo (61–63). Soluble TDP-43 is also degraded by the UPS (64, 65) and motor neuron-specific knockout of Rpt3/S6b results in an ALS-like phenotype with TDP-43 mislocalization in mice (66). Proteasome inhibition in cultured human neurons also leads to TDP-43 mislocalization (67). UPS impairment may represent an early event in disease pathogenesis, but the precise roles of the UPS in G-PD pathology remain to be delineated.

**UBB\(^+\) Alters TDP-43 Aggregation and Cytotoxicity In Vitro**

TDP-43 pathology is present in G-PD (32, 33), pointing to a detrimental role for the protein in the disease. Furthermore, accumulation of UBB\(^+\) and several UPS components in neurons of G-PD patient brains suggests that UPS activity is affected in G-PD (20). Because the UPS might interact with TDP-43, it is of great interest to elucidate the relationship between the cellular accumulation of UBB\(^+\) and TDP-43 pathology.

UBB\(^+\) accumulation was previously shown to impair cell survival in aging yeast (31), as well as in neuroblastoma cells (68). In line with these findings, UBB\(^+\) was shown to accelerate the cytotoxicity of disease-associated proteins, like huntingtin, in yeast (24) and other experimental models (39, 69). However, UBB\(^+\) accumulation can also be protective in cells, potentially by inducing cellular stress response mechanisms, such as the heat shock response (70). As such, UBB\(^+\) could act as a proteostasis regulator that alters the composition of the proteostasis network. In agreement with a protective role of UBB\(^+\), UBB\(^+\)ameliorated \(\beta\)-amyloid plaque load in transgenic mice (57). Thus, UBB\(^+\) may play both detrimental and protective roles in human proteinopathies.

To test whether UBB\(^+\) can modify the aggregation and toxicity of TDP-43, a yeast TDP-43 proteinopathy model was used. By analyzing the formation of TDP-43-EGFP foci, it was found that UBB\(^+\) does not modify the aggregation of TDP-43 in wild-type yeast cells. However, UBB\(^+\) did mitigate TDP-43-triggered growth deficits in these cells. Thus, UBB\(^+\) turned out to be protective in wild-type cells: TDP-43 toxicity was lowered, although TDP-43 aggregation was not reduced in the presence of high levels of UBB\(^+\). In cells with constitutively increased UPS capacity (\(\Delta\)ubr2), UBB\(^+\) expression reduced both TDP-43 aggregation and TDP-43 toxicity, underlining the potential protective effect of UBB\(^+\) on TDP-43 pathology. As these effects depend on Rpn4, the master transcriptional activator of the UPS in yeast, the data suggest that UBB\(^+\) triggers a protective transcriptional response at the level of the UPS. This response may counteract the cellular accumulation of cytotoxic TDP-43 aggregates, either by preventing their formation and/or by increasing their clearance. Interestingly, it has been found that Rpn4 cooperates with the UPR to promote ER stress resistance in yeast cells (71).

It is important to note that, besides a protective effect of UBB\(^+\) in wild-type and \(\Delta\)ubr2 cells, we observed that UBB\(^+\) can also promote TDP-43 aggregate formation in cells lacking the ubiquitin gene \(UB14\) (Supplementary Data Fig. S2). In \(\Delta\)ubi4 cells, the ratio of nonfunctional mutant versus functional wild-type ubiquitin (UBB\(^+\)/ubiquitin) is increased due to depletion of wild-type ubiquitin. Under these conditions, the protective role of UBB\(^+\) switches to a detrimental one.

As aforementioned, the presence of UBB\(^+\) in G-PD brains might be independent from TDP-43 pathology. Whether UBB\(^+\) also controls the aggregation and toxicity of other proteins, such as tau, needs to be addressed in further experiments. We stress that more detailed examinations are required to link the different findings at the neuropathological level. In addition, it will be critical to repeat these experiments in other model systems. Regardless, these data in yeast support a pivotal role for ubiquitin-dependent pathways in modulating TDP-43 pathology.

**Components of the Autophagy-Lysosomal Pathway Are Deposited in G-PD**

To determine whether components of the autophagy pathway are accumulated in G-PD brains, immunohistochemical analyses were performed on hippocampal sections. Immunohistochemistry revealed deposition of autophagy-related proteins ATG8 and ATG12 in G-PD. Accumulation of these ATG proteins indicated alterations in autophagosome formation. Also, p62, a protein targeting cargo for autophagy (44), was accumulated in G-PD brain sections. Remarkably, p62 was not detected in pyramidal neurons in the CA1 region of the hippocampus. This might indicate different vulnerability of this region in G-PD, that is the CA1 region is more intensely affected than other hippocampal areas. Proteostatic differences between cells could explain differential sensitivity to degeneration.

These findings on autophagy proteins in G-PD have several implications. Abnormal autophagy has been linked to neurodegeneration and probably plays a role in G-PD
pathogenesis as well. Protein aggregates colocalize with autophagy markers in brain and disease-linked mutations may alter the activity of autophagy (42). Lysosomal proteases were previously found to be abundant in NFTs in G-PD patient brains (72). Significantly, autophagy mediates both TDP-43 and tau degradation (60, 64, 65). In stressed cells, for example cells in which the UPS is impaired or energy metabolism is abnormal, UPR signaling increases autophagy to restore proteostasis (73, 74), but insufficient capacity of the autophagy pathway can result in degenerative changes (Fig. 5). For these reasons, autophagy may represent a promising therapeutic target to mitigate neurodegeneration (42, 43, 55, 75).

Conclusion
In this study, we provide evidence for activation of the UPR in G-PD brains. Although initial activation of the UPR in G-PD is presumably neuroprotective, sustained activation of the UPR may initiate or mediate neurodegeneration. In addition, components of the UPS and autophagy-lysosome system, two major proteolytic systems, were found to accumulate in G-PD brains, suggesting a role for these proteostasis mechanisms in G-PD pathogenesis. Specifically, a mutant ubiquitin (UBB+) was detected in G-PD brains and was shown to be a modifer of disease-associated protein aggregation and toxicity. The findings suggest that proteostasis pathways are closely related to abnormal protein aggregation in G-PD. Finding out how different protein clearance systems collaborate to dispose of misfolded proteins may result in the identification of therapeutic targets to prevent or stop neurodegeneration in G-PD and other neurodegenerative disorders.

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