# Supporting Information

## Hamada et al. 10.1073/pnas.1409730111

### SI Materials and Methods

**Materials.** All-trans retinoic acid (RA), *N,N*-dimethylated casein, 2-aminoethoxydiphenyl borate (2-APB), and αactin antibodies were purchased from Sigma-Aldrich. Zwittergent 3–14, ionomycin, and calpain I from porcine erythrocytes were purchased from Calbiochem. The enhanced chemiluminescence (ECL) reagents and HRP-conjugated streptavidin were purchased from GE Healthcare. The 5-biotinamido-pentylamine (BP), bis[sulfosuccinimidyl] suberate (BS), 1,8-bis-maleimido-diethyleneglycol (BM), Tris(2-carboxyethyl)phosphine (TCEP), and avidin proteins (NeutrAvidin) were purchased from Thermo Scientific. Inositol 1,4,5-trisphosphate ($IP_3$), fura-2, and fura-2 acetoxymethyl ester (AM) were purchased from Dojindo. The α transglutaminase type 2 (αTG2) antibody (transglutaminase II Ab-1, CUB 7402) was purchased from LabVision. αLC3 was purchased from Medical & Biological Laboratories (MBL, Japan). Purified guinea pig TG2, bovine thyroglobulin, xestospongin C (xest), and lysyl-endopeptidase (Lep) were purchased from Wako Pure Chemical. DMEM, penicillin–streptomycin mixed solution, acetonitrile for HPLCy (HPLC), dimethyl sulfoxide (DMSO), and Triton X-100 were purchased from Nacalai. Ethanol was purchased from Junsei. FBS was purchased from Equitech-Bio. Tetramethylrhodamine cadaverine (TMR-cad) and pcDNA3.1 were purchased from Invitrogen. The site-specific inhibitor of TG2, Z-DON–Val–Pro–Leu–OMe (ZDON), was purchased from Zedira. The $F(ab')_2$ fragment of rabbit anti-human IgM was purchased from Jackson ImmunoResearch. Human neuroblastoma SH-SY5Y, human gliocytoma U87-MG, and mouse neuroblastoma Neuro2a cells were purchased from the American Type Culture Collection. PC12 cells with an inducible expression of full-length huntingtin (Htt) containing 145 polyglutamine repeats (Htt145Q–RFP) and plasmids containing full-length Htt and 23 (Htt23Q–myc) or 145 polyglutamines (Htt145Q–myc) were obtained from Coriell Institute.

**Cell Culture.** PC12 cells were seeded in collagen IV-coated 6-cm dishes and cultured in DMEM containing 15% (vol/vol) horse serum (Gibco), 2.5% (vol/vol) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mg/mL zeocin (InvivoGen), and 0.1 mg/mL G418 (Nacalai). Neuro2A inducibly expressing Htt exon1 with polyQ (eHtt16Q– or eHtt150Q–EGFP) was cultured in DMEM containing 10% (vol/vol) heat-inactivated FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin, 0.4 mg/mL zeocin, and 0.2 mg/mL G418 (Nacalai) and routinely differentiated by treatment with 5 mM dbc AMP (Nacalai) (1). Expression of eHtt16Q– or eHtt150Q–EGFP was induced by adding 1 μM ponasterone A (pon A, Invitrogen) to the culture medium. All cells were maintained in 5% (vol/vol) $CO_2$ at 37 °C.

**Chemical Cross-Linking of $IP_3R1$.** Chemical cross-linking between $IP_3$ receptor type 1 ($IP_3R1$) subunits was initiated by incubation of the P2/P3 fractions (1.5 mg/mL) in a reaction buffer containing 25 mM Hepes–NaOH, pH 7.5, 75 mM NaCl, 1 mM TCEP, 0.5 mM EDTA, and BS. SDS sample buffer was added, and the samples were incubated for 30 min on ice to terminate the reaction; the resulting samples were subjected to agarose gel electrophoresis (AGE) analysis. The membranes of the P2/P3 fractions were solubilized in 1% (wt/vol) detergents and then centrifuged at 20,000 × *g* for 15 min at 4 °C. A solution of 500 μM BS was added to the solubilized fraction, followed by incubation on ice for 30 min.

**Fractionation of Endogenous Cross-Linking Activity.** The supernatant fraction obtained after ultracentrifugation of the homogenates was applied onto an anion exchanger column (TSKgel BioAssist Q, 50 mm × 4.6 mm i.d., Tosoh) that was pre-equilibrated with 10 mM Tris buffer (pH 7.5) containing 50 mM NaCl. The fractions containing cross-linking activity were eluted using a linear gradient from 50 to 750 mM NaCl at a flow rate of 1 mL/min. A total of 20–30 fractions (1 mL each) were collected to estimate cross-linking activity.

**Analysis of $IP_3R1$ Cross-Linking in SH-SY5Y and U87-MG Cells.** Human SH-SY5Y or U87-MG cells harvested with scrapers from 3.5-cm dishes were heated at 55 °C for 15 min, followed by sonication twice for 5 s each. The resulting samples were subjected to AGE, and human $IP_3R1$ was detected with 4C11.

**Immunocytochemistry.** We plated cells on sterilized coverslips and transfected siRNA or expression vectors. The cells were fixed in 3% (wt/vol) paraformaldehyde for more than 15 min and were then treated with 50 mM $NH_4Cl$ in PBS for 10 min. After washing the cells twice, we permeabilized them by incubation with 50 μg/mL digitonin in 0.1% gelatin–PBS for 10 min, as described previously (2). The cells were then incubated with α-LC3 (1:1,000, MBL) and α-LAMP1 (1:100, Santa Cruz Biotechnology, H4A3) for 1 h. After washing three times, we incubated the cells with AlexaFluor488–IgG and/or AlexaFluor594–IgG (1:1,000) for 1 h. After washing, we observed fluorescence signals under an FV1000 (Olympus).

**Limited Proteolysis.** Before digestion by calpain or Lep, the cerebellar membranes or TG2-treated membranes were washed with cross-linking buffer three times by centrifugation at 20,000 × *g* followed by resuspension. Digestion was initiated by incubation of the washed membrane with various concentrations of calpain or Lep at 37 °C. After the addition of 1/50th the volume of 100 mM $CaCl_2$ to the mixture, digestion by calpain was initiated.

**Analysis of Peptides and Isopeptides.** HPLC was performed on an HP1100 system (Agilent Technologies) equipped with a photodiode array UV detector. The resulting reaction mixture was injected onto a $C_8$ column (Superoctyl TSKgel, 100 mm × 2.0 mm i.d., 2-μm resin, Tosoh) using a 10- or 200-μL loop. The peptide was eluted using a linear gradient from 10% (vol/vol) to 50% (vol/vol) acetonitrile containing 0.1% TFA for 15 min at a flow rate of 200 μL/min.

**Protein Assay.** The protein concentrations of the P2/P3 fractions, cytosol fractions, or purified enzymes were measured in a U-0080D photodiode array spectrophotometer (Hitachi) according to the Bradford method using BSA as a standard.

**Inactivation of TG2 by NEM or Boiling.** We inactivated TG2 as described previously (3). TG2 was incubated with 5 mM *N*-ethylmaleimide (NEM) in 20 mM Hepes–NaOH (pH 7.5) for 30 min at room temperature. The reaction was terminated by incubation with 5 mM DTT for 30 min at room temperature. The resulting mixture was successively passed through two spin columns (Bio-Rad) to remove free NEM. Inactive TG2 was also prepared by boiling for 5 min in 20 mM Hepes–NaOH (pH 7.5).

**Measurement of TG2 Activity.** TG2 activity was measured in an F-2500 fluorescence spectrometer (Hitachi) by monitoring the velocity of

the florescence increase during TG2-catalyzed incorporation of monodancyl–cadaverine into *N, N*-dimethylcasein (4).

**Measurement of TG2 Expression Level.** SH-SY5Y and HeLa cells harvested from 3.5-cm, 6-cm dishes, or 12-well plates were boiled for 3 min or heated at 55 °C for 15 min in SDS sample buffer and subjected to SDS/PAGE analysis. TG2s were detected using αTG2 antibody (CUB 7402). Quantification of TG2 was performed by densitometric analysis of Western blots (WBs) using Image J software (National Institutes of Health).

**Analysis of Autophagosome Dynamics by Fluorescence Microscopy.** To track the fluorescent vesicles, time-lapse images were obtained on the FV1000D at 5-s intervals for 2.5 min using a 60× oil-immersion objective lens; the images were analyzed with the Image J software. To obtain color-coded time-lapse images, we used the Z-code stack plug-in and Z-project on Image J.

**In Vitro Transamidation of $IP_3R1$ by TG2.** The P2/P3 fraction was diluted with a transamidation buffer (pH 7.5) containing 50 mM Hepes–NaOH, 150 mM NaCl, 1 mM EGTA, and 2 mM TCEP. Transamidation by TG2 was performed by incubating the reaction mixture containing 1 mg/mL protein of cerebellar membrane, 2 mM $CaCl_2$, and various concentrations of TG2 for 30 min at 37 °C. Transamidated $IP_3R$ was detected using 4C11 unless stated otherwise.

**In Vitro Deamidation of $IP_3R1$ by TG2.** The P2/P3 fraction (1 mg/mL) from cerebella was incubated with TG2 (0, 1, 3, 10, 30, and 100 ng/mL) at 37 °C for 90 min in Hepes buffer containing 50 mM Hepes–NaOH, 150 mM NaCl, 1 mM EGTA, 3 mM $CaCl_2$, and 2 mM TCEP.

**Transfection of Plasmids.** The plasmids for EGFP–$IP_3R1$, EGFP–Q2746E, Htt23Q–myc, Htt145Q–myc, Htt exon1 including 18 polyglutamines (eHtt18Q–EGFP), eHtt60Q–EGFP, and eHtt150Q–EGFP were used to transfect COS-7, HeLa, or Neuro2a cells using FuGENE (Promega). Primary cultured rat striatal neurons were transfected with eHtt18Q–EGFP or eHtt150Q–EGFP using Lipofectamine LTX (Invitrogen).

**Measurement of Intracellular $Ca^{2+}$ in PC12 and Neuro2a Cells.** PC12 cells were seeded in poly-L-lysine–coated 3.5-cm glass-bottom dishes and cultured for 1 d in 2 mL of DMEM containing 15% (vol/vol) horse serum, 2.5% (vol/vol) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mg/mL zeocin, and 0.1 mg/mL G418 (Nacalai). To induce the full-length Htt mutant in the PC12 cells, we added 10 μM pon A to the medium. Neuro2a cells were seeded in 3.5-cm glass-bottom dishes and cultivated for 1 d in 2 mL of DMEM containing 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and then transfected with Htt23Q–myc, Htt145Q–myc, eHtt18Q–EGFP, or eHtt150Q–EGFP using FuGENE. The 2 d after induction or transfection, we loaded fura-2AM into Neuro2a or PC12 cells by incubation with balanced salt solution (BSS) containing 5 μM fura-2AM for 30 min at room temperature and evaluated intracellular concentration of $Ca^{2+}$ ($[Ca^{2+}]i$) using AQUACOSMOS as described previously (5). To monitor $Ca^{2+}$ release from the endoplasmic reticulum (ER), Neuro2a or PC12 cells were perfused with $Ca^{2+}$-free BSS for 2 min immediately before $[Ca^{2+}]i$ measurements. Induced expression of mutant Htt in PC12 cells was confirmed by fluorescence of RFP fused with carboxyl-terminus of Htt and also verified by WB using αhtt (clone EP867Y, Abcam). After $Ca^{2+}$ imaging, we particularly fixed Neuro2a cells with 3% (wt/vol) paraformaldehyde and immunostained them with αmyc (clone 9E10, Santa Cruz) to measure $[Ca^{2+}]i$ in only Neuro2a cells expressing Htt23Q–myc or Htt145Q–myc. The regions of interest (ROIs) were put within the αmyc-stained Neuro2a cells, and in the case of PC12 cells, ROIs were selected within the sites that responded to agonist stimuli in the presence of extracellular $Ca^{2+}$. The primary cultures of striatal neurons were prepared and subjected to $Ca^{2+}$ imaging as described previously (6). For eHtt18Q–EGFP, eHtt60Q–EGFP, or eHtt150Q–EGFP overexpression, we measured $[Ca^{2+}]i$ in GFP-positive Neuro2a cells or primary culture neurons.

**Identification of BP-Incorporated $IP_3R1$ in HeLa and SH-SY5Y Cells.** SH-SY5Y cells were trypsinized, evenly suspended, and then counted using trypan blue staining. The SH-SY5Y cells were then seeded at a density of $0.3 \times 10^6$ in 10-cm dishes and cultivated for 6 additional days in culture medium containing 20 μM RA or DMSO, which was replaced every 2 d. We also seeded HeLa cells in 10-cm dishes. We added 2 mM of BP to the medium 24 h before harvesting with cell scrapers. The cells were centrifuged and washed with PBS by centrifugation. The resulting cell precipitate was rapidly frozen in liquid nitrogen and stored at –80 °C. Frozen cells in Eppendorf tubes were treated with 0.5 mL Hepes buffer containing 1% Triton X-100 (HE-T), suspended, and rotated for 30 min at 4 °C. After centrifugation for 15 min at 20,000 × *g* at 4 °C in a MX-300 centrifuge (Tomy), the supernatant was mixed with pep6-Ab (7) bound to Protein A–Sepharose 4B and incubated for 2 h at 4 °C. The Sepharose beads were washed with 0.5 mL HE-T buffer three times and were then subjected to SDS/PAGE. Immunoprecipitated $IP_3R1$ was separated on a 5% (wt/vol) gel, transferred to a PVDF membrane, and incubated with avidin–HRP (1:2,000 dilution, GE Healthcare). The biotin–avidin complex was detected using ECL kits.


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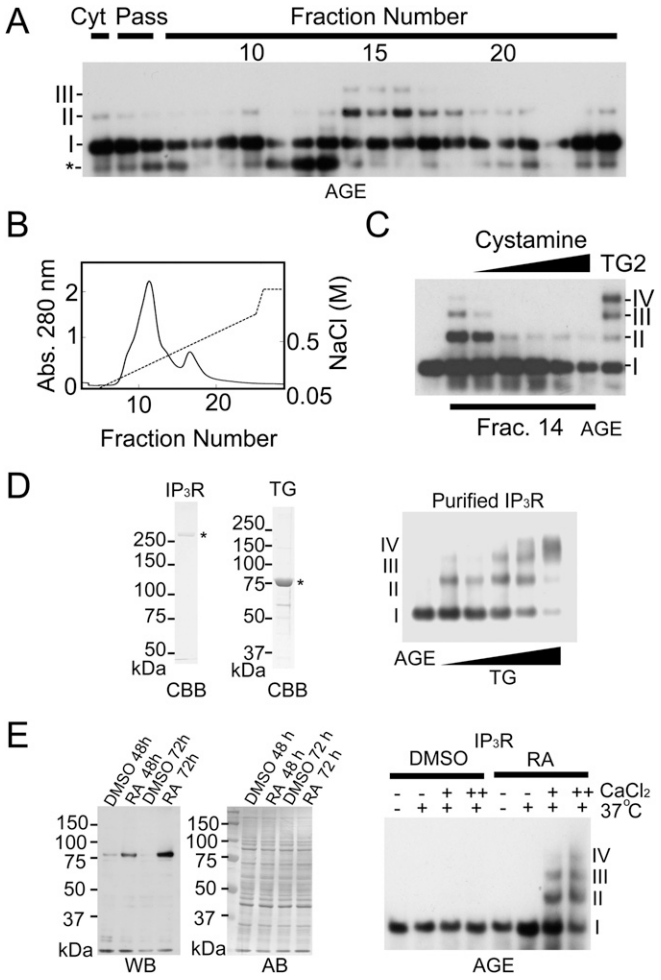



**Fig. S1.** TG2 covalently modifies $IP_3R1$. (*A*) The cross-linked subunits (*I–IV*) on AGE were judged by thyroglobulin monomer (335 kDa) and dimer (670 kDa). Each eluate, cytosol (Cyt), or pass-through (pass) on an anion exchanger chromatography was incubated with cerebellar P2/P3 membranes. An asterisk is a fragment of $IP_3R1$ cleaved by $Ca^{2+}$-dependent enzymes. (*B*) Chromatogram of brain lysates on an anion exchanger column. (*C*) A peak fraction (no. 14) on the anion exchange chromatography or purified TG2 was incubated with cerebellar P2/P3 fractions in the absence or presence of cystamine (2, 4, 10, 20, and 40 mM). (*D*) Coomassie brilliant blue (CBB) staining shows purified $IP_3R1$ or TG2 (asterisk). The AGE gel shows that purified TG2 directly cross-links $IP_3R1$. (*E*) Cross-linking activity of HeLa cells pretreated with DMSO or RA. WBs with αTG2 and amido black (AB) stains show cellular levels of TG2 and total proteins, respectively. Shown in *Right* is the result of $Ca^{2+}$-dependent cross-linking activity using AGE.

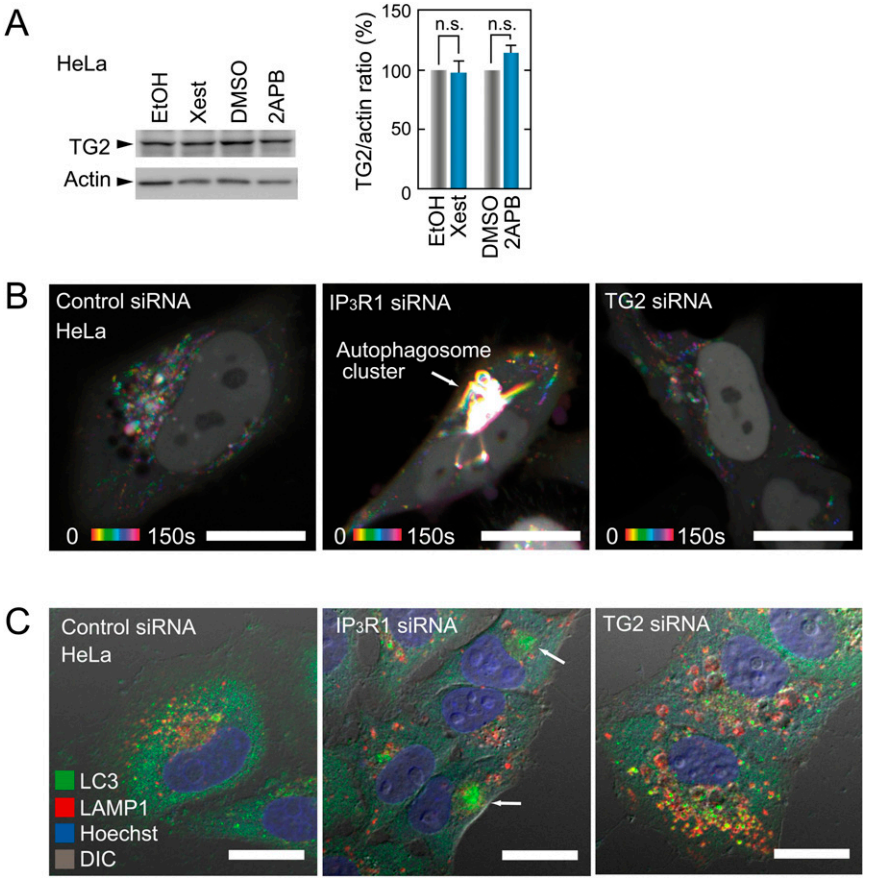



**Fig. S2.** TG2 negatively regulates $IP_3R1$-mediated autophagy processes. (*A*) Expression levels of TG2 in HeLa cells were evaluated by Western blotting with αTG2 and αactin. We added 1 μL of ethanol (EtOH), 2 mM xest in EtOH, DMSO, or 100 mM 2-APB in DMSO to 1 mL of culture medium and cultivated HeLa cells for an additional 42 h under the same condition as Fig. 3*A*. Left gels show representative results of Western blotting, and a bar graph summarizes the mean + SEM of TG2 contents in HeLa cells from three culture wells. Effects of these inhibitors were not statistically significant (n.s.). $P = 0.78$ (xest), $P = 0.27$ (2-APB). (*B*) Autophagosome dynamics upon $IP_3R1$ or TG2 knockdown. Color-coded time-lapse images indicate that loss of $IP_3R1$ results in large autophagosome clusters (white arrow). (*C*) Endogenous autophagosomes and lysosomes in HeLa cells transfected with control, $IP_3R1$, or TG2 siRNA. White arrows indicate the clustered autophagosomes in the image merged with LC3 (green), LAMP1 (red), nuclear stain (blue), and transmitted light DIC (gray).

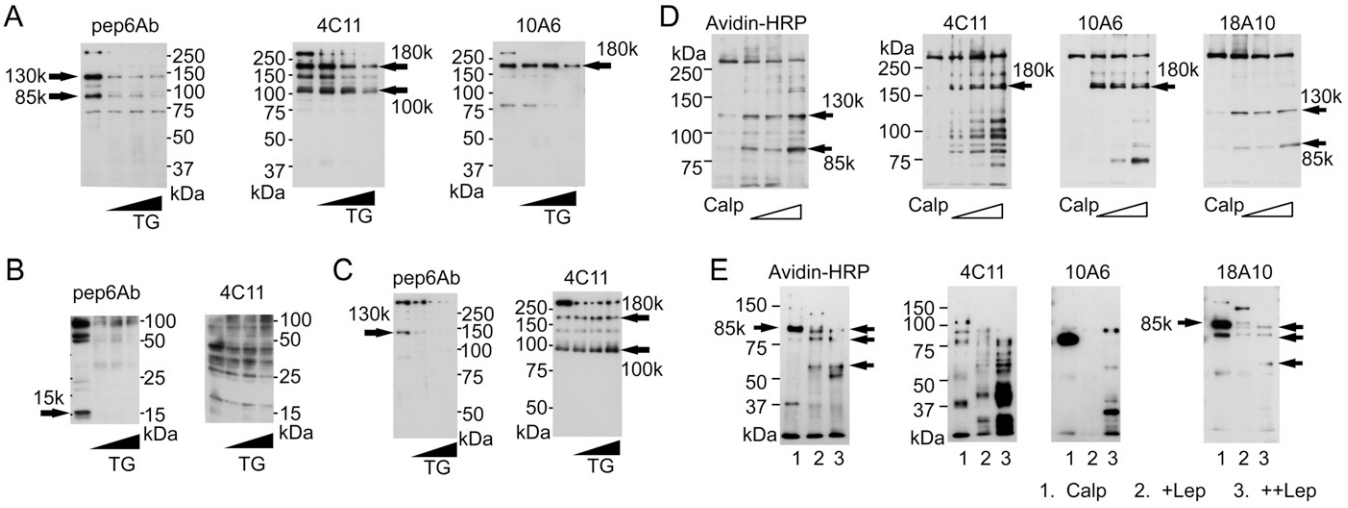
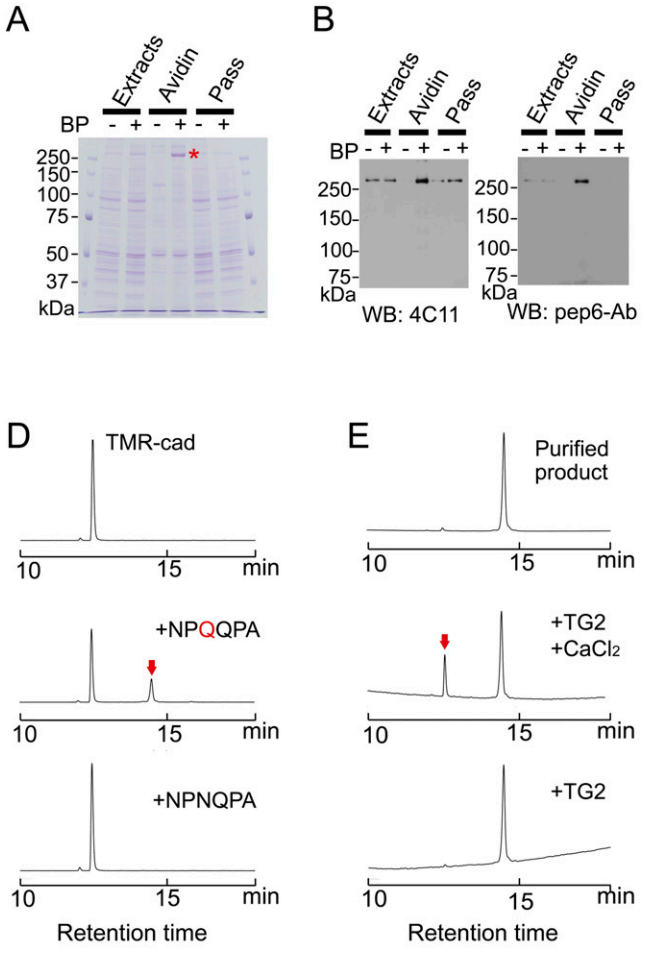



**Fig. S3.** Cross-linking sites were localized in C-terminal fragments generated by calpain or Lep digestion. The cerebellar $IP_3R1$ was cross-linked by TG2 (0, 5, 10, and 20 μg/mL) and then digested by 50 μg/mL Calp (*A*) or 1 μg/mL Lep (*B*). In *C*, the cerebellar $IP_3R1$ was digested by 50 μg/mL Calp and then cross-linked by TG2 (0, 5, 10, and 20 μg/mL). Purified $IP_3R1$ that was incorporated with BP was digested by various concentrations of calpain (Calp) in *D* and *E* or lysyl-endopeptidase (Lep) in *E*. Resultant fragments were detected with avidin-HRP, 4C11, 10A6, or 18A10. Arrows indicate major fragments.




C

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 151 LDEAGNEGSW FYIQPFYKLR SIGDSVVIGD KVVLNPVNAG QPLHASSHQL
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 301 SLFRFKHLAT GHYLAAEVDP DFEEECLEFQ PSVDPDQDAS RSRLRNAQEK
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 601 NNRKLLEKHI TAAEIDTFVS LVRKNREPRF LDYLSDLCVS MNKSIPVTQE
 651 LICKAVLNPT NADILIETKL VLSRFEFEGV STGENALEAG EDEEEVWLFW
 701 RDSNKEIRSK SVRELAQDAK EGQKEDRDIL SYYRYQLNLF ARMCLDRQYL
 751 AINEISGQLD VDLILRCMSD ENLPYDLRAS FCRLMLHMHV DRDPQEQVTP
 801 VKYARLWSEI PSEIAIDDYD SSGTSKDEIK ERFAQTMEFV EEYLRDVVCQ
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1001 FDESNSQSSE TSSGNSSQEG PSNVPGALDF EHIEEQAEGI FGGRKENTPL
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1101 QLLVTSQDVD NYKQIKQDLD QLRSIVEKSE LWVYKGQGPD EPMDGASGEN
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2001 ETLQFLDCIC GSTTGGLGLL GLYINEKNVA LINQTLESLT EYCQGPCHEN
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**Fig. S4.** TG2 modifies the C terminus of $IP_3R1$. (*A*) Pull-down of TG2 substrates from the mouse cerebellum. The asterisk indicates a major band stained with CBB, which was identified as $IP_3R1$ by mass spectrometry and WB in *B*. (*C*) Identification of $IP_3R1$ and its biotinylated site using mass spectrometry. Peptides, identified as mouse $IP_3R1$, are shown in blue, and a biotinylated peptide is in red bold. The shown data are a representative result from two independent experiments that consistently revealed that the C-terminal peptide consisting of 18 amino acids (in red) was exclusively modified by TG2. Sequence coverage was reproducibly 45–48%. (*D*) Gln2746 is sufficient for isopeptide formation and hydrolysis by TG2. The panels show the peaks of rhodamine-labeled cadaverine (TMR-cad) and a product of the TG2-catalyzed reaction of TMR-cad and the NPQQPA peptide (red arrow). (*E*) *Top* shows the purified product, and *Middle* shows a hydrolyzed product (red arrow) by TG2 and $CaCl_2$.

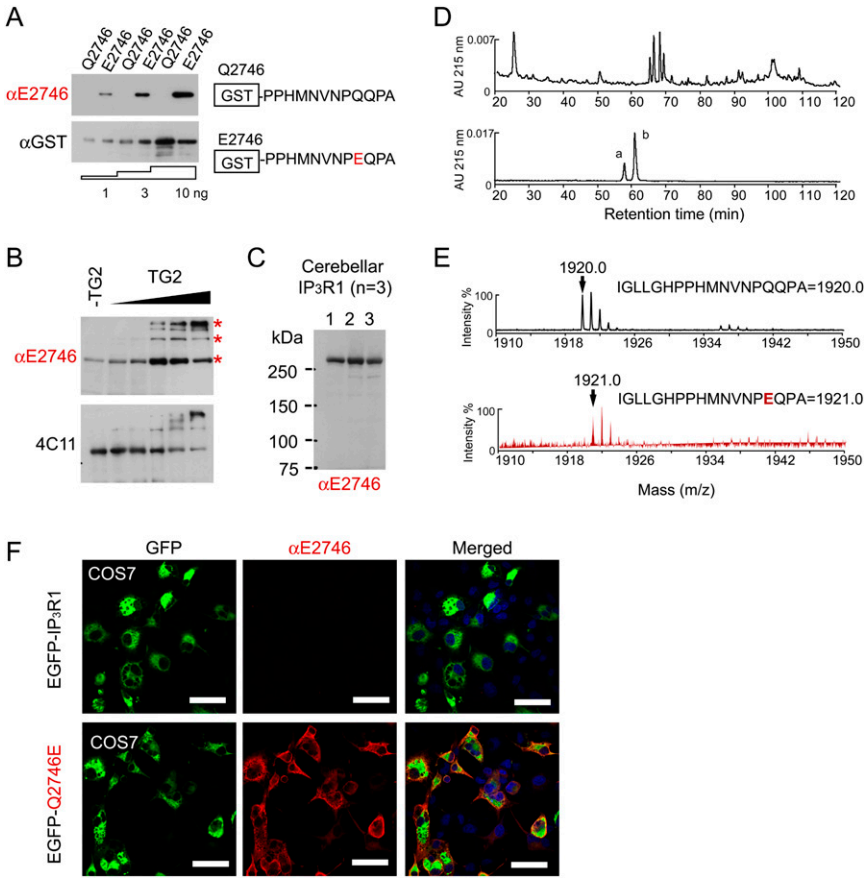


**Fig. S5.** Specificity of our developed antibody that recognizes Gln2746 modification. (*A*) The scheme indicates GST fusions of the control (Q2746) or a mutant with Gln2746 to glutamate substitution (E2746). The blotted Q2746 or E2746 proteins were detected with αE2746 and αGST antibodies. (*B*) Enzymatic deamidation of Q2746 in vitro. Mouse cerebellar $IP_3R1$ was pretreated with TG2 for 90 min and detected using αE2746 and 4C11. (*C*) Deamidated $IP_3R1$ in brain samples. Gel showing the results obtained from three mice (lanes 1–3). (*D*) Separation of the deamidated peptide by reversed-phase chromatography. *Upper* and *Lower* show chromatograms of trypsin-digested $IP_3R1$ and the synthetic control (peak a) or the deamidated peptide (peak b), respectively. (*E*) Identification of deamidated $IP_3R1$ by mass spectrometry. *Upper* and *Lower* represent the mass spectra of nonmodified (black line) and deamidated (red line) peptides, respectively, of $IP_3R1$ purified from mouse brains. Note that a 1.0-Da shift in mass spectrum is clearly detected. (*F*) EGFP–$IP_3R1$- or EGFP–Q2746E-expressing COS-7 cells were fixed and stained with αE2746 antibody and Heochst 33342. Fluorescence of EGFP, αE2746/αrabbit Alexa594, and Heochst 33342 was presented in green, red, and blue, respectively. Bars in rows indicate 50 μm. The EGFP–$IP_3R1$ or EGFP–Q2746E plasmids were transfected to COS-7 cells using FuGENE (Promega).

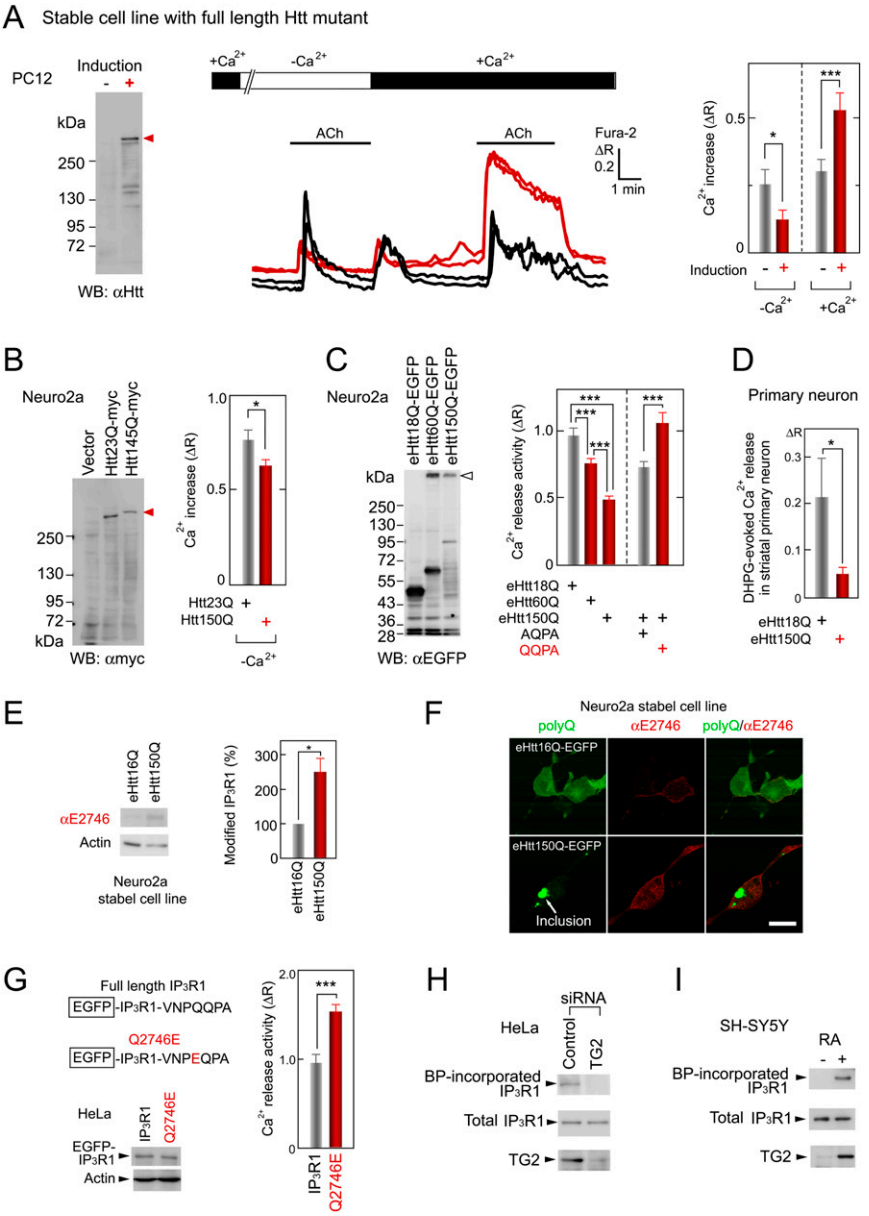


**Fig. S6.** Functional and structural modifications of $IP_3R$ in living cells. (*A*) A left gel shows the mutant Htt expression in PC12 cells (red arrow head), as detected by WB with the αHtt. We added EtOH instead of pon A to control cells. The representative traces of the fura-2 ratio are shown in black (control) and red (induction). Bar chart summarizes the mean + SEM of ΔR peak amplitudes in response to 100 μM ACh in nominally $Ca^{2+}$-free BSS (no induction, $n = 21$; induction, $n = 17$) or 2 mM $Ca^{2+}$ BSS (no induction, $n = 15$; induction, $n = 14$). The peak amplitude (ΔR) was calculated by subtracting an average of the fura-2 ratio for 1 min before ACh stimulation from a peak of the fura-2 ratio during the stimulation. $^*P = 0.045$, $^{***}P = 0.0040$. (*B*) The left gel shows Htt proteins in Neuro2a cells transfected with a vector (pcDNA3.1zeo), full-length Htt23Q–, or Htt145Q–myc. Bradykinin (BK)-evoked $Ca^{2+}$ release in Neuro2a cells transiently expressing full-length Htt23Q– or Htt145Q–myc. The bar charts represent the mean + SEM of peak amplitudes in response to 100 nM BK under a $Ca^{2+}$-free condition (23Q, $n = 68$; 145Q, $n = 68$). The peak amplitude (ΔR) was calculated by subtraction of an average of the fura-2 ratio before ACh stimulation from a peak of the fura-2 ratio during the stimulation. $^*P = 0.022$. (*C*) The transiently expressing Htt exon1 including polyglutamines, eHtt18Q–, eHtt60Q–, or eHtt150Q–EGFP in Neuro2a cells were confirmed by WB with αEGFP. An arrowhead indicates the gel top. The bar charts represent the mean + SEM of peak amplitudes (18Q, 60Q, 150Q, $n = 105$; AQPA, QQPA, $n = 60$). $^{***}P = 0.0045$ (18Q vs. 60Q), $^{***}P = 1.3 \times 10^{-12}$ (18Q vs. 150Q), $^{***}P = 6 \times 10^{-8}$ (60Q vs. 150Q), $^{***}P = 0.00044$ (AQPA vs. QQPA). *P* values after multiple comparisons were corrected by the Bonferroni method. As DsRed was fused with AQPA or QQPA, in the case of coexpression with eHtt150Q–EGFP, $Ca^{2+}$ release was observed in Neuro2a cells colabeled with EGFP and dsRed fluorescence. (*D*) Neurons were confirmed by responses to depolarization with 60 mM KCl. The bar chart represents the mean + SEM of $Ca^{2+}$ release induced by 100 μM DHPG in striatal primary neurons expressing eHtt18Q–EGFP ($n = 6$) or eHtt150–EGFP ($n = 11$). $^*P = 0.018$. The bar charts represent the mean + SEM of peak amplitudes. (*E*) Modification of $IP_3R1$ in Neuro2a stable cell lines. Endogenous $IP_3R1$ in control (eHtt16Q induced by pon A) or HD model Neuro2a cells (eHtt150Q induced by pon A) was detected with the αE2746 antibody. Bar charts summarize the means + SEM of $IP_3R1$ modification that are normalized by β-actin contents. $^*P = 0.022$ ($n = 3$). (*F*) Effects of inducible eHtt16Q and eHtt150Q on Gln2746 in Neuro2a cells. Cells were transfected with the HA-tagged C terminus (100 amino acids) of $IP_3R1$ to enhance αE2746 signals. (*G*) A scheme shows the wild-type and the Q2746E mutant, and their expression levels were confirmed by WB. Fura-2 ratio images of living HeLa cells expressing EGFP–$IP_3R1$ or EGFP–Q2746E mutant were obtained under nominally $Ca^{2+}$-free conditions. Bar chart summarizes the mean + SEM of ΔR peak amplitudes in response to 3 μM ATP (control $IP_3R1$, $n = 15$; Q2746E, $n = 22$). $^{***}P = 0.00036$. Total and BP-incorporated $IP_3R1$s in HeLa (*H*) or SH-SY5Y (*I*) cells are detected by 4C11 and avidin–HRP, respectively. (*I*) *Top* shows a marked incorporation of BP within $IP_3R1$ in SH-SY5Y cells differentiated by RA or not. *Middle* and *Bottom* show the amount of $IP_3R1$ and TG2. Each lane was loaded with 3 μg of protein.