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Abnormal Dentatorubral-Pallidoluysian Atrophy (DRPLA) Protein Complex Is Pathologically Ubiquitinated in DRPLA Brains

Ikuru Yazawa,*,†,[1] Hirofumi Nakase,*,† and Hiroshi Kurisaki‡

*Department of Neurology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan; †Okinaka Memorial Institute for Medical Research, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan; and ‡Department of Neurology, National Tokyo Hospital, 3-1-1 Takeoka, Kiyose-shi, Tokyo 204-8585, Japan

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Dentatorubral-pallidoluysian atrophy (DRPLA) is caused by expansion of a glutamine repeat in DRPLA protein. DRPLA protein undergoes greater complex formation in DRPLA brain tissue, and expanded glutamine repeat enhances complex formation of DRPLA protein. Immunoblots with and without reduction show that the DRPLA protein complex is ubiquitinated only in DRPLA brain tissue. Moreover, immunoblots of regional DRPLA brain tissues reveal that pathological ubiquitination of DRPLA protein complex is found selectively in affected lesions. Double-labeling immunohistochemical studies with antibodies against DRPLA protein and ubiquitin demonstrate that the DRPLA protein is co-localized with ubiquitin in DRPLA neurons and show characteristic neuronal cytoplasmic inclusions with ubiquitinated DRPLA protein complex in the center. Our findings suggest that DRPLA protein undergoes abnormal complex formation with expanded glutamine repeat, and then the complex is pathologically ubiquitinated in DRPLA brain tissue. Pathological ubiquitination of abnormal DRPLA protein complex plays a role in DRPLA pathology.

Dentatorubral-pallidoluysian atrophy (DRPLA) is a hereditary neurodegenerative disorder caused by expansion of a glutamine repeat in DRPLA protein (1–3). Similar expansions have been reported in seven other neurodegenerative disorders: spinobulbar muscular atrophy (SBMA), Huntington's disease (HD), spinocerebellar ataxia 1 (SCA1), Machado-Joseph disease (MJD), SCA 2, SCA 6, and SCA 7, referred to as glutamine repeat diseases (4–12). Development of neuronal intranuclear inclusions in HD transgenic mice with expansion of glutamine repeats is suspected to have a role in generating the HD phenotype (13). Intranuclear inclusions also have been reported in MJD, SCA1, and DRPLA brain tissue (14–16), and in SBMA spinal cord tissue (17), and neuronal intranuclear inclusions and dystrophic neurites in HD brain tissue (18). Although intranuclear inclusions were reported to show immunoreactivity with the antibodies against gene products in glutamine repeat diseases and ubiquitin, it remains unclear whether the intranuclear inclusions are part of pathological processes leading to neuronal degeneration.

We have recently shown that DRPLA gene product (DRPLA protein) forms a high molecular weight complex with disulfide bondings, using immunoblotting of human brain tissue samples under non-reducing conditions with two anti-DRPLA protein antibodies against N- and C-termini of DRPLA protein, and that more of the disulfide-bond complex of DRPLA protein was present in DRPLA brain tissue than in control brain tissue based on immunoblot data obtained under non-reducing conditions (19). This result indicates the expansion of glutamine repeat affects and produces larger amounts of DRPLA protein complex. Abnormal complex formation of DRPLA protein plays a crucial role in the pathological processes leading to DRPLA. Abnormal accumulations of ubiquitinated protein in neurons are found in various neurodegenerative disorders (20). A human ubiquitin-conjugating enzyme (hE2-25K) was reported to interact with HD protein using a yeast two-hybrid system, but the expressions of hE2-25K in HD and control brain tissue revealed no difference, indicating the interaction between HD protein and hE2-25K is not modulated by CAG repeat expansion in a HD gene (21). In this study, using immunoblotting with and without reduction and double-labeling immunohistochemical methods, we have shown what role abnormal DRPLA protein complex formation plays in pathological process in DRPLA and have determined the role of ubiquitination of DRPLA protein in DRPLA brain tissue. We discuss the

[1] To whom correspondence should be addressed. Fax: 813-3582-7068. E-mail: yazawa-tky@umin.ac.jp.

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ubiquitination of abnormal complex formation in DRPLA protein and show similarity in ubiquitination of protein complex between DRPLA and the other neurodegenerative disorders.

MATERIALS AND METHODS

Sample preparation. Postmortem brain tissue samples from five DRPLA patients (24–69 years old), whose disease had been diagnosed genetically by the PCR analysis and confirmed pathologically as were, and samples from four control patients (59–79 years old) were examined (3). Tissue samples (2 g) of cerebra from human control and from DRPLA brains were each homogenized in 5 volumes of tris-saline buffer with protease inhibitors (TSpi) (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 μg/ml aprotinin, 1 mM EDTA, 10 μg/ml Leupeptin, 0.5 mM Pefabloc SC, and 10 μg/ml Pepstatin). Samples were stored at −80°C until used for gel electrophoresis. For comparing complex formations among affected lesions and unaffected DRPLA brain tissues, tissue samples of severely affected lesions of brain tissues (dentate nucleus and globus pallidus), mildly affected lesions of brain tissues (cerebrum and cerebellum), unaffected brain tissues (pons and thalamus) and the other non-nervous organ tissues (ovary and lymphoblastoid cells) from a DRPLA patient were homogenized in 5 volumes of TSpi and examined using immunoblotting.

Reducing/non-reducing electrophoresis and immunoblots. Anti-DRPLA protein polyclonal antibody (C580) that had been raised in rabbits against synthetic peptides corresponding to the C-terminal end of human DRPLA protein (amino acids 1171–1184) was purified by affinity chromatography as described elsewhere (3, 22). After protein was determined by the bicinchoninic acid (BCA) protein assay (Pierce), samples of the brain tissues were mixed with equal volumes of sodium dodecyl sulfate (SDS) sample buffer (4% SDS, 160 mM Tris-HCl, pH 6.8, 20% glycerol) with and without a reducing agent (10% 2-mercaptoethanol, ME). The acrylamide concentration of the stackings was 5% and that of the running gels 7.5% in 0.1% SDS. Samples (10 μg each) were electrophoresed in the gel, then the gels were subjected to immunoblotting. Proteins were transferred electrophoretically to a polyvinylidene difluoride (Immobilon) membrane (Millipore), which then was blocked with 4% nonfat milk and stained overnight at 4°C with C580 and anti-ubiquitin polyclonal antibody (DAKO). The Immobilon membrane was incubated for 1 hour at room temperature with anti-rabbit secondary antibodies and the reaction was made visible with an enhanced chemiluminescence (ECL) western blotting system (Amersham).

Double-labeling immunohistochemistry. Blocks of tissue from three human control brains and three DRPLA brains were fixed in 10% formalin. Sections from 10% formalin-fixed blocks were embedded in paraffin then immunostained with anti-DRPLA protein (C580) and anti-ubiquitin antibodies. The sections which had been incubated for 30 minutes in 0.3% hydrogen peroxide in methanol, were incubated with the first sequence of primary antibody against ubiquitin at a dilution of 1:1500 for overnight at 4°C. They were preincubated with secondary biotinylated secondary antibody, and incubated avidin-biotinylated horseradish peroxidase complex (Vectastain). 3,3′-diaminobenbenzidine (DAB, Sigma) was used as the first reaction substrate and reaction product appeared as brown color. After brown visualization, the sections were washed with 100 mM Tris-HCl solution, pH 2.2. The sections were reacted with the second sequence of primary antibody against DRPLA protein (C580) overnight at 4°C and consecutively stained with secondary anti-rabbit antibody (Nichirei Corporation), using standard indirect peroxidase and streptavidin biotin techniques (Histofine SAB-POR Kit, Nichirei Corporation). 3,3′,5,5′-tetramethylbenzidine (TMB), was used as the second reaction substrate and reaction products were made visible as blue color by True Blue Peroxidase Substrate Kit (KPL).

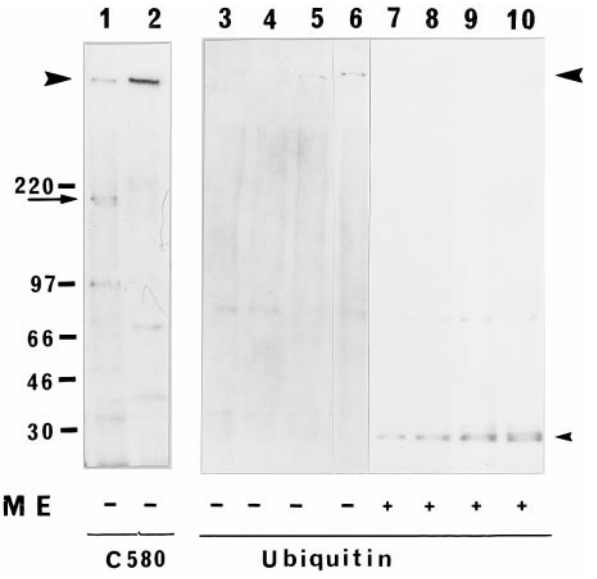

FIG. 1. Immunoblots of the total homogenates of cerebra from control brain tissue from two individuals (lanes 1, 3, 4, 7, 8) and brain tissue from two DRPLA patients (lanes 2, 5, 6, 9, 10) (10 μg protein per lane) under the non-reducing (lanes 1–6) and reducing (lanes 7–10) conditions, stained with anti-DRPLA protein antibody (C580) (lanes 1, 2) and anti-ubiquitin antibody (lanes 3–10). Immunoblots of control and DRPLA brain tissue samples stained with C580 under non-reducing conditions (lanes 1, 2) show a complex band located at the top of the stacking gel (large arrowhead), but only the immunoblot of control brain tissue (lane 1) shows a DRPLA protein band with the apparent molecular weight of approximately 200 kDa (arrow). Immunoreactivity of the complex band is greater on the immunoblots of DRPLA brain tissue than on those of control brain tissue. Immunoblots of DRPLA brain tissue stained with anti-ubiquitin antibody under non-reduced conditions show the same complex band (large arrowhead) as that stained with C580 located at the top of the stacking gel (lanes 5, 6), whereas immunoblots of control brain tissue show no immunoreactivity at the top of stacking gel (lanes 3, 4). Immunoblots of control brain tissue and DRPLA brain tissue samples under reducing conditions stained with anti-ubiquitin antibody show equal immunoreactivities of multiple ubiquitin bands (small arrowhead), revealing absence of immunoreactivity of the complex band (lanes 7–10). ME is 2-mercaptoethanol. The molecular weight markers are indicated to the left.

RESULTS

Pathological ubiquitination of DRPLA protein complex in DRPLA brain tissue. The total homogenates of human control brain tissues and DRPLA brain tissues were analyzed in SDS-polyacrylamide gels under reducing and non-reducing conditions. The gels were immunoblotted using anti-DRPLA protein antibody (C580), an affinity-purified polyclonal antibody against the C-terminus of DRPLA protein (3), and anti-ubiquitin antibody. On the immunoblots of cerebra of control and DRPLA brain tissues under non-reducing conditions stained with C580, DRPLA protein migrated in a band of apparently higher molecular weight at the top of the stacking gel (Fig. 1, lanes 1, 2). Immunoblots under non-reducing conditions stained with

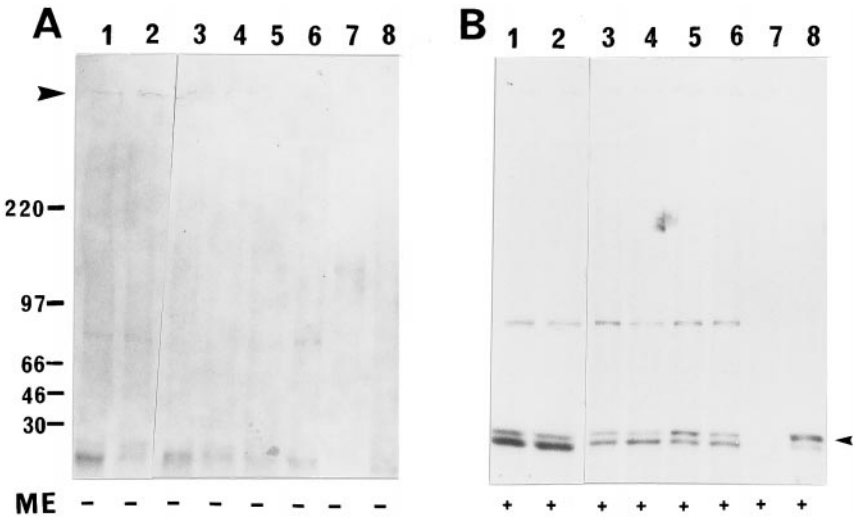

FIG. 2. Immunoblots of severely affected lesions of brain tissues (globus pallidus and dentate nucleus; lanes 1, 2), mildly affected lesions of brain tissues (cerebrum and cerebellum; lanes 3, 4), unaffected brain tissues (pons and thalamus; lanes 5, 6), and the other non-nervous organ tissues (ovary and lymphoblastoid cells; lanes 7, 8) from a DRPLA patient, under non-reducing (A) and reducing (B) conditions. (A) Immunoblots of severely and mildly affected lesions stained with anti-ubiquitin antibody under non-reducing conditions show the ubiquitinated bands of DRPLA protein complexes at the top of the gel (large arrowhead), which possess stronger immunoreactivities in severely affected lesions than in mildly affected lesions (lanes 1–4). Immunoblots of unaffected brain tissues and non-nervous organ tissues under non-reducing conditions show little ubiquitin-immunoreactivity of DRPLA protein complex at the top of the gel (lanes 5–8). (B) Immunoblots of all brain tissues (lanes 1–6) under reducing conditions stained with anti-ubiquitin antibody show equal immunoreactivity of ubiquitin bands (small arrowhead), but the DRPLA protein complex band is absent at the top of the gel. Immunoblots of non-nervous organ tissues (lanes 7, 8) under reducing conditions show different patterns of ubiquitin bands.

C580 show immunoreactivity of DRPLA protein complex in DRPLA brain tissue is greater than in control brain tissue, revealing conclusively that the extent of disulfide bond formation by DRPLA protein differs in control brain tissue and DRPLA brain tissue, showing that DRPLA protein forms more disulfide bonds in DRPLA brain tissue than control brain tissue. Immunoblots of cerebra of DRPLA brain tissues under non-reducing conditions stained with anti-ubiquitin antibody show a band of apparently higher molecular weight at the top of the stacking gel with the same electrophoretic mobility as that of DRPLA protein complex (Fig. 1, lanes 5, 6). In contrast, on the immunoblots of cerebra of control brain tissue under non-reducing conditions stained with anti-ubiquitin antibody, there is no immunoreactivity of apparently higher molecular weight at the top of the stacking gel (Fig. 1, lanes 3, 4). Immunoblots of control brain tissue and DRPLA brain tissue samples under reducing conditions stained with anti-ubiquitin antibody show equal immunoreactivities of multiple ubiquitin bands (Fig. 1, lanes 7–10).

To understand whether the ubiquitin-positive DRPLA protein complex formation relates to DRPLA pathology, we have examined and compared DRPLA protein complex formation among severely affected lesions of brain tissues (dentate nucleus and globus pallidus), mildly affected lesions of brain tissues (cerebrum and cerebellum), unaffected brain tissues (pons and thalamus) and non-nervous organ tissues (ovary and lymphoblastoid cells) from a DRPLA patient. Immunoblots of severely and mildly affected lesions stained with anti-ubiquitin antibody under non-reducing conditions show the ubiquitinated bands of DRPLA protein complexes at the top of the gel, which reveal stronger immunoreactivities in severely affected lesions than in mildly affected lesions in DRPLA brain tissues (Fig. 2A). Immunoblots of unaffected brain tissues and non-nervous organ tissues under non-reducing conditions show little ubiquitin-immunoreactivity of DRPLA protein complex at the top of the gel (Fig. 2A). In contrast, immunoblots of all brain tissues under reducing conditions show equal immunoreactivities of ubiquitin bands but DRPLA protein complex band is absent at the top of the gel, although those of non-nervous organ tissues show different patterns of ubiquitin bands (Fig. 2B). These results indicate that disease-specific ubiquitinated DRPLA protein complex is only detectable in the affected lesions using immunoblotting but not detectable in the unaffected brain tissues, and that extent of DRPLA protein complex formation relates to the severity of involvement in DRPLA brain tissue.

Immunohistochemical analysis of DRPLA protein and ubiquitin. DRPLA protein and ubiquitin in control brain tissue and DRPLA brain tissue are distinguished by different colored reaction products of the individual chromogens used in double-labeling immu-

nohistochemical staining stained with two antibodies against DRPLA protein (C580) and ubiquitin. Peroxidation of 3,3′-diaminobenbenzidine (DAB), a reaction substrate for ubiquitin, resulted in a brown color, while 3,3′,5,5′-tetramethylbenzidine (TMB) for DRPLA protein gave a blue color. Using double-labeling immunohistochemical study, neurons in control brain tissues appear as a blue color with cluster formation in part of the cytoplasm, showing neurons have immunoreactivity of C580 but little immunoreactivity of anti-ubiquitin antibody (Fig. 3A). In contrast, using the same technique to study immunoreactivities of two antibodies against C580 and ubiquitin, neurons in DRPLA brain tissue appear as a light black color (color combination of brown and blue) with a apparent granular and fibrous pattern, showing both immunoreactivities of two antibodies against C580 and ubiquitin (Fig. 3B). This indicates that DRPLA protein is co-localized with ubiquitin in DRPLA neurons. These immunohistochemical results suggest that DRPLA protein complex undergoes pathological ubiquitination only in DRPLA brain tissue. Moreover, neuronal cytoplasmic inclusions are identified in DRPLA dentate nuclei (Fig. 3C), appearing as a brown color with a light black-colored core in center (Fig. 3C, arrow), indicating the ubiquitinated DRPLA protein complex in the center of the inclusions, and the ubiquitin-conjugated proteins in the area surrounding the ubiquitinated DRPLA protein complex. Double-labeling immunohistochemical studies show that the intranuclear inclusions appeared as a brown color, indicating that ubiquitinated intranuclear inclusions do not show immunoreactivity with C580 in DRPLA brain tissue (Fig. 3D, arrowhead).

DISCUSSION

DRPLA protein forms a high molecular weight complex and that more of the DRPLA protein complex was present in DRPLA brain tissue than in control brain tissue based on immunoblot data obtained under non-reducing conditions (19). As there was quantitatively more DRPLA protein complex in DRPLA brain tissue than in control brain tissue, expansion of glutamine repeat affects and produces abnormally large amounts of complex formation in DRPLA brain tissue. We demonstrate that the DRPLA protein complex shows a qualitative difference between in control and DRPLA brain tissue in addition to a quantitative difference. We have identified ubiquitin as a component of DRPLA protein complex on the immunoblots of DRPLA brain tissue under non-reducing conditions, but could not identify ubiquitin on the immunoblots of control brain tissue. Our immunoblotting data indicate that only DRPLA protein complex in the diseased brain tissues is pathologically ubiquitinated. We previously reported that immunoblots of regional DRPLA brain tissues stained with C580 showed that DRPLA protein is ubiquitously expressed in DRPLA brain and the amounts of DRPLA protein are relatively larger in cerebrum and cerebellum than in the other brain tissues, indicating that the regional distribution of DRPLA protein is not related to DRPLA pathology (3). Here, immunoblots of regional DRPLA brain tissues stained with anti-ubiquitin antibody show that pathological ubiquitination of DRPLA protein complex is found selectively in the affected lesions of DRPLA brain tissues, providing that pathological ubiquitination play an important role in DRPLA pathology. Double-labeling immunohistochemical studies are consistent with the immunoblotting findings. The immunoreactive C580-staining product with cluster formation, which corresponds to the complex formation of DRPLA protein in immunoblotting, shows immunoreactivity with anti-ubiquitin antibody in DRPLA neurons, but revealing the absence of ubiquitin-immunoreactivity in control neurons. In addition, identification of characteristic neuronal cytoplasmic inclusions in DRPLA dentate nuclei, appearing as abnormal DRPLA protein complex in the center of the inclusions (Fig. 3C, arrow) and the ubiquitin-conjugated proteins in the area surrounding the ubiquitinated DRPLA protein complex, provides the evidence of the disease-specific abnormal ubiquitination of DRPLA protein complex. Our data indicate patients with DRPLA undergo neuronal degeneration caused by two pathological processes in DRPLA brains; abnormal complex formation and pathological ubiquitination of DRPLA protein.

Ubiquitin, a 76-amino acid residue protein, is a component of a protein posttranslational modification catalyzed by a specific ATP-dependent enzyme pathway (23, 24). Most proteins known to be polyubiquitinated are targeted to the 26S proteasome: a complex, multicatalytic cytoplasmic and nuclear protease (24). Abnormal accumulations of ubiquitinated proteins are found in intracellular inclusions in various neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and motor neuron diseases (25–29). Thus, ubiquitin accumulations are related to the process of neurodegeneration (20). There seems to be much similarity between these ubiquitin-accumulated neurodegenerative diseases and DRPLA. In both ubiquitin-accumulated neurodegenerative diseases and DRPLA, cytoplasmic inclusions in neurons have been identified using immunohistochemical studies. In DRPLA, pathological ubiquitination is not found when DRPLA protein is separated into a monomer form, but found when DRPLA protein forms greater amounts of abnormal complex in DRPLA brain tissue. Ubiquitination may be a common process in neurodegenerative disorders and ubiquitin may activate the proteolysis of abnormal protein complexes and accumulated inclusions, although ubiquitin is not usually found conju-

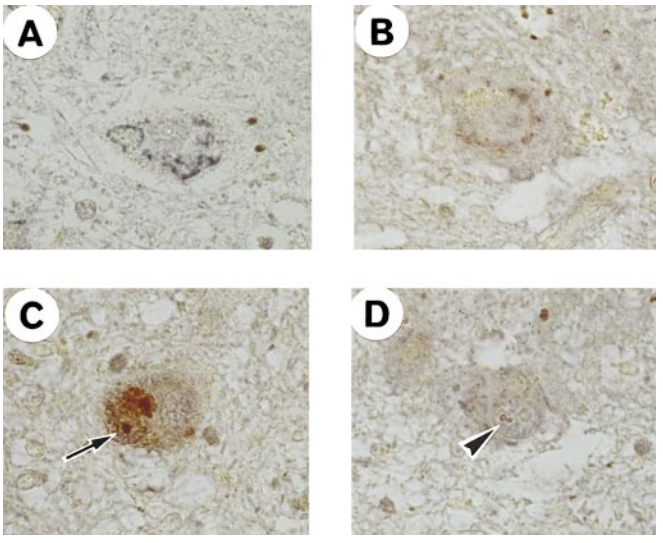

FIG. 3. Four photographs of neurons in dentate nuclei in control brain tissue (A) and DRPLA brain tissue (B–D), using double-labeling immunohistochemical methods. (Magnification A–D; ×328) (A) Neurons in control brain tissue appear as a blue color with cluster formation in part of the cytoplasm, showing that neurons have immunoreactivity of C580 but little immunoreactivity of anti-ubiquitin antibody. (B) Neurons in DRPLA brain tissue appear as a light black color (color combination of brown and blue) with an apparent granular and fibrous pattern, revealing both immunoreactivities of two antibodies against C580 and ubiquitin. DRPLA protein is co-localized with ubiquitin in DRPLA neurons. (C) Neuronal cytoplasmic inclusion appears as a brown color with a light black-colored core in the center in DRPLA dentate nuclei (arrow) and the ubiquitin-conjugated proteins in the area surrounding the DRPLA protein complex. (D) Intranuclear inclusions appear as a brown color (arrowhead).

gated to cytoskeletal proteins from which inclusion bodies are derived (20). Thus, the abnormal complex formation of DRPLA protein may be the primary process in DRPLA neurodegeneration. As DRPLA protein complex is observed in control and DRPLA brain tissue, our immunoblotting data clearly shows that the extent and character of DRPLA protein complex formation is important in the disease-specific ubiquitination. Disulfide bonds are important to structural folding and the stability of many proteins (30, 31), and our previous research indicated that the disulfide bonds may be important to the folding and stability of DRPLA protein (19). As previously shown in rat DRPLA protein, homologous to the amino acid sequence of human DRPLA protein, the structure of DRPLA protein that relates to the glutamine repeat influences the amounts of rat and human DRPLA protein complex formations (32, 33). Our findings indicate that the structural folding and stability of DRPLA protein, which is influenced by the expansion of glutamine repeat in DRPLA brain tissue, is important in pathological ubiquitination. Pathological ubiquitination of abnormal DRPLA protein complex plays a role in DRPLA pathology.

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