Supporting information

Materials and Methods

Electron microscopy

Electron microscopy analysis was performed as described previously (1). Samples were fixed in 2.5% glutaraldehyde overnight at 4℃, and then sectioned on a Leica EM UC6/FC6 Ultramicrotome. Sections were stained by toluidine blue to assure the fixed position. Ultrathin sections were transferred to carbon/formvar coated copper grids and double stained with uranyl acetate and lead acetate (2).

Western blot analysis

Head tissues from 5-day-old GMR-GAL4>UAS-GFP, GMR-GAL4>UAS-uN2C-GFP and GMR-GAL4>UAS-uN2CpolyG-GFP male flies were lysed with RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 1% SDS (pH 7.4), 1x protease inhibitor cocktail (Roche)]. Lysates of fly or cell samples were analyzed by western blotting with specific antibodies against GFP (Proteintech Group 66002-1-Ig), Beta-Actin (Proteintech Group 66009-1-Ig), TIM23 (Proteintech Group 11123-1-AP), TOM20 (Proteintech Group 11802-1-AP), Histone-H3 (Proteintech Group 17168-1-AP), GAPDH (Proteintech Group 60004-1-Ig), LRPPRC (Proteintech Group 21175-1-AP).

Patients, muscle biopsies and brain autopsy

The study was approved by the Ethics Committee of the Peking University First Hospital. All methods were carried out in accordance with relevant guidelines and regulations. We examined muscle biopsy samples of NIID patients and age-matched control subjects. All clinical materials used in this study were obtained for diagnostic purpose after obtaining informed consent. All samples had previously been examined by routine histology techniques and electron microscopy. Fresh frozen samples were kept at −80 °C until used. Brain specimen from an autopsied NIID patient was fixed with 20% buffered formalin, and multiple tissue blocks were embedded in paraffin.

Cell culture and transfection

SH-SY5Y cells were cultured in Dulbecco's modified Eagle medium (DMEM-F12; Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/ mL penicillin, and 100 lg/mL streptomycin in a humidified incubator at 37℃ under 5% CO_2/95% air as previously reported (3). Viral infection was performed using adenovirus expressing GFP, uN2C-GFP and uN2CpolyG-GFP.

Immunofluorescence

Serial frozen sections (8 μm) were performed on muscle samples and fly eyes. Brain samples were fixed with formalin, embedded in paraffin and sectioned. Selected sections were immunostained with primary antibodies against N2CpolyG (4D12) (4), COX IV (Proteintech Group 11242-1-AP; 1:200), ubiquitin (Proteintech Group 10201-2-AP; 1:200) and p62 (Abcam, ab56416, anti-mouse; 1:200).

Cells were rinsed with 1× phosphate-buffered saline (PBS), fixed with 4% polyformaldehyde in 1× PBS at 24h post-transfection with GFP, uN2C-GFP and uN2CpolyG-GFP. Mitochondria were stained by mitotracker Red (Invitrogen).

Immuno-electron microscopy

For immune-EM, brain tissue from NIID patient was fixed in fix solution containing 2% polyformaldehyde and 0.2% glutaraldehyde (pH7.2) in PBS for 3 hours at room temperature. After rinses and post-fixation treatment, blocks were prepared in 2.3 M sucrose at 4℃. Ultrathin frozen sections were obtained at 120℃ using dry diamond knives. Following blocking, sections were immunostained with monoclonal anti-mouse-uN2CpolyG antibody (1:100) and 6 nm-colloidal gold conjugated secondary antibody.

Immunoprecipitation

SH-SY5Y cells were used for the infections and analyses of protein-protein interactions. The experiments were performed 24 hours after infection. The harvested cells were washed with phosphate-buffered saline (PBS) once and then lysed on ice in the lysis buffer (50mM Tris, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, pH7.4, 1x protease inhibitor cocktail (Roche)) for 30 minutes. The soluble fraction of cell lysates was used for immunoprecipitation with anti-GFP antibody and protein A-agarose (Roche) at 4°C.

RNA-seq and bioinformatic analysis.

RNA sequencing was carried out in collaboration with Oebiotech (Shanghai, China). Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) following the manufacturer's protocol. RNA-seq and bioinformatic analysis were carried out as in a previous study (2).

Oxygen consumption rate

For each group, five alive flies were weighed and transferred to a glass Teflon Dounes homogenizer filled with 500 μL of cold respiration medium (3mM MgCl2, 60mM Lactobionic acid, 20mM Taurine, 10mM KH2PO4, 20mM HEPES,110mM D-sucrose, 1 g/L BSA and 0.5mM EGTA) and were homogenized on ice for 20 strokes.

Mitochondrial respiratory function was measured in a two-channel titration injection respirometer (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). The homogenate was transferred separately to oxygraph chambers. After a brief stabilization period, the chambers were closed, and the data were recorded using DatLab software 5.2 (Oroboros Instruments, Innsbruck, Austria).

To evaluate *Drosophila* mitochondrial respiratory function, we applied specifically designed substrate-uncoupler-inhibitor titrations in an extended experimental protocol. When the respiration was stable, routine respiration (no additives, Routine) was measured. The respiratory leak state of complex I (CI Leak) was determined after titration with glutamate (G, 5 mM) and malate (M, 2 mM) in the absence of ADP. The oxidative phosphorylation capacity of complex I (CI OXPHOS) was examined after adding 5 mM ADP. Succinate (Suc, 100 mM) was added to induce maximal OXPHOS capacity via convergent input through both CI and complex II (CII, CI+II OXPHOS). Subsequently, the maximal uncoupled respiratory capacity of the electron transfer system (ETS) was obtained by titrating FCCP (injected stepwise up to 0.5 μM) (CI+II ETS). After added rotenone (Rot, 0.5 μM), the CII-supported uncoupled respiratory function (CII ETS) was measured. Finally, residual oxygen consumption was evaluated after the addition of antimycin A (Ama, 2.5 μM).

ATP assay

The total cellular ATP level was measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's instruction. Briefly, for flies, equal amounts of fly homogenate were incubated with CellTiter-Glo substrate at 37℃ for 10 minutes. The reaction mixtures were transferred to another opaque 96-well plate to measure luminescence. For cells, 24 hours before assay, the GFP, N2C-GFP or N2CpolyG-GFP expressing SH-SY5Y cells were seeded in 96-well plates. Following removal of the culture media and cell lysis, reaction mixtures were transferred to another opaque 96-well plate for 10 min at 37℃ to measure luminescence.

Luminescent signal values were normalized by the protein amount in each group to determine the ATP levels. The remaining samples were dissolved in RIPA buffer (Beyotime) and the protein concentration measured by BCA assay (LABLEAD). The ATP concentration was determined using a standard curve for the luciferase assay and then normalized to the protein concentration.

Supplementary Figures

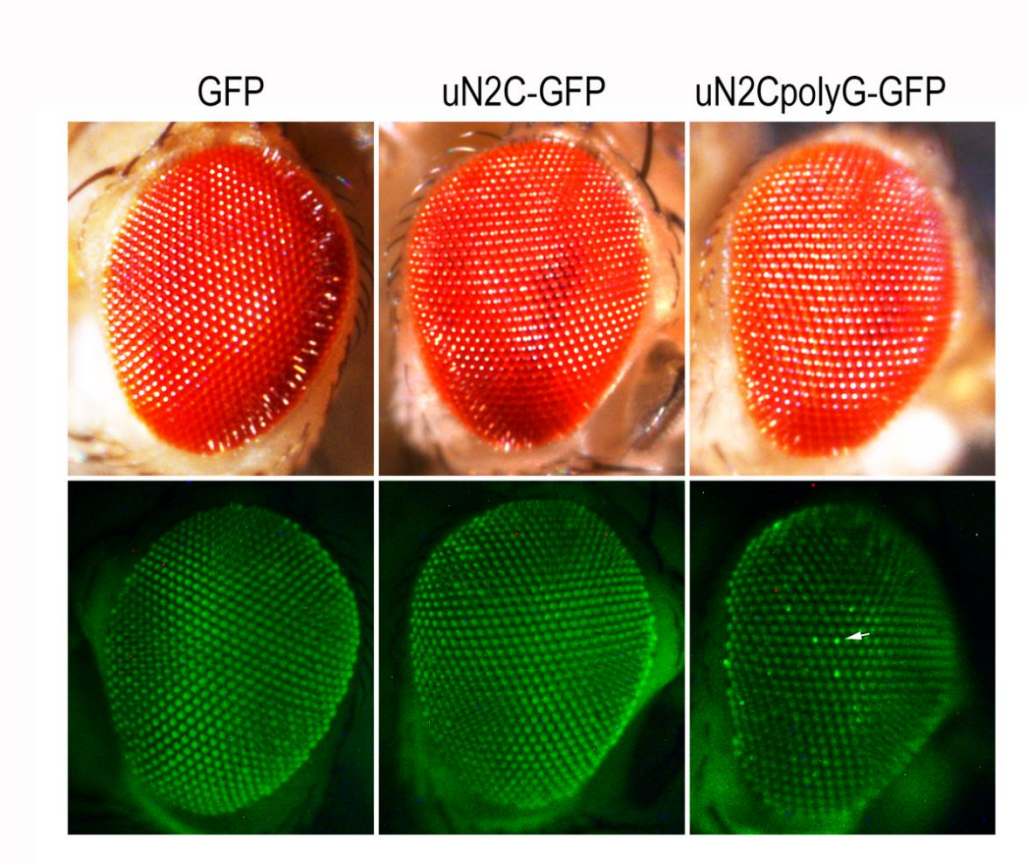

Figure S1 Microscopy images of fly eyes expressing GFP, uN2C-GFP and uN2CpolyG-GFP. Expression of uN2CpolyG do not cause obvious eye surfaces degeneration compared to controls. GFP inclusions in ommatidia from flies expressing uN2CpolyG-GFP (marked by arrow), but not GFP or uN2C-GFP. The top panel: light microscopy images, the bottom panel: immunofluorescence microscopy images. Fly genotypes: GFP: GMR-GAL4>UAS-GFP, uN2C: GMR-GAL4>UAS-N2C-GFP and uN2CpolyG: GMR-GAL4>UAS-N2CpolyG-GFP.

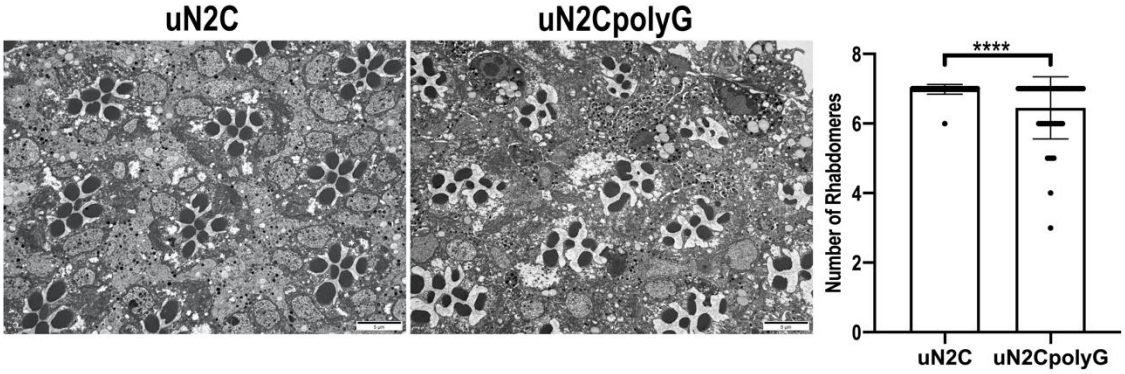

Figure S2 Expression of uN2CpolyG without GFP fusion causes progressive neurodegeneration in the eyes of Drosophila. EM micrographs show intact ommatidial structures with 7-rhabdomere female adult fly expressing uN2C protein without GFP fusion. Whereas the rhabdomere structures are damaged or severely lost in the fly eyes expressing uN2CpolyG protein without GFP fusion.

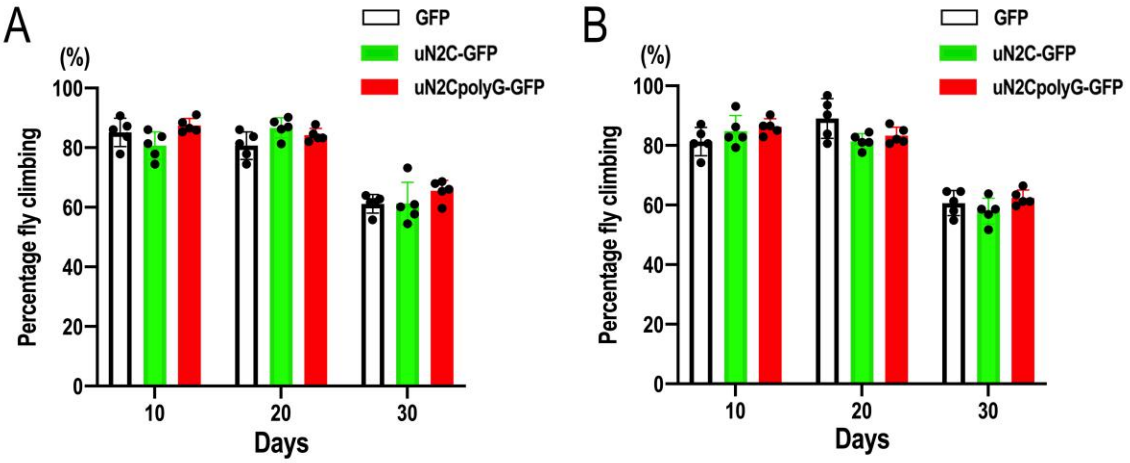

Figure S3 Neuron-specific expression of uN2CpolyG did not affect locomotor ability. The locomotor index was measured in adult flies at different time point. Expression of uN2CpolyG protein in adult flies by Elav-GAL4 driver led to no significant locomotor deficits in both male flies (A) and female flies (B) at day 30. (Log-rank Mantel–Cox test; n = 31-134/genotype for female flies and n = 86-156/genotype for male flies).

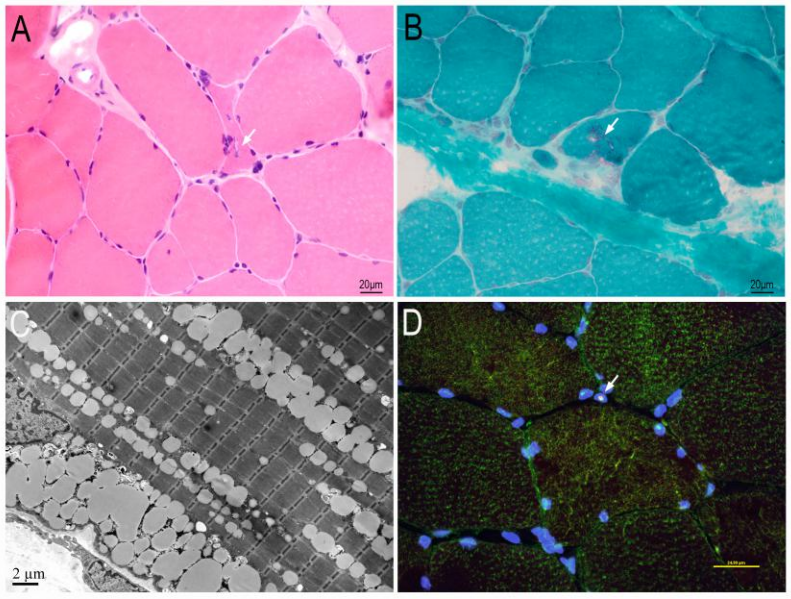

Figure S4 Muscle pathological changes of the NIID patients. (A) Haematoxylin and eosin (HE) staning and (B) modified Gomori trichrome (mGT) staining of muscle sections from NIID Patient, which revealed dystrophic change with variation in fiber size and endomysial fibrosis, and fibers with rimmed vacuoles (marked by arrow). (C) Transmission electron microscopy (TEM) revealed lipid droplets accumulated in the muscle sample from NIID patient. (D) Immunofluorescence showed ubiquitin and uN2CpolyG co-localized in the intranuclear inclusion of NIID muscle biopsies.

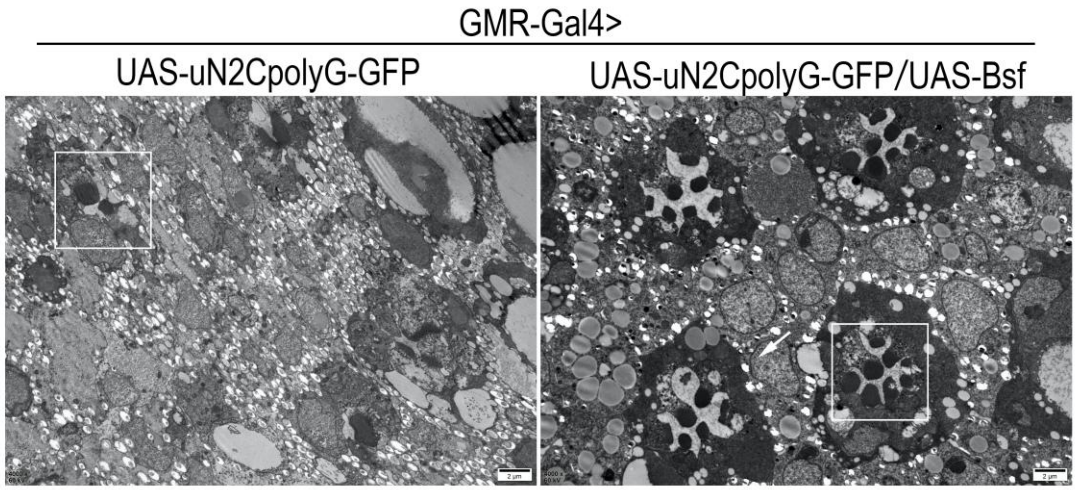

Fig. S5 Overexpressing Bsf in uN2CpolyG transgenic flies partially rescued the retinal degeneration. Electron micrographs show that the rhabdomere structures are

severely damaged or completely lost in the retinae of GMR-Gal4>UAS-uN2CpolyG-GFP flies, while the retinal degeneration was partially restored in GMR-Gal4>UAS-uN2CpolyG-GFP/UAS-Bsf flies. Bar= 2μm.

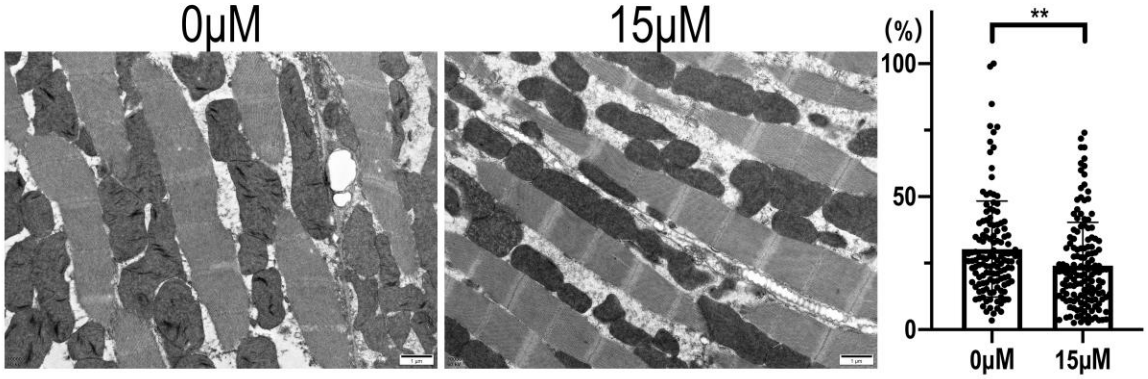

Fig. S6 Electron microscopy analysis showing reduced mitochondrial size in muscle samples from uN2CpolyG expressing flies fed with a 15 μM dose of IDB. (A) Muscle fibers were intact in uN2CpolyG-GFP expressing flies fed with 0 μM or 15 μM IDB at day 15. While reduced mitochondrial size was observed in flies fed with 15 μM IDB compared to flies fed with 0 μM IDB. (B) Statistical analysis of mitochondrial size in uN2CpolyG-GFP expressing flies fed with 0 μM and 15 μM IDB at day 15. A 15 μM dose of IDB significantly reduced mitochondrial swelling in uN2CpolyG-expressing flies. Bar= 1μm (A); **P < 0.01.

Table S1 The primer pair sequences used for RT-qPCR reactions

Actin-F	5’-TCACCCACACTGTGCCCATCTACGA-3’
Actin-R	5’-TCGGTGAGGATCTTCATGAGGTA-3’
ND2-F	5’-ATTCCATCCACCCTCCTCTC-3’
ND2-R	5’-TGGGGTGGGTTTTGTATGTT-3’
ND6-F	5’-GGGTGGTGGTTGTGGTAAAC-3’
ND6-R	5’-CCCCGAGCAATCTCAATTAC-3’
COX1-F	5’-CGATGCATACACCACATGAA-3’

COX1-R	5’-AGCGAAGGCTTCTCAAATCA-3’
NDUFA7-F	5’-GATCTCCAAGCGAACTCAGC-3’
NDUFA7-R	5’-TGTTGGAGAGCTTGTGGCTA-3’
NDUFA3-F	5’-CGCGGAGACAAAGATGGCTG-3’
NDUFA3-R	5’- GACGGGCACTGGGTAGTTGT-3’
NDUFA6-F	5’-CTTCTACCGCCAGCACCTTC-3’
NDUFA6-R	5’-GGTCTGTGACATGGGCATTCTTC -3’
NDUFV3-F	5’-AGCCCAGGTGTTTCGAGGAC-3’
NDUFV3-R	5’-CTGGCGGGTAAGAACTGGGT-3’

Reference

1. J. Deng et al., FUS interacts with ATP synthase beta subunit and induces mitochondrial unfolded protein response in cellular and animal models. Proc Natl Acad Sci U S A 115, E9678-e9686 (2018).
2. J. Deng et al., Expansion of GGC Repeat in GIPC1 Is Associated with Oculopharyngodistal Myopathy. Am J Hum Genet 106, 793-804 (2020).
3. J. Yu et al., The GGC repeat expansion in NOTCH2NLC is associated with oculopharyngodistal myopathy type 3. Brain 144, 1819-1832 (2021).
4. M. Boivin et al., Translation of GGC repeat expansions into a toxic polyglycine protein in NIID defines a novel class of human genetic disorders: The polyG diseases. Neuron 109, 1825-1835 e1825 (2021).