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Polyglutamine expansion disturbs the endoplasmic reticulum formation, leading to caspase-7 activation through Bax

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ABSTRACT

The endoplasmic reticulum (ER) plays a pivotal role in cellular functions such as the ER stress response. However, the effect of the ER membrane on caspase activation remains unclear. This study reveals that polyglutamine oligomers augmented at ER induce insertion of Bax into the ER membrane, thereby activating caspase-7. In line with the role of ER in cell death induced by polyglutamine expansion, the ER membrane was found to be disrupted and dilated in the brain of a murine model of Huntington's disease. We can conclude that polyglutamine expansion may drive caspase-7 activation by disrupting the ER membrane.

1. Introduction

Apoptosis is necessary for development and tissue homeostasis, but its dysfunction can initiate disorders such as cancer and neurodegenerative diseases [1]. Apoptosis is mediated by caspases, which are activated by signals from the plasma membrane and mitochondria. Caspase-9 activity is regulated by the apoptosome that contains Apaf-1 and cytochrome c, whose release from mitochondria is controlled by members of the Bcl-2 family [2]. Research reveals that the endoplasmic reticulum (ER) is a site that initiates caspase activation [3,4], as demonstrated in the experiment where translocation of cell death abnormal (CED)-4 protein to the nuclear envelope was shown to be crucial for CED-3 activation in the nematode worm *Caenorhabditis elegans* [5]. Caspase-7 (gene symbol *CASP7*) is an effector caspase, whose activated form is observed in the microsomal fraction after apoptotic stimuli (incidentally, an inactivating mutation of *CASP7* is found in human cancers) [6,7].

Polyglutamine (polyQ) expansion is a mutation that increases the length of a polyglutamine sequence in a normal protein. The unusual length of polyQ causes conformational changes in the protein, and consequently pathological changes within the cell. PolyQ expansion is observed in some diseases, such as Huntington's disease (HD). PolyQ expansion may be caused by several aberrations in axonal transport, the ubiquitin–proteasome system, or transcription [8]. These anomalies cause neuronal cell death through caspase activation initiated via aggregation of the polyQ-containing protein [9] and via p53-mediated mitochondrial mechanism of apoptosis (cytochrome c release) [10]. An HD-related protein, huntingtin (gene symbol *HTT*), contains a short sequence targeting it to ER [11]. A study on laboratory animals revealed that concanavalin A immunoreactivity of ER membranes is increased *in vitro* in striatal neurons that express mutant Htt^{Q111} [12]. At present, the role of the ER membrane in caspase activation by polyglutamine remains obscure.

In this study, we tested whether polyglutamine expansion affects conformation of the ER membrane and its ability to regulate caspase-7 activity.

2. Materials and methods

2.1. Materials

We purchased the following antibodies and chemicals: anti-FLAG (M2) and anti-α-tubulin antibodies were from Sigma Aldrich (St. Louis, MO); thapsigargin was from Sigma Aldrich and Research Biochemicals International (Natick, MA); anti-BiP (grp78) antibody was from Stressgen (Victoria, BC, Canada); anti-calreticulin

Abbreviations: Apaf-1, Apoptotic protease activating factor 1; cb5, cytochrome b5; polyQ79, polyQ82, polyglutamine repeats containing proteins with 79 or 82 glutamine residues; xbp-1, x-box binding protein 1; FRET, fluorescence resonance energy transfer.

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antibody was from Abcam (Cambridge, MA); anti-cleaved caspase-7 and anti-lamin A/C antibodies were from Cell Signaling Technology (Beverly, MA); anti-huntingtin (mEM48) antibody was from Chemicon (Temecula, CA); anti-Bax antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-SRP54 antibody was from Proteintech Group (Chicago, IL); anti-HA (HA.11) antibody was from COVANCE (Richmond, CA); HRP-conjugated anti-rat, anti-mouse, and anti-rabbit IgG (H + L) antibodies were from Southern Biotech (Birmingham, AL); and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (H + L) and Cy3-conjugated anti-chicken IgY antibodies were from Jackson ImmunoResearch (West Grove, PA). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemical (Tokyo, Japan), and Sigma Aldrich. The R6/2 HD exon 1 transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

2.2. DNA constructs

The transmembrane domain of cb5 (amino acids 100–134, AF007108), which contains a static ER retention signal [13], was fused to murine Bcl-xL lacking its C-terminal region (amino acids 1–211, L35049) to generate ER-targeted Bcl-xL (ER-Bcl-xL). ER-Bcl-xL tagged with FLAG was subcloned into the pBabe-Puro retroviral vector. ER-targeted Bax (ER-Bax) was generated by fusing human Bax lacking its C-terminal region (amino acids 1–171, L22473) to cb5, which had been subcloned into the pcDNA6/myc-His (Invitrogen, Carlsbad, CA) or pEGFP-N3 (Clontech, Mountain View, CA) vector. ER-polyQ82 was generated by fusing polyQ82 to cb5, which had been fused to mRFP. polyQ82 was subcloned into the pEYFP-N1 and pECFP-C1 vectors (Clontech) for the FRET assay. The vector pmRFP-Mito was generated by substituting mRFP for EYFP in pEYFP-Mito (Clontech). The polypeptide pQ79-APER is a chimeric type I transmembrane protein that contains the N-terminal 79 repeat polyglutamine (pQ79), the transmembrane region of the amyloid precursor protein (APP), YFP, a nuclear localization signal (NLS), and the ER retrieval motif of p28Bap31 at the cytosolic side (APER). APER, which lacks the pQ79 part of pQ79-APER, was used as a control. Seventy-nine-CAG repeat was inserted within the 3′ untranslated region of the pEGFP vector to generate a CAG repeat RNA expression plasmid, named GFPstopCAG80.

2.3. Reverse transcription PCR (RT-PCR)

RT-PCR was performed according to a previously described procedure [14]. PCR of xbp-1 and β-actin was performed for 25 cycles. Splicing of xbp1 mRNA was detected by electrophoresis using a 3% agarose 21 gel (Wako). The following PCR primer pairs were used: rat xbp-1, 5′-GCTTGTGATTGAGAACCAGG-3′ and 5′-GAGGCTTGGTGTATACATGG-3′ and β-actin, 5′-GTTTGAGACCTTCAACACC-3′ and 5′-GTGGTGGTGAAGCTGTAG-3′.

To detect mRNA of transfected plasmids, total RNA was purified from HEK293T cells after transfection with GFPstopCAG80 or GFP-GST and ER-mRFP, and then treated with RNase-free DNase (1 U; Promega, Madison, WI) for 30 min at 37 °C. We synthesized cDNA by reverse transcription using an oligo$(dT)_{20}$ primer. The following primer pair was used in the PCR to amplify GST and CAG-repeat cDNAs (GST cDNA from the GFP-GST plasmid; 80 CAG-repeat cDNA from the GFPstopCAG80 plasmid): 5′-TGCCCGACAACCACTACCTGAG-3′ and 5′-TAAAGCAAGTAAAACCTCTAC-3′.

2.4. Cell culture and induction of cell death

Induction of cell death was performed as described previously [3]. To generate PC12 cells stably expressing ER-Bcl-xL, the cells were infected with the viral supernatant generated by transfection of the pBabe-Puro vector (control) and ER-Bcl-xL/pBabe-Puro vector (ER-Bcl-xL) in the presence of 8 μg/ml polybrene (Sigma). Infected cells were selected via growth in the presence of 1.5 μg/ml puromycin (Sigma). Time lapse analysis was performed 6 h after transfection with polyQ82-GFP and ER-mRFP using an inverted fluorescence microscope (Axio Observer.Z1 with AxioCaMRm and PM-S1 incubator system; Carl Zeiss, Oberkochen, Germany). HEK293 Tet-Off Advanced Cell Line (Clontech) was transfected with 18 μg of pTRE-Tight-HA-polyQ82-GFP and 0.9 μg of pTK-Hyg by the calcium phosphate method. Selection of stable cell clones was initiated 50 h after the transfection using hygromycin B (250 μg/ml; Wako). For induction of HA-polyQ82-GFP, the cells were washed twice with HBSS (−), and then incubated in doxycycline free medium.

2.5. Immunostaining

Immunocytochemical and immunohistochemical analyses were performed as described previously [15]. PolyQ82-GFP and ER-mRFP were cotransfected into HEK293T cells and rat primary hippocampal neurons using a Lipofectamine 2000 and by the calcium phosphate DNA precipitation method, respectively. Mouse brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) followed by incubation in 15% sucrose. Sections (14 μm) were incubated with mEM48 and anticalreticulin antibodies, followed by FITC-conjugated anti-mouse or Cy3-conjugated anti-chicken immunoglobulin antibodies. Samples were examined under an inverted fluorescence microscope (Axiovert 200; Carl Zeiss) or a confocal microscope (LSM510; Carl Zeiss).

2.6. Western blotting

Western blotting was performed as described previously [14]. Quantification of signals was performed using a LAS-4000 (Fujifilm).

2.7. Transmission electron microscopy

Mouse brains (control and R6/2 mice) were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After postfixation with 1% osmium, samples were embedded in Epon-812. Ultrathin sections were obtained with glass knives on a Porter Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Norwalk, CT) and collected onto copper mesh grids. The sections were analyzed using transmission electron microscopy (Hitachi H-800) after staining with 0.1% uranyl acetate in sodium acetate buffer and lead salts.

To combine fluorescence and electron microscopy, polyQ82-GFP-transfected HEK293T cells, which were cultured on coverslips with grids, were fixed in 1.5% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min. After examination using confocal microscopy, the cells were fixed with 1% osmium and 1.5% potassium ferrocyanide for 50 min on ice, dehydrated, and the embedded in Epon-812.

2.8. The FRET assay

HEK293T cells were sonicated in cold PBS (−) supplemented with protease inhibitors 24 h after transfection with CFP-polyQ82, CFP-YFP fusion, or CFP-polyQ82 plus polyQ82-YFP plasmids by the calcium phosphate DNA precipitation method. Supernatants were separated by centrifugation. Congo red (100 μM) was added to the supernatant 6 h after the transfection. The reported FRET value is the ratio of emission at 538 nm to emission at 485 nm after excitation at 440 nm on a fluorometer (Fluoroskan Ascent).

2.9. Alkaline treatment

The microsomal fraction was harvested and treated with 100 mM sodium carbonate pH 11.5 at 0 °C for 30 min, and

centrifuged at 105,000*g* for 1 h. The pellets were dissolved in SDS sample buffer containing 8 M urea and subjected to SDS–PAGE.

2.10. Statistical analysis

We used the KaleidaGraph software (Synergy Software) to determine the statistical significance of differences by means of unpaired, 2-tailed Student's *t* tests. The data were calculated as mean ± SEM from 3 independent experiments.

3. Results

3.1. Disruption of the ER membrane is exacerbated during the progressive stage of a mouse model of Huntington's disease

To identify the subcellular site of caspase-7 activation, we examined the localization of caspase-7 by Western blotting of 2subcellular fractions of PC12 cells (Fig. S1A). In line with the previous findings [7], caspase-7 was detected in the membranous and cytosolic fractions. Using a previously validated ER-targeted expression construct [16] of Bcl-xL (designated ER-Bcl-xL), we found that thapsigargin-, but not serum deprivation-, induced cell death and caspase-7 activation in PC12 cells were prevented by stable expression of ER-Bcl-xL (Figs. S1B and S1C). These results suggested that caspase-7 activation was regulated by the ER.

Caspase-7 activation was observed (Fig. 1A) and polyglutamine was colocalized with an ER-localizing protein, calreticulin (Fig. 1B) in R6/2 HD exon 1 transgenic mice [17]. Therefore, we first analyzed morphology of the ER membrane in a model of polyglutamine expansion disease using transmission electron microscopy (TEM; Fig. 1C–E). Rough ER luminal space in the neurons of wild-type mice was 31.2 ± 0.9 nm wide (Fig. 1C, $n = 132$ rough ER), whereas that in the neurons of the R6/2 mice was 56.3 ± 2.2 nm wide (Fig. 1C, $n = 118$ rough ER). This difference was significant

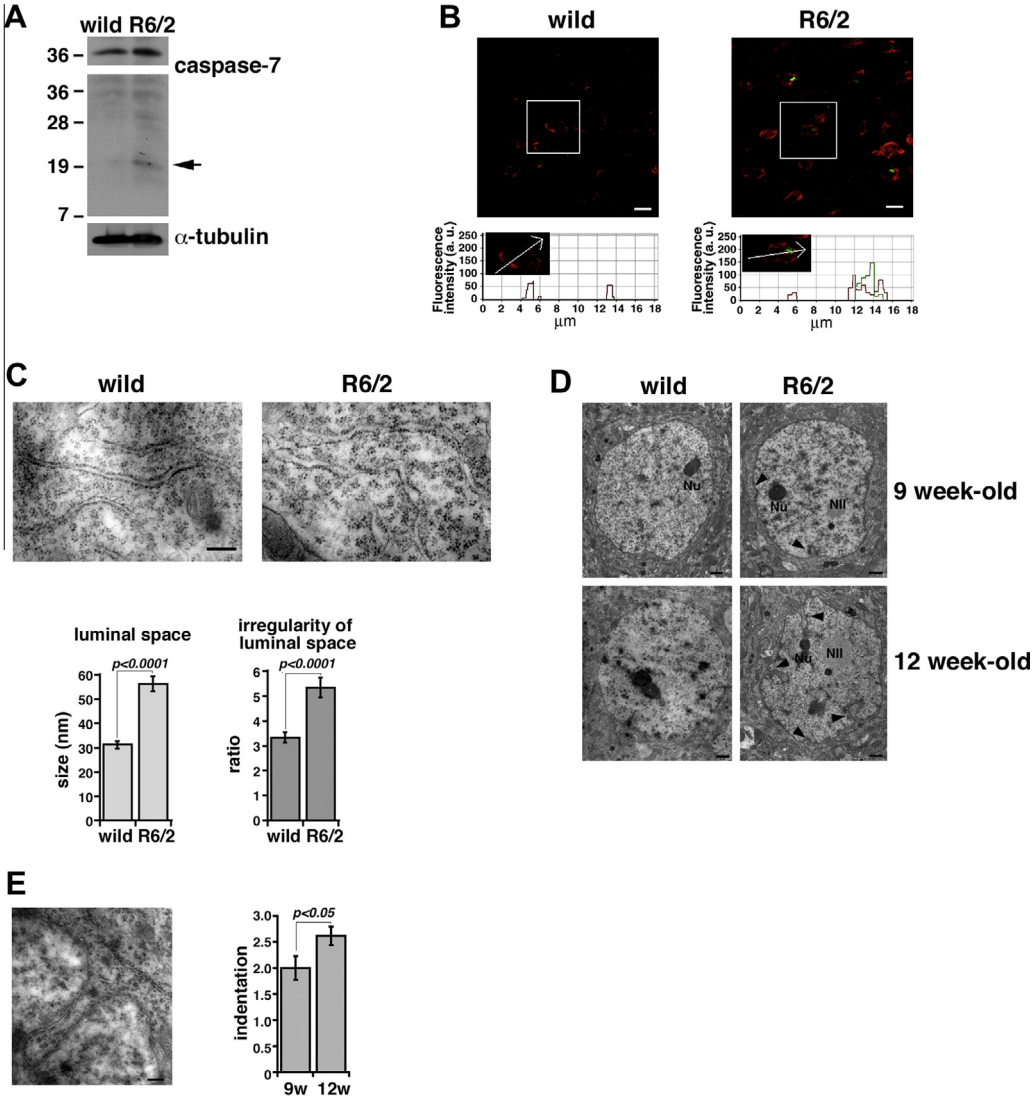

Fig. 1. ER membrane is affected in the mouse model of Huntington's disease. (A) A Western blot of active caspase-7 in the striatum of a 13-week-old R6/2 mouse brain (active caspase-7: arrow, middle). Top: full-length caspase-7; bottom: α-tubulin. (B) The upper panel: confocal images reveal that the signal of ER stained with an anti-calreticulin antibody (red) is increased, which accumulates around polyglutamine aggregates that are stained with a monoclonal EM48 antibody (green). The lower panel: the fluorescence intensity profile (arbitrary units) along the arrow is shown. (C) An irregularity of the ER lumen is observed in neurons of 9-week-old R6/2 mice but not in wild-type mice according to transmission electron microscopy (upper panels). The size and irregularity of the luminal space, as determined by the ratio of the widest to the narrowest lumen, are significantly increased in R6/2 mice (lower panels, $p < 0.0001$, $n = 132$ for ER in wild type; $n = 118$ for ER in R6/2 mice). (D) Severe indentations of the nuclear membrane in 12-week-old R6/2 mice. Arrowheads indicate indentations in the nuclear membrane. Nu: nucleolus; NII: neuronal intranuclear inclusion. (E) Higher magnification of the nuclear membrane in a neuron of an R6/2 mouse. Indentations in the nuclear membrane are significantly more evident in 12-week-old than in 9-week-old R6/2 mice ($p = 0.04$, 13 neurons from each stage).

(lower left panel in Fig. 1C, $p < 0.0001$). Furthermore, an irregularity of the rough ER luminal space was observed in the R6/2 mice: the ratio of the widest to the narrowest luminal space in ER was 3.3 ± 0.1 in wild-type mice versus 5.3 ± 0.3 in the R6/2 mice (lower right panel in Fig. 1C, $p < 0.0001$).

Indentations in the nuclear envelope and in the accompanying outer nuclear membrane composed of ER became more evident around the entire nuclear membrane during the progressive stage of the HD phenotype in the R6/2 mice (Fig. 1D and E). These results suggested that polyglutamine caused a conformational change in

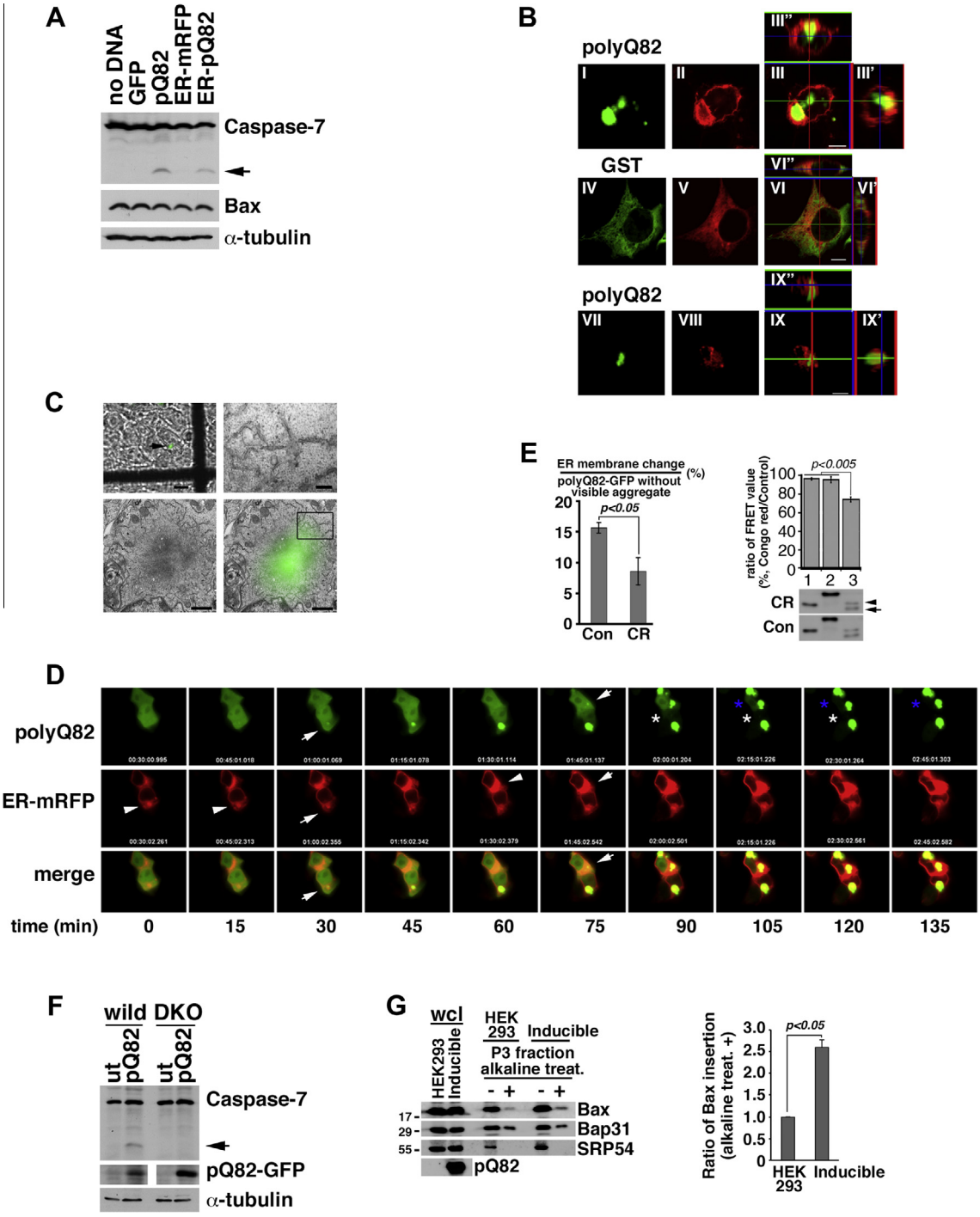

Fig. 2. Polyglutamine expansion-induced accumulation of the ER membrane and caspase-7 activation. (A) ER-localizing polyQ82 activates caspase-7. The arrow: an active form of caspase-7. (B) polyQ82 aggregates partially colocalize with the ER membrane in HEK293T cells (I–III) and rat primary hippocampal neurons (VII–IX). I, IV, and VII: GFP; II, V, and VIII: ER-mRFP; III, VI, and IX: merged; III′, III″, VI′, VI″, IX′, and IX″: an ortho image under a confocal microscope. The scale bar is 5 μm. (C) Analysis by combination of confocal and transmission electron microscopy (TEM). The arrow: a polyQ82-GFP-transfected HEK293T cell, which was examined using TEM. The lower right panel: merged image of confocal and TEM; the upper right panel: higher magnification of the box area. The scale bar is 10 μm (upper left, confocal), 5 μm (bottom, EM), and 200 nm (upper right, EM). (D) Time lapse analysis reveals that ER membrane accumulates (arrowhead) before the formation of visible polyQ82 aggregates (arrow). *Nucleus. (E) The polyglutamine oligomer affects ER. The left panel: a percentage of accumulated ER membrane with diffusely expressed polyQ82-GFP. Con: control, CR: 100 μM Congo red. The right panel: FRET value. 1: CFP-polyQ82, 2: CFP-YFP, and 3: CFP-polyQ82 and polyQ82-YFP. The lower panel: Western blot (arrow: polyQ82-YFP, arrowhead: CFP-polyQ82. (F) Bax and Bak are required for caspase-7 activation induced by polyglutamine expansion. DKO, Bax/Bak double-knockout MEF cells. (G) Insertion of Bax into the ER membrane was enhanced by polyglutamine expansion. pQ82: HA-tagged polyQ82, SRP54: ribonucleoprotein that associates with the ER membrane, Bap31: p28Bap31, P3: microsomal membrane fraction, wcl: whole cell lysate, Inducible: polyQ82-GFP inducible cells.

the ER membrane, and we hypothesized that this change could activate caspase-7.

3.2. Expression of a protein containing polyglutamine expansion affects the ER membrane, coinciding with Bax insertion and caspase-7 activation

To assess the effects of polyQ82 on ER, we tested whether ER-targeted polyQ82 activates caspase-7. The ER-targeted polyQ82 was constructed by combining polyQ82 and monomeric red fluorescent protein (mRFP) fused with cb5, and the construct was subsequently named ER-polyQ82. We found that ER-polyQ82 induced caspase-7 activation similarly to polyQ82 (Fig. 2A), suggesting that caspase-7 was activated on or near the ER membrane that was attached to polyglutamine. PolyQ$_{82}$ tagged with GFP was transfected into HEK293T cells and rat primary neurons using an ER marker (ER-mRFP). Confocal images revealed that polyQ82 aggregated whereas partially localized to ER in HEK293T cells and in rat primary neurons.

ER was accumulating around the aggregates (Fig. 2B). Analysis by combination of confocal and transmission electron microscopy revealed that the ER membrane was disrupted by the polyQ82

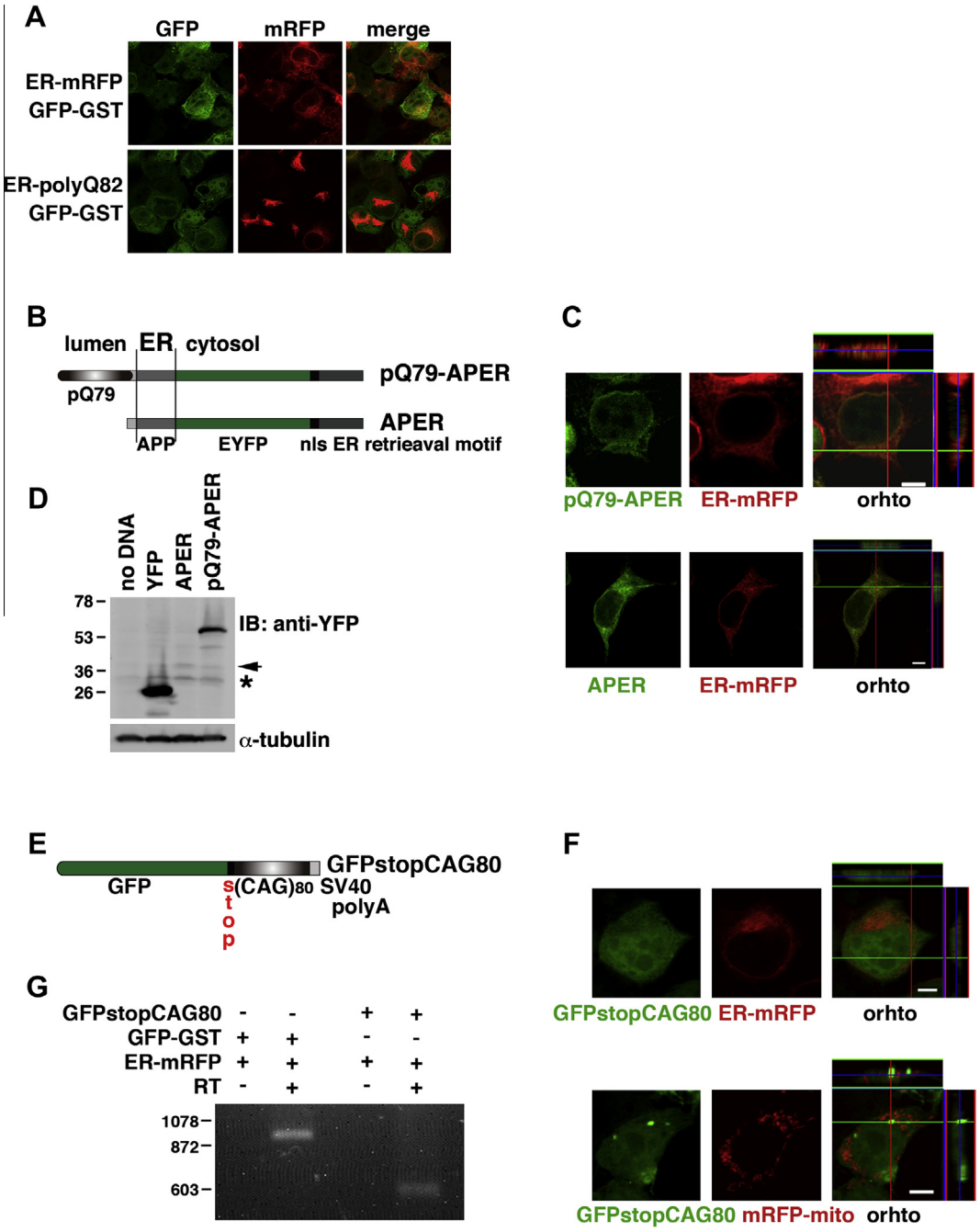

Fig. 3. The ER membrane is affected by polyQ82 expression in cytosol. (A) Confocal images reveal the accumulation of the ER by ER-polyQ82. Upper panels: ER-mRFP (red), GST fused to GFP (green). Lower panels: ER-polyQ82. (B) Schematic representation of pQ79-APER and APER. (C) Confocal images were obtained after transfection of ER-mRFP and pQ79-APER or APER into HEK293T cells. Top panels: pQ79-APER, bottom panels: APER. The scale bar is 5 μm. (D) Western blot with an anti-YFP antibody. The arrow indicates a cleaved product of pQ79-APER. *a nonspecific signal. (E) Schematic representation of GFPstopCAG80. (F) The morphological change was detected by confocal microscopy. Small GFP aggregates were observed in a limited number of cells. (G) RNA containing a CAG repeat within the 3′ untranslated region was amplified by RT-PCR.

aggregate (Fig. 2C), suggesting that the ER membrane was directly affected by polyglutamine expansion. This result is consistent with previous reports that revealed aberrant concanavalin A immunoreactivity in mutant Htt^{Q111}-expressing striatal neurons [12] and linker cell death in *C. elegans* [18].

Time lapse analysis was performed to determine the timeframe of polyQ82 aggregation and the ER membrane accumulation. PolyQ82 aggregates rapidly formed in the cytosol (arrow in Fig. 2D). Polyglutamine aggregates appeared in the location where the mRFP signal of ER was strong (arrowhead in Fig. 2D), suggesting that polyglutamine oligomers were responsible for changes in the ER membrane. To confirm the influence of polyglutamine oligomers on the ER membrane, we tested whether Congo red prevented accumulation of ER. Evaluation using confocal microscopy revealed that the percentage of accumulated ER around diffuse polyQ82-GFP signals (invisible aggregates) decreased with the addition of Congo red (left panel in Fig. 2E). This result was in agreement with the FRET value obtained after transfection with CFP-polyQ82 and polyQ82-YFP plasmids (right panel in Fig. 2E), and with inhibition of caspase-7 activation by Congo red during polyQ82-induced cell death (data not shown). These findings indicated that polyglutamine oligomers may affect the ER membrane.

Although the ER membrane was significantly disrupted after polyQ82 transfection in Bax/Bak double knockout MEF (mouse embryonic fibroblast) cells (Fig. S2), we did not detect caspase-7 activation (Fig. 2F). Therefore, we tested whether Bax is involved in polyQ82-induced caspase-7 activation. Alkaline treatment of the microsomal membrane revealed that Bax insertion increased after induction of polyQ82 expression (Fig. 2G). Consistently, ER-targeted Bax (designated as ER-Bax and constructed in a manner similar to that described for ER-polyQ82) induced caspase-7 activation in HEK293T cells, which was inhibited by ER-localizing Bcl-xL (Fig. S3). These results suggested that polyQ82 controlled activation of caspase-7, primarily in the ER membrane via insertion of Bax.

3.3. The cytosolic side of polyglutamine likely contributes to accumulation of ER membrane

Because accumulation of the ER membrane was observed in the experiment with ER-targeted polyQ82 (Fig. 3A), we tested whether the polyQ82 in the ER lumen or mRNA carrying CAG expansion affects ER morphology. We generated a plasmid that produced polyglutamine expansion in the ER lumen (Fig. 3B), and found that the ER membrane was not affected by the expression of polyQ79 inside the ER (Fig. 3C and D). Next we generated a construct expressing a 79-CAG repeat RNA, which was confirmed by RT-PCR (Fig. 3E and G). The ER membrane was not affected by the expression of the 79-CAG repeat RNA (Fig. 3F). These results indicated that neither polyQ79 in the ER lumen nor the 79-CAG repeat RNA disturbed the ER membrane. Although further studies are required to determine the precise mechanism of the disruption of the ER membrane by polyglutamine, we concluded that the ER membrane is possibly affected by polyglutamine on the cytosolic side.

4. Discussion

In this study, we revealed that the ER membrane is affected by polyglutamine expansion *in vivo* and *in vitro*, and that polyglutamine expansion activates caspase-7 by causing insertion of Bax into the ER membrane. Integrity of the ER membrane is maintained by several proteins, including ER membrane proteins such as reticulon, DP1, CLIMP-63, and atlastin [19–22], and mutations in these proteins lead to changes in ER morphology [22] that often cause disease [23].

Our results reveal that indentations in the nuclear membrane were exacerbated during the progressive stage of the HD phenotype of the R6/2 mice, coinciding with activation of caspase-7. Because polyQ82 fused with cb5 (which inserts into the ER membrane from the cytosolic side) disrupted ER and caused activation of caspase-7, it is possible that polyglutamine expansion causes pathological changes in the ER membrane. Analysis by means of the combination of confocal and electron microscopy indicated that the ER membrane is affected by polyglutamine expansion in HEK293T cells after polyQ82-GFP transfection. Moreover, the ER membrane accumulated prior to the formation of microscopically visible polyglutamine aggregates, whereas Congo red, which inhibits the growth of polyglutamine oligomers [24,25], prevented ER accumulation. These results suggest that polyglutamine oligomers may affect directly on the ER membrane.

Because disruption of the ER membrane by polyQ82 was observed in Bax/Bak double-knockout MEF cells, Bax was not necessary for those morphological changes to occur. On the other hand, Bax was indispensable for caspase-7 activation. Similar to ER stress, which leads to the insertion of Bax into the ER membrane [4], polyglutamine expansion also appears to cause this phenomenon.

It is believed that the ER membrane is dynamically expanded to alleviate ER stress independently of an increase in ER chaperone levels in yeast [26]. Similarly, because the number of short ER components was increased in thapsigargin-treated HEK293T cells (Fig. S4) and polyglutamine expansion induced ER accumulation, the ER membrane may also dynamically change its morphology in *mammalian* cells. Because ER stress induces a conformational change of Bax in ER [4], the disruption of the ER membrane by polyglutamine expansion may also influence Bax conformation, thereby triggering caspase-7 activation. Blum et al. recently revealed that nonapoptotic developmental cell death in *C. elegans* requires a polyglutamine repeat protein, which may contribute to nuclear crenellation [18]. In our experiment, polyglutamine-induced cell death was partially prevented by ER-localizing Bcl-xL even though caspase-7 activation was completely inhibited (data not shown). These results suggest that apoptotic as well as nonapoptotic cell death signals may be involved in polyglutamine expansion related diseases through ER membrane disruption.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.114.

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