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Glucocorticoid Modulatory Element-Binding Protein 1 Binds to Initiator Procaspases and Inhibits Ischemia-Induced Apoptosis and Neuronal Injury

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Running title: endogenous procaspase regulatory protein

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Caspases are divided into two classes: initiator caspases, which include caspase-8 and -9 and possess long prodomains; and effector caspases, which include caspase-3 and -7 and possess short prodomains. Recently, we demonstrated that glucocorticoid modulatory element-binding protein 1 (GMEB1) interacts with the prodomain of procaspase-2, thereby disrupting its autoactivation and the induction of apoptosis. Here we show that GMEB1 is also capable of binding to procaspase-8 and -9. GMEB1 attenuated the Fas-mediated activation of these caspases and the subsequent apoptosis. The knockdown of endogenous GMEB1 using RNA interference revealed that cells with decreased GMEB1 expression are more sensitive to stress and undergo accelerated apoptosis. Transgenic mice expressing a neurospecific GMEB1 had smaller cerebral infarcts and less brain swelling than wild-type mice in response to transient focal ischemia. These results suggest that GMEB1 is an endogenous regulator that selectively binds to initiator procaspases and inhibits caspase-induced apoptosis.

Human caspases are the primary executioners of apoptosis and are divided into two classes, initiators and effectors. The effector caspases, which include caspase-3 and -7, possess short prodomains. The initiator caspases, which include caspase-8 and -9, possess long prodomains that consist of interaction domains essential for their activation. In response to the binding of ligands to death receptors, such as Fas, the adaptor protein FADD and procaspase-8 are recruited to the activated receptor and are incorporated into a death-inducing signaling complex (DISC) (1). The interaction between FADD and procaspase-8 is mediated by the death effector domains (DED) of both proteins (2-4). A second molecule of procaspase-8 then binds to the complex, thus facilitating the autocatalytic activation of caspase-8.

Procaspase-9 is activated by a variety of internal and external death stimuli that promote cytochrome *c* release from mitochondria. In the cytosol, cytochrome *c* binds the adaptor protein Apaf-1 in a dATP-dependent fashion. The binding of cytochrome *c* and dATP induces a conformational change in Apaf-1 that allows it to associate with procaspase-9 via their

respective caspase recruitment domains (CARD) (5, 6). The oligomerization of these procaspases while associated with these specific adapter molecules leads to autoactivation of the caspases (7). The enzymatic activation of initiator caspases leads to proteolytic activation of downstream effector caspases, the subsequent caspase-dependent cleavage of many vital proteins, and ultimately to programmed cell death. Thus, the regulation of initiator caspases is critical in determining whether a cell lives or dies.

Caspases are synthesized in the cell as inactive precursors (procaspases), and their activation is strictly controlled by a variety of regulatory proteins, including Bcl-2, heat-shock proteins (HSPs), and the inhibitor of apoptosis (IAP) family of proteins (8-13). Recently, we showed that glucocorticoid modulatory element-binding protein 1 (GMEB1) is capable of binding to the prodomain of caspase-2, thereby disrupting its autoactivation (14). In the present study, we tested whether GMEB1 also interacts with other procaspases. Our results show that GMEB1 interacts selectively with initiator caspases such as procaspase-8 and -9, and attenuates their activation in response to treatment with monoclonal antibody specific for Fas. These results suggest that GMEB1 functions as an endogenous regulator of caspase activation.

MATERIALS and METHODS

Materials - Full-length GMEB1, and caspase-3, -7, and -8 cDNA and several deletion mutants were amplified from human neuroblastoma cells by RT-PCR. The caspase-3 (C163S), -7 (C186S) and -8 (C360S) mutants were constructed by PCR as described previously (15). For the yeast two-hybrid assay, cDNA encoding the

full-length caspases were subcloned along with cDNA encoding full-length or truncated fragments of GMEB1 into pAS2-1 or pACT2 (Clontech, Palo Alto, CA), respectively. The cDNAs encoding caspase-8 (C360S) and caspase-9 (C287S) were subcloned into the mammalian expression vector pCR3.1 (Invitrogen, Carlsbad, CA). For the *in vitro* protease assay, full-length GMEB1 was subcloned into pGEX-6P-1 (Amersham Biosciences, Uppsala, Sweden). The pNSE was a kind gift from Prof. Ora Bernard (16).

Yeast Two-hybrid Assay - The interactions between GMEB1 and the caspases were confirmed using a yeast two-hybrid assay as described (14). GAL4-BD/p53 and GAL4-AD /SV40 T-antigen were used as positive controls (17, 18).

Preparation of siRNAs - The following siRNA sequences specific to human GMEB1 were used; 5'-GAAUAAACGUGAA GUGUGUdTdT-3', 5'-GCACACACAU UUGGCCUAAAdTdT-3', 5'-GGGCAAA GCAGUCAUCUUGdTdT-3' and 5'-CAA CAGAA GGAACCAAGUAdTdT-3'. The EGFP -specific siRNA 5'-GAACGG CAUCAAGGUGAACdTdT-3' was used as a control. All siRNAs were designed by B-Bridge International, Inc. (Sunnyvale, CA) and were synthesized by Dharmacon, Inc. (Lafayette, CO) using 2'-bis (acetoxymethoxy)-methyl ether (ACE) protection chemistry. The SH-SY5Y cells were transfected with 200 pg of siRNA using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. After 30 h, the cells were subjected to hypoxic stress or treatment with 5 ng/ml TNF- α .

Immunoprecipitation and Immunoblotting - The 293T cells were transfected with caspase-8 (C360S), caspase-9 (C287S), or

caspase-7 plus wild-type or mutant (1-517) GMEB1-FLAG expression vectors for 48 h. The cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na₃VO₄, 1 mM NaF, 1 mM DTT, and 1% NP-40] at 4°C for 20 min. The cells were then disrupted by 15 passages through a 25-gauge needle. The lysates were centrifuged, and the supernatants were immunoprecipitated using anti-FLAG (Sigma, St. Louis, MO), anti-caspase-8, or anti-GMEB1 antibodies and protein G-Sepharose (Amersham Biosciences). To study the endogenous interactions between GMEB1 and procaspases, the immunoprecipitates were subjected to Western blotting with mouse anti-FLAG mAb (M2), anti-caspase-8 mAb (5D3, MBL, Tokyo, Japan), mouse anti-caspase-9 mAb (F-7, Santa Cruz, CA), or anti-caspase-7 pAb (Cell Signaling, MA) (19-23). Endogenous caspase-2 and α-tubulin proteins were extracted from SH-SY5Y cells and subjected to Western blotting with goat anti-caspase-2 pAb (C-20: Santa Cruz Biotechnology, CA) and mouse anti-α-tubulin mAb (Zymed Laboratories, San Francisco, CA), respectively.

Cell Death Assay - HeLa K cells were stably transfected with the expression plasmids indicated in the figure legends. Anti-Fas antibody (CH-11, MBL) was added and cells were harvested, washed in cold phosphate buffered saline (PBS), fixed with 1% glutaraldehyde, and stained using Hoechst 33258 to assess the nuclear morphology of the cells. Apoptotic cells were counted in randomly chosen fields (23-25).

DISC formation - HeLa K cells (2×10^7) were stimulated with 50 ng/ml anti-Fas

(CH11, mouse IgM) for a variable amount of time (Fig. 4C), and then incubated in lysis buffer [50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na₃VO₄, and 1% NP-40] at 4°C for 30 min. Fas was immunoprecipitated with anti-mouse IgM antibody. Immunoprecipitates were subjected to 12% SDS-PAGE, and immunoblotted with anti-FADD (1F7) and anti-caspase-8 (5D3) mAbs (26).

In vitro Protease Assay - HeLa K cell lysates and reaction mixtures were prepared using a Caspase Fluorometric Protease Assay Kit (MBL) according to the manufacturer's protocol. The samples were measured using a fluorometer (Fluoroscan Ascent; LabSystems, Franklin, MA) at wavelengths of 400 nm for excitation and 505 nm for emission. GST-fused GMEB1 proteins and GST-fused control proteins were purified with a GST Purification Module (Amersham Biosciences) according to the manufacturer's protocol. The 293T cells were harvested by centrifugation at 1800 x g for 10 min and washed with cold buffer A [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1mM DTT, and 0.1 mM PMSF]. The cell pellet was resuspended in one volume of buffer A and incubated on ice for 20 min. The cells were disrupted by 15 passages through a 25-gauge needle, and the extracts were clarified by centrifugation at 16000 x g for 30 min. NaCl was added to the resulting supernatants to a final concentration of 50 mM, and the solution was stored at -80°C. For the cell-free experiments, 293T cytosolic extracts were incubated with GST or GST fusion protein (5 µg) for 1 h at 4°C and were activated by adding cytochrome *c* (10 µM) and dATP (1 mM) for 30 min at 37°C.

Generation of Transgenic Mice - The NSE-GMEB1 transgene was constructed by cloning a cDNA fragment containing the full-length HA-tagged GMEB1 protein downstream of the rat *neuron-specific enolase (NSE)* promoter and upstream of an SV40 polyadenylation signal. Transgenic mice were generated by microinjection of the NSE-GMEB1-HA DNA construct into the pronuclei of fertilized C57BL/6J mice (16). Genomic DNA obtained from tail biopsies was amplified by PCR using the sense primer 5'-AAGCTTATGGCTAATGCAGAAGTGAAG-3', and the antisense primer 5'-CCATCGATTCAAGCGTAATCTGGAAAT-3'. This reaction generated a 1750-bp PCR product.

Ischemic model - Adult male wild-type (C57BL/6J) and GMEB1-transgenic (line 36) mice weighing 20-30 g were housed under diurnal lighting conditions and were used for all experiments. Anesthesia was induced by inhalation of 2.0% isoflurane (Merck, Whitehouse Station, NJ), and was maintained with 1% isoflurane in 70% N₂O and 30% O₂ using a general anesthesia machine (Soft Lander: Sin-ei Industry Co. Ltd., Saitama, Japan). The body temperature of the mice was maintained between 37.0 and 37.5°C using a heating pad and heating lamp. A filament occlusion of the left middle cerebral artery (MCA) was performed as previously described (27, 28). Briefly, the left MCA was occluded with an 8-0 nylon monofilament (Ethicon, Somerville, NJ) coated with a mixture of silicone resin (Xantopren; Bayer Dental, Osaka, Japan). This coated filament was introduced into the internal carotid artery through the common carotid artery, and was fed into the anterior cerebral artery *via* the internal carotid artery, resulting in occlusion of the MCA. At 90 min after ischemia, the animals were briefly re-anesthetized with

isoflurane, and the filament was withdrawn through the common carotid artery. The removal of the filament resulted in the reperfusion of the MCA, posterior communicating artery, and common carotid artery. Two 2 µl of Z-VAD-FMK (40 ng) were injected intracerebroventricularly (i.c.v.: bregma: 0.9 mm lateral, 0.1 mm posterior, 3.1 mm deep), one 15 min before ischemia and the other immediately after reperfusion (28).

Brain infarction and swelling - Twenty-four hours after occlusion, the forebrain was divided into five 2-µm coronal sections using a mouse brain matrix (RBM-2000C; Activational Systems, Warren, MI). The sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO). The infarcted areas were imaged using a digital camera (COOLPIX4500; Nikon, Tokyo, Japan), and Image J (<http://rsb.info.nih.gov/ij/download/>), and the degree of injury was calculated as described previously (28). Brain swelling (%) was calculated according to the following formula: [(infarct volume + ipsilateral undamaged volume - contralateral volume)/contralateral volume] x 100 (27). Physiological parameters were recorded as previously described (28). Briefly, a polyethylene catheter was inserted into the femoral artery, and arterial blood pressure, pO₂, pCO₂, pH, and plasma glucose were measured 15 min after the start of ischemia.

In vivo caspase activity - The activity of caspase-8 and -9 were measured in ischemic brains by a Caspase-Glo Assay Kit (Promega, Madison, WI) that provided a proluminescent substrate containing a tetrapeptide sequence such as LETD (caspase-8) or LEHD (caspase-9). Cytosolic

fractions from brain tissue (10 mg, cortex) were prepared by Dounce homogenization in extraction buffer (25 mM Hepes [pH 7.5], 5 mM MgCl₂, 1 mM EGTA, 1mM Pefablock, and 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) (29) and then centrifuged at 15,000 rpm for 20 min at 4°C. The cytosolic protein concentration was adjusted to 1 mg/ml with the same buffer and an equal volume of reagents. The samples were incubated for 30 min at room temperature. The luminescence of each samples was measured in a luminometer (Lumat LB 9507, Berthold, Bad Wildbad, Germany).

Statistical analysis - The data are presented as the mean ± S.E.M. Statistical comparisons were performed using one- or two-way ANOVA followed by Student's *t*-test, or the Mann-Whitney U-test (using STAT VIEW v. 5.0; SAS Institute Inc., Cary, NC). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Selective interaction between GMEB1 and procaspase-8 or -9 - To determine whether GMEB1 binds to caspases other than procaspase-2 (14), we performed a yeast two-hybrid assay. We found that procaspase-8 and -9 interacted with GMEB1 in yeast (Fig. 1A). We then analyzed the domains required for the interaction between procaspase-8 or -9 and GMEB1. Procaspase-8 contains two repeated DED domains in the N-terminal region. Both DED domains of procaspase-8 interacted with GMEB1, whereas neither the N-terminal DED domain alone nor the p20/p10 domain was sufficient for the interaction (Fig. 1B). Similar to procaspase-2 (14), procaspase-9 required the CARD domain to interact with

GMEB1 (Fig. 1B). The C-terminal region of GMEB1 (amino acid sequence 375-563), which contains a putative α -helical coiled-coil structure, was necessary for both procaspase-8 and -9 to interact with GMEB1 (Fig. 1C). This same domain was previously shown to be required for GMEB1 interaction with procaspase-2 (14).

GMEB1 can bind to procaspase-8 and -9 in mammalian cells - To demonstrate an interaction of GMEB1 with procaspase-8 and -9 *in vivo*, wild-type or mutant (1-517) FLAG-tagged GMEB1 was transiently transfected together with procaspase-8 or -9 into human 293T cells. Both procaspase-8 and -9 were specifically co-immunoprecipitated with GMEB1 (Fig. 2A). To confirm these findings, we examined the binding ability of a mutant GMEB1 (1-517) that lacks the putative C-terminal binding motif. Neither caspase-8 nor caspase-9 interacted with the mutant. We then examined the endogenous interaction between GMEB1 and procaspase-8 and -9 using non-transfected 293T cells. Both procaspase-8 and -9 were detected following immunoprecipitation of 293T cell lysates with an anti-GMEB1 antibody (Fig. 2B). Caspase-7, an effector caspase, did not bind to GMEB1 (Fig. 2C). These results indicated that endogenous GMEB1 associates with initiator caspases such as procaspase-8 and -9 in quiescent cells. In addition, immunocytochemical analysis revealed that endogenous GMEB1, as well as caspase-8 and -9, localizes in both the nucleus and cytosol (Fig. 3).

Attenuation of Fas-mediated apoptosis by GMEB1 - To test the effects of GMEB1 on apoptosis, we first investigated death receptor signal-dependent apoptosis using anti-Fas antibody treatment. In HeLa K cells, the

stimulation of Fas triggered a striking change in nuclear morphology characterized by typical apoptotic features such as nuclear condensation and fragmentation (Fig. 4A). The overexpression of wild-type GMEB1, but not the C-terminal-deleted mutant of GMEB1 (1-517), reduced Fas-mediated apoptosis in a concentration-dependent manner (Fig. 4B). Next, we addressed how GMEB1 regulates Fas-induced signaling. Treatment with Fas ligand or antibody stimulated Fas aggregation, conformational changes in the cytoplasmic domain, and ultimately a death-inducing signaling complex (DISC) (30, 31). To investigate the effect of GMEB1 on Fas antibody-induced DISC formation, HeLa K cells were treated with 50 ng/ml CH11 for a variable amount of time (Fig. 4C). Fas was immunoprecipitated with anti-mouse IgM antibody and then the precipitates were examined by Western blot analysis with anti-FADD and anti-caspase-8 antibodies. FADD was weakly associated with Fas 5 min after treatment, peaked at 15 min, and gradually decreased hereafter. Caspase-8 was significantly appeared within the DISC 15 min after stimulation in mock-transfected cells. Although there was also an association between FADD and Fas in GMEB1-transfected cells, caspase-8 was not observed within the DISC in these cell types (Fig. 4C), suggesting that GMEB1 disrupts the interaction of FADD and caspase-8, but not that of Fas and FADD. Therefore, it is likely GMEB1 inhibits caspase-8 activation and subsequent apoptosis in response to Fas antibody via prevention of DISC formation by binding to the DED domain in procaspase-8.

GMEB1 inhibits the enzymatic activity of caspases in vivo and in vitro - To further determine whether the inhibitory mechanisms of GMEB1 depended on the inhibition of

caspase activation, we measured the cleavage of the caspase peptide substrates IETD (for caspase-8), LEHD (for caspase-9), DEVD (for caspase-3 and -7), and VDVAD (for caspase-2). HeLa K cells were treated with anti-Fas antibody for 4 or 8 h, and caspase activation was measured in the cytosolic extracts. A significant level of Fas-mediated caspase activation was observed in mock-transfected HeLa K cells, whereas this activation was lower in GMEB1-transfected cells (Fig. 5A). Because these results were performed with the lysates prepared from cells overexpressing GMEB1, we confirmed the inhibitory effects of GMEB1 using recombinant GMEB1 protein in a cell-free system. There have been reports that the addition of cytochrome *c* into lysates stimulated caspase activation through caspase-9 (32, 33). In this study, the addition of cytochrome *c* to detergent extracts prepared from 293T cells caused a rapid increase in the peptide-specific protease activity *in vitro*. The recombinant GMEB1 protein dramatically reduced the cytochrome *c*-dependent protease activity of the LEHD-, DEVD-, and VDVAD-specific enzymes (Fig. 5B).

Changes in sensitivity to hypoxia in GMEB1 knockdown cells - As GMEB1 has been implicated in the regulation of caspase activity and the subsequent apoptosis, we investigated the effect of reducing the level of endogenous GMEB1 in cells by using siRNA. The knockdown of GMEB1 accelerated the appearance of apoptotic cells in response to not only hypoxic stress but also TNF- α (Fig. 6).

Mice that express human GMEB1 specifically in neurons - To limit the expression of GMEB1 to neuronal cells *in vivo*, human HA-tagged GMEB1 cDNA was

ligated to the NSE promoter. Total RNA and cell lysates were prepared from the brains of transgenic offspring and were analyzed for the expression of GMEB1 protein by RT-PCR and Western blotting. Six mouse lines expressed high levels of human GMEB1 mRNA (data not shown). Among these six lines, the level of GMEB1 expression in the brain was highest in line 36 (Fig. 7), and these mice were used for subsequent experiments.

Attenuation of brain infarction and swelling in GMEB1-expressing transgenic mice - To test the function of GMEB1 in neuronal cells, we performed MCA occlusion in 9 transgenic and 11 wild-type mice. The mice were scored for neurological damage at 30 min and 24 h after occlusion. There were no significant differences in the neurological scores between the wild-type and GMEB1-transgenic (TG) mice. However, neurological damage was somewhat prolonged in wild-type mice as compared with the TG mice, which showed signs of improvement by 24 h after the occlusion (Table 1). At 24 h, the mice had developed infarcts affecting the cortex and striatum. The area and volume of the infarcts were smaller in GMEB1-TG mice than in wild-type mice (Fig. 8. A-E). Brain swelling at 24h after the occlusion was $45.2 \pm 4.0\%$ in wild-type mice and $26.1 \pm 5.2\%$ in GMEB1-TG mice ($p < 0.05$). There were no significant differences in the other physiological parameters measured (body temperature, arterial blood pressure, pO_2 , pCO_2 , pH, and plasma glucose) between the wild-type and GMEB1-TG mice. To test whether the *in vivo* protective effect of GMEB1 could be explained by the inhibition of caspase activation, we investigated the effect of Z-VAD-FMK, a general caspase inhibitor, on infarct and swelling formation, and the

activity of both caspase-8 and -9 in wild-type and GMEB1-TG mice. As shown in Fig. 8F and 8G, the inhibitor significantly decreased both infarct and swelling formation in wild-type, but had little to no effect on GMEB1-TG mice. Ischemia led to increased activity of both caspase-8 and -9 in the ipsilateral hemisphere, but not in the contralateral hemisphere. The activation of these caspases was significantly lower in GMEB1-TG mice (Fig. 8H).

DISCUSSION

The purpose of this study was to elucidate whether GMEB1 functions as an anti-apoptotic protein. In a previous study, we reported that GMEB1 could bind to procaspase-2 and inhibit caspase-2-dependent apoptosis (14). In the present study, we found that GMEB1 also interacts with procaspase-8 and -9, suggesting that GMEB1 interacts with pro-form initiator caspases such as procaspase-2, -8, and -9. The complete DED domain of procaspase-8 and the CARD domain of procaspase-9 were required for the interaction with GMEB1 (Fig. 1). The C-terminal region of GMEB1, which contains a putative α -helical coiled-coil domain (amino acid sequence 375-563), was required for GMEB1 to bind to procaspase-8 and -9 (Fig. 1C). This is the same region previously shown to be required for binding to procaspase-2. The GMEB1 protein lacks DED, CARD, and BIR domains, but has the putative α -helical coiled-coil domain in its C-terminal, which is critical for its interaction with procaspases. Acidic amino acid residues in the C-terminal region of GMEB1 may be important for its binding to procaspases, similar to the requirement for acidic amino acids in the CARD domains of

procaspase-2 and the adaptor protein RAIDD for their interaction with each other (34). Immunoprecipitation and Western blot analysis showed that GMEB1 interacts with both procaspase-8 and -9, but not procaspase-7 (an effector caspase), in resting mammalian cells (Fig. 2). Based on these findings, we conclude that endogenous GMEB1 can bind to initiator procaspases in quiescent cells.

GMEB1 expression attenuated apoptosis in response to the Fas ligand in HeLa K cells. Fas-stimulated caspase activity was also significantly abrogated by GMEB1 expression (Fig. 5A). Moreover, we demonstrated that GMEB1 abrogates the appearance of caspase-8 within DISC that forms in response to anti-Fas mAb in HeLa K cells (Fig. 4C). These results suggest that GMEB1 overexpression prevents the activation of certain initiator caspases by hindering complete DISC formation in response to Fas-mediated signals by interacting with their prodomains. In addition, consistent with this conclusion, *in vitro* cytochrome *c*-induced caspase activity in a cell-free system was significantly reduced in the presence of recombinant GMEB1 (Fig. 5B). Thus, GMEB1 seems to bind to the prodomains of initiator caspases *in vivo* and *in vitro*, thereby inhibiting the activation of these caspases and the downstream effector caspases. However, we did not propose direct evidence of how GMEB1 disrupts the caspase activation by addition to cytochrome *c*. In this regard, caspase-9 activated by cytochrome *c* may stimulate downstream caspases such as caspase-3, -7, and -2. Unfortunately, we could not determine the effect of GMEB1 on apoptosome formation including procaspase-9, Apaf-1, and dATP. It is of interest to elucidate whether GMEB1 suppresses apoptosome formation induced by apoptotic stimuli or cytochrome *c* release

from mitochondria.

We previously found that the level of endogenous GMEB1 decreased during hypoxia-induced apoptosis in a caspase-independent manner (14), suggesting that GMEB1 itself is not a substrate for caspases. The lack of cleavage sites for caspases in the GMEB1 structure also suggests that other proteinases are responsible for the decrease in GMEB1 during apoptosis. The knockdown of endogenous GMEB1 protein using siRNA rendered the cells more sensitive to hypoxic stress and TNF- α treatment (Fig. 6), indicating that a lack of interaction between GMEB1 and procaspases accelerates procaspase oligomerization and subsequent autoactivation. To confirm the function of GMEB1 *in vivo*, we created a transgenic mouse that constitutively expresses human GMEB1 in neurons. The GMEB1-TG mice suffered less neuronal tissue injury than the wild-type mice, further suggesting that GMEB1 functions as an endogenous regulator of caspase activation (Figs. 7 and 8). Many reports have shown that neuronal cell death is characterized by apoptosis or necrosis (35, 36). In this study, we detected a significant increase in caspase-8 and -9 activation in response to focal ischemia *in vivo*. However, the activities toward caspases were partially attenuated in GMEB1-TG mice. Furthermore, treatment with the caspase inhibitor Z-VAD-FMK did not completely block infarction and swelling formation by focal cerebral ischemia. These findings suggest that there might be two pathways leading to neuronal cell death: one that is caspase-dependent and GMEB1-sensitive, and another that is caspase-independent and therefore GMEB1-insensitive. In this regard, a caspase-dependent pathway may trigger apoptosis-like cell death while a

caspase-independent pathway might lead to necrosis-like death.

However, the amino acid sequence 412-563 in GMEB1 is sufficient for the expression of intrinsic trans-activation activity (37). In the present study, we demonstrated that the C-terminus 373-563 containing the trans-activation domain is critical for interactions with procaspase, suggesting that this C-terminal may function as both a transcriptional factor and a procaspase inhibitor through binding. In addition, HSP27 also interacts with GMEB1 via the transactivation domain (38). In contrast, the binding region of GMEB1 and GMEB2, glucocorticoid receptor, or cAMP-response element-binding protein (CREB)-binding protein (CBP) is different from that in procaspase or HSP27. We have shown here that the deletion mutant of GMEB1 lacking the putative binding site to procaspases does not suppress Fas-induced apoptosis (Fig. 4B). Therefore, it is likely that the C-terminal region of GMEB1 may function as both trans-activation domain and a procaspase- or HSP27-binding domain. Because GMEB1 interacts with the molecular chaperone HSP27 in cytosol, and GMEB in cytosol solution prepared from hepatoma tissue culture (HTC) and PC12 cells bind GME in a gel-shift assay (39), we

concluded that at least some GMEB1 exists in the cytosolic fraction, giving it the ability to associate with procaspase and HSP27 and to participate in apoptosis. However, we do not exclude the possibility that GMEB1 overexpression may modulate caspase function via newly expressed proteins that are sensitive to transcriptional activity and thereby contribute to the inhibition of caspase activation. Although cytosolic GMEB1 may be involved in the attenuation of caspase activation via direct binding, how GMEB1 might block caspase activation via expressing new genes remains unknown.

In summary, GMEB1 appears to be a novel regulator of initiator procaspase activation. This intrinsic protein interacts with several types of initiator caspases and thereby is capable of inhibiting the death signal triggered by apoptosis-inducing stimuli. GMEB1 is also expressed in cell lines derived from immunocytomas, which are associated with the Fas/Fas ligand system. Thus, this molecule may also be crucial for the proper functioning of the immune system. Owing to its novel ability to inhibit the activation of initiator caspases, GMEB1 may also participate in various regulatory processes in concert with other regulating molecules such as FLIP (40) and ARC (41), to play a role in embryogenesis.

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FOOTNOTES

The abbreviations used are GMEB1, glucocorticoid modulatory element-binding protein 1; CARD, caspase recruitment domain; DED, death effector domain; GST, glutathione S-transferase; mAb, monoclonal antibody; pAb, polyclonal antibody

Figure Legends

Fig. 1. Determination of the domains required for the interaction between procaspase-8 or -9 and GMEB1. (A) Association of caspases with GMEB1. The expression vectors encoding wild-type GMEB1 or various mutants fused to the GAL4 DNA activation domain were co-transformed into cells together with various caspase subtypes whose catalytic cysteine was replaced with serine, or with various mutants fused to GAL4 DNA binding domain in Y190 cells. Each transformant was assayed for β -galactosidase activity. (-) Color development was not observed after 12 h of incubation at 30°C. (+) Blue color started to develop within 30 min

of incubation in all positive samples. (B, C) Yeast two-hybrid assays between several mutants of GMEB1 and procaspase-8 or -9. The domain of GMEB1 that interacts with procaspase-8 or -9 was determined. KDWK indicates the KDWK domain; S/T and Q indicate serine/threonine-rich and glutamine-rich regions, respectively; α indicates the putative α -helical coiled-coil domain. The β -galactosidase activity was determined for 3-day-old Y190 yeast transformants containing the indicated plasmids using a filter assay. Three to five independent yeast transformations showed the same results in the interaction assay.

Fig. 2. GMEB1 interacts with caspase-8 and -9 in cells. (A, C) HEK 293T cells were transfected with pCR3.1-GMEB1-Flag or pCR3.1-GMEB1(1-517)mutant-FLAG and pCR3.1-caspase-8 (C360S), pCR3.1-caspase-9 (C287S) or pCR3.1-caspase-7. The indicated antibodies were used for immunoprecipitation (IP) and immunoblotting (IB). (B) Endogenous interaction between GMEB1 and caspase-8 and -9. Co-immunoprecipitation was performed in HEK293T cells. Total cell lysates were immunoprecipitated with normal rabbit IgG or anti-GMEB1 pAb. The immune complex bound to protein G-Sepharose beads was washed with lysis buffer and washing buffer, resolved by SDS-PAGE and analyzed by Western blotting using an anti-caspase-8 mAb (top panel), or an anti-caspase-9 mAb (second panel), respectively.

Fig. 3. Cellular localization of GMEB1 and the procaspases. SH-SY5Y cells were fixed and subjected to indirect immunofluorescence staining with anti-GMEB1 pAb and anti-caspase-8 or caspase-9 mAb, respectively. The green signal (GMEB1) was obtained with anti-rabbit IgG Alexa 488-conjugated secondary Ab, and the red signals (caspases) were obtained with anti-mouse IgG Alexa 594-conjugated secondary Ab. Nuclei were stained with Hoechst 33258.

Fig. 4. GMEB1 reduces Fas-mediated apoptosis. HeLa K cells were transiently transfected with 2 μ g of pCR3.1 (mock), pCR3.1-GMEB1-Flag, or pCR3.1-GMEB1(1-517)mutant-FLAG. (A) The cells were treated with vehicle (left) or anti-Fas antibody (50 ng/ml; center and right) for 8 h and were stained using Hoechst 33258. The filled arrows indicate apoptotic cells. (B) The total amount of each transfected plasmid was adjusted to 2 μ g using the mock-transfected plasmid as a standard. Transfection and anti-Fas antibody stimuli were as described above. The percentages of specific apoptosis were determined as described in the Materials and Methods. Data represent the mean \pm S.E. (n = 5). Significant differences between groups were determined using the Dunnett test. (C) Effect of GMEB1 on DISC formation stimulated by anti-Fas antibody treatment. Kinetic analysis of Fas-mediated DISC formation was investigated in 2×10^7 HeLa K cells transfected with GMEB1. The cells were stimulated with 50 ng/ml CH-11 for the indicated amount of time. Then, cell lysates were prepared and Fas was immunoprecipitated with anti-mouse IgM antibody. Immunoprecipitates were resolved in SDS-PAGE and immunoblotted with anti-FADD and anti-caspase-8 Abs.

Fig. 5. GMEB1 attenuates the activation of caspases *in vivo* and *in vitro*. (A) Stably mock (-) or pCR3.1-GMEB1-Flag-transfected (+) HeLa K cells were treated with anti-Fas antibody (50 ng/ml). After the indicated times, the cells were harvested, and cell extracts were prepared. Proteolytic activity was measured as described in the Materials and Methods. Each stably

transfected cell (GMEB1 + or -) without anti-Fas antibody treatment was used as a control. Data represent mean \pm S.E.M. (n = 3). Significantly different values from those of mock-transfected cells were determined using Student's *t*-test. (B) The 293T cell extracts were treated with glutathione S-transferase (GST) or GST-fused GMEB1 (GMEB1) and then with cytochrome *c* and dATP. Proteolytic activities were measured as described in the Materials and Methods. Cell extracts without GST or GMEB1 were used as controls. Data represent the mean \pm S.E.M. (n = 3). Significantly different values from those of GST-treated samples were determined using Student's *t*-test.

Fig. 6. Inhibition of endogenous GMEB1 using siRNA results in the hyper-sensitivity of cells to apoptosis-inducing stress. The siRNA specific for human GMEB1 or an siRNA specific for EGFP (control) were transfected into SH-SY5Y cells and the expression level of GMEB1 was estimated by Western blot analysis (A). The cells were then subjected to hypoxic stress or TNF- α treatment (5 ng/ml) for 24 h. The cells exposed to hypoxia for 18 and 24 h were stained with a DNA-specific fluorochrome Hoechst dye 33258 and observed by fluorescence microscopy (B). Apoptotic cells were quantified by counting cells with condensed nuclei from six randomly chosen fields. The percentage of apoptotic cells was represented as the ratio of total cells to apoptotic cells (C). Values represent the mean \pm S.E.M. of quadruplicate cultures run in parallel. $**p < 0.01$, $***p < 0.001$: indicates significant differences from the hypoxia-exposed control siRNA-transfected cells (ANOVA followed by a Bonferroni test).

Fig. 7. Confirmation of human GMEB1 expression in transgenic mice. (A) PCR genotyping of transgenic line. (B) Characterization of GMEB1 expression in the brains of transgenic mice. Western blot analysis was performed on lysates from brain sections as indicated. (C) Immunohistochemistry of cerebral cortex sections from transgenic and wild-type mice. The red signal (NeuN, a neuronal marker) was obtained with anti-mouse IgG Alexa 594-conjugated secondary Ab; the green signals (GMEB1-HA) were obtained with anti-rabbit IgG Alexa 488-conjugated secondary Ab.

Fig. 8. Protection from MCA occlusion-mediated infarct and swelling formation in GMEB1-transgenic mice compared with wild-type mice. Images show TTC staining of coronal brain sections (2 mm) from representative mice at 24 h after MCA occlusion. Damaged tissue is shown as white areas. Left and right brains are shown for a wild-type mouse (A and C) and a GMEB1-transgenic mouse (B and D). GMEB1-transgenic mice had reduced infarct compared with wild-type mice. (E) Brain infarct area at 24 h after MCA occlusion in wild-type mice and GMEB1-transgenic mice. The brains were removed, and the forebrain was sliced into five coronal 2-mm sections. The infarct areas in the brain slices were stained with 2% TTC. $*p < 0.05$, $**p < 0.01$ indicates statistically significant differences between transgenic and wild-type mice, n=9 or 11. (F, G) Effects of the caspase inhibitor Z-VAD-FMK on infarct (F) and swelling (G) formation induced by focal ischemia in wild-type and GMEB1-TG mice. Z-VAD-FMK (40 ng) was injected intracerebroventricularly (icv) twice (2 μ l per dose; bregma: 0.9 mm lateral, 0.1 mm posterior, 3.1 mm deep) 15 min before ischemia (90 min) and immediately after reperfusion. $*p < 0.05$, $**p < 0.01$ indicates statistically significant differences between transgenic and wild-type mice, n=4 - 8. (H) Effects of GMEB1 on caspase activation.

The activities of caspase-8 and -9 in samples prepared from the contralateral or ipsilateral hemisphere 1 h after reperfusion were detected as described under “Experimental Procedures”. ** $p < 0.01$ indicates statistically significant differences between transgenic and wild-type mice, n=6.

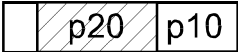
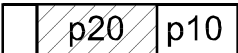

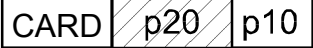
Table 1

Table 1. Neurological scores in wild-type mice and GMEB1-transgenic mice 30 min and 24 h after MCA occlusion.





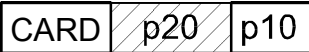

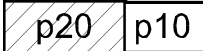
Treatment (Time after reperfusion)	n	Neurological score				Mean \pm S.E.M.
		0	1	2	3	
Wild-type mice (30 min)	9	0	4	5	0	1.6 \pm 0.18
GMEB1 transgenic mice (30 min)	11	0	4	7	0	1.6 \pm 0.15
Wild-type mice (24 h)	9	0	2	3	4	2.2 \pm 0.28
GMEB1 transgenic mice (24 h)	11	1	4	4	2	1.6 \pm 0.28

Neurological grading: 0, no neurological deficits; 1, failure to extend the right forepaw; 2, circling to the contralateral side; 3, loss of walking or righting reflex.

A

		Interaction with GMEB1
Caspase-3		-
Caspase-7		-
Caspase-8		++
Caspase-9		+

B

		Interaction with GMEB1
Caspase-8 WT		++
1-101		-
1-210		++
211-479		-
Caspase-9 WT		+
1-138		+
139-418		-

C



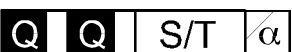





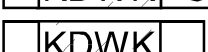
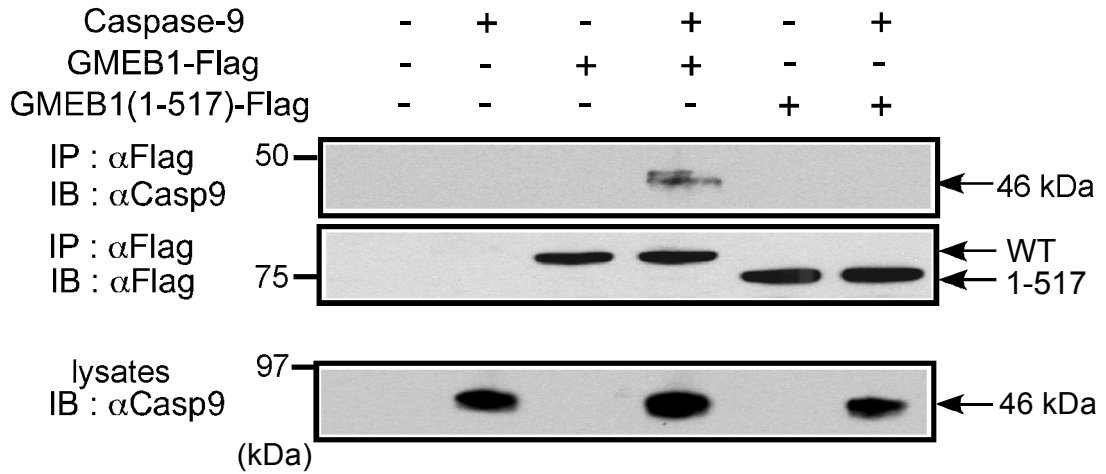
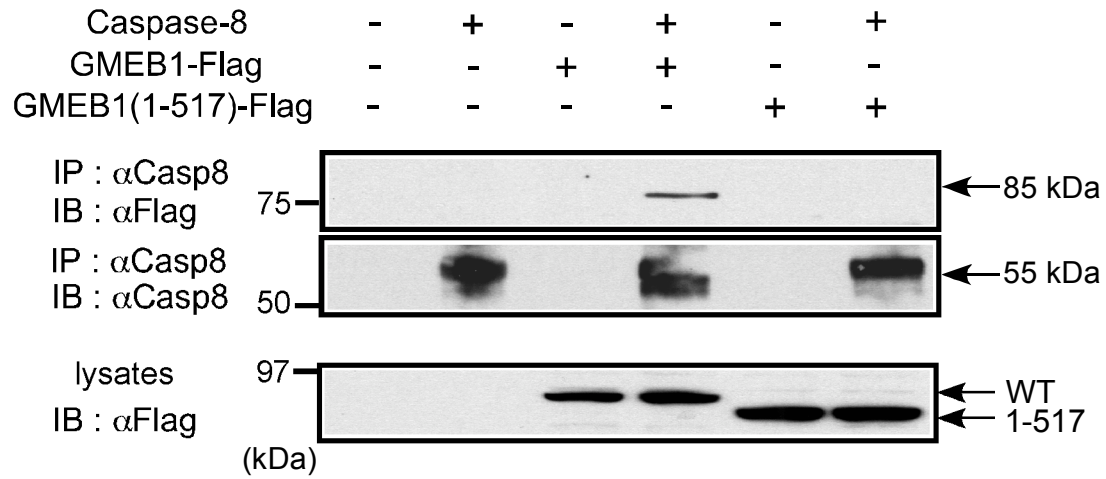
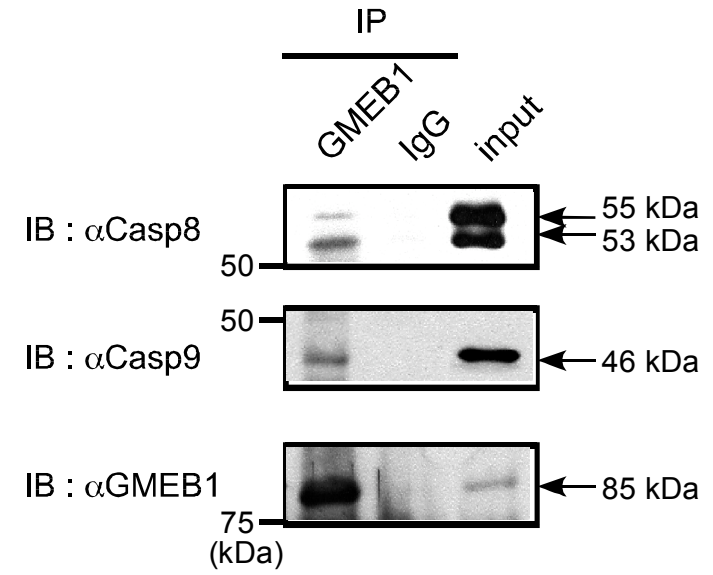
	GMEB1	Interaction with caspase-8	Interaction with caspase-9
AA 1-563 (WT)		++	+
187-563		++	+
267-563		+++	+
375-563		+++	+
509-563		-	-
1-517		-	-
1-362		-	-
1-297		-	-
1-217		-	-

Figure 1. Tsuruma et al.

A



B



C

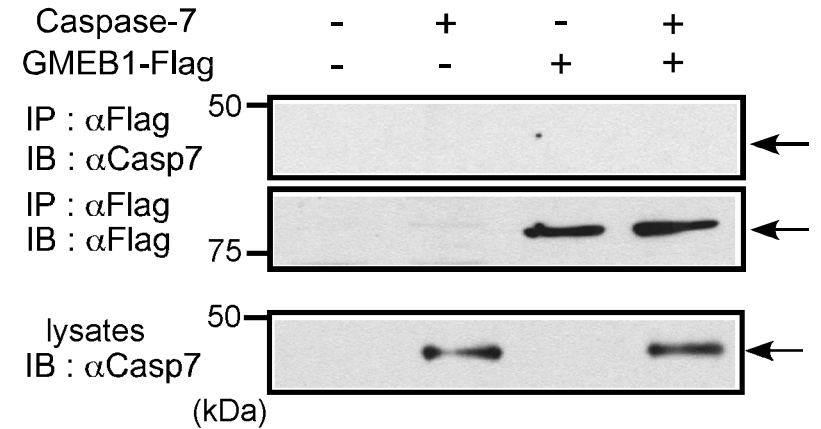
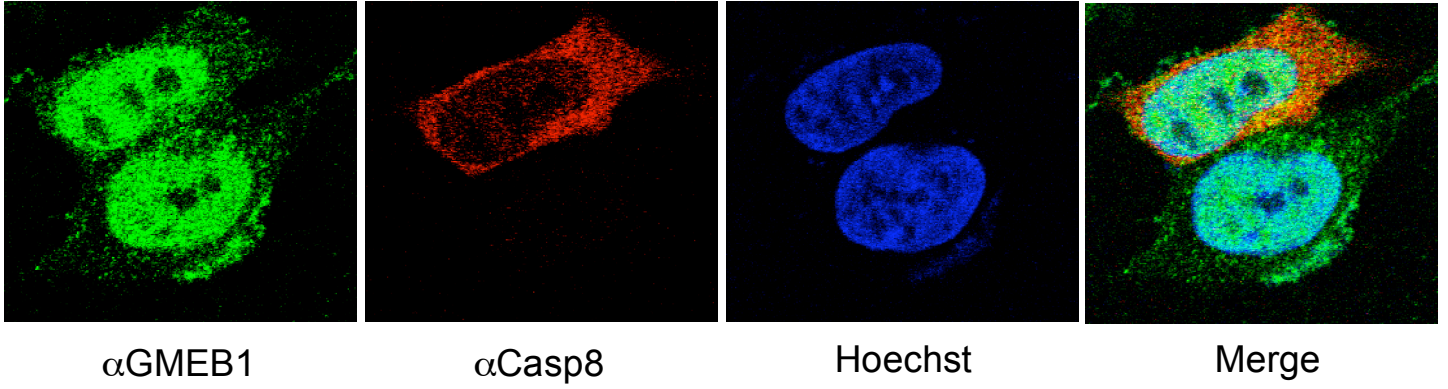


Figure 2. Tsuruma et al.

A



B

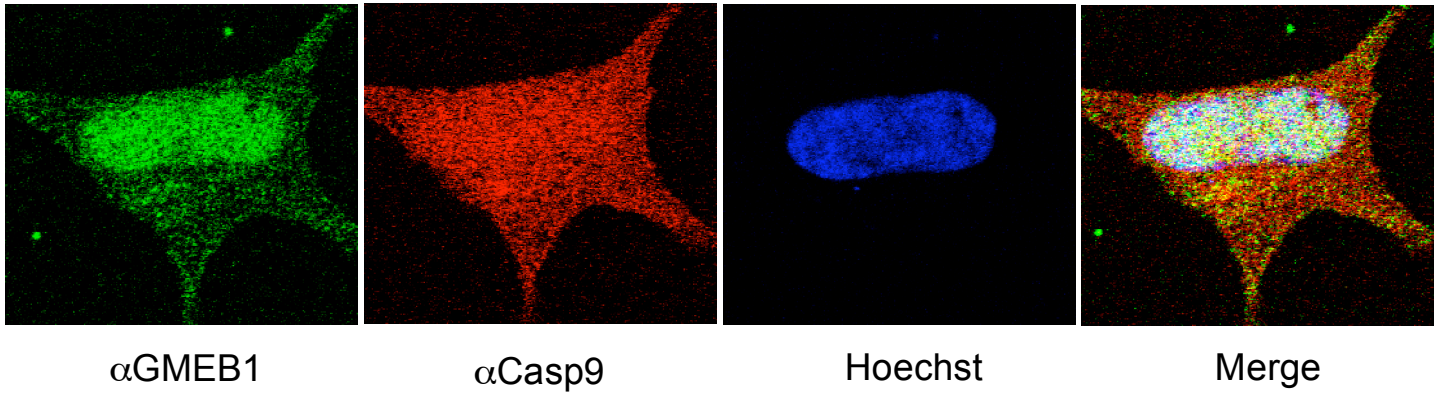
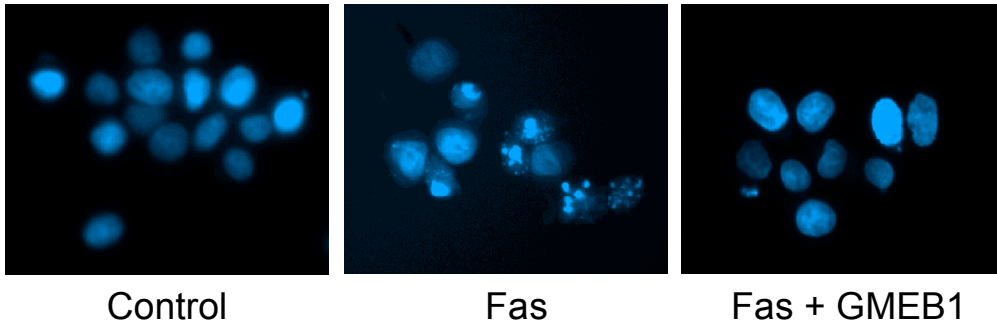
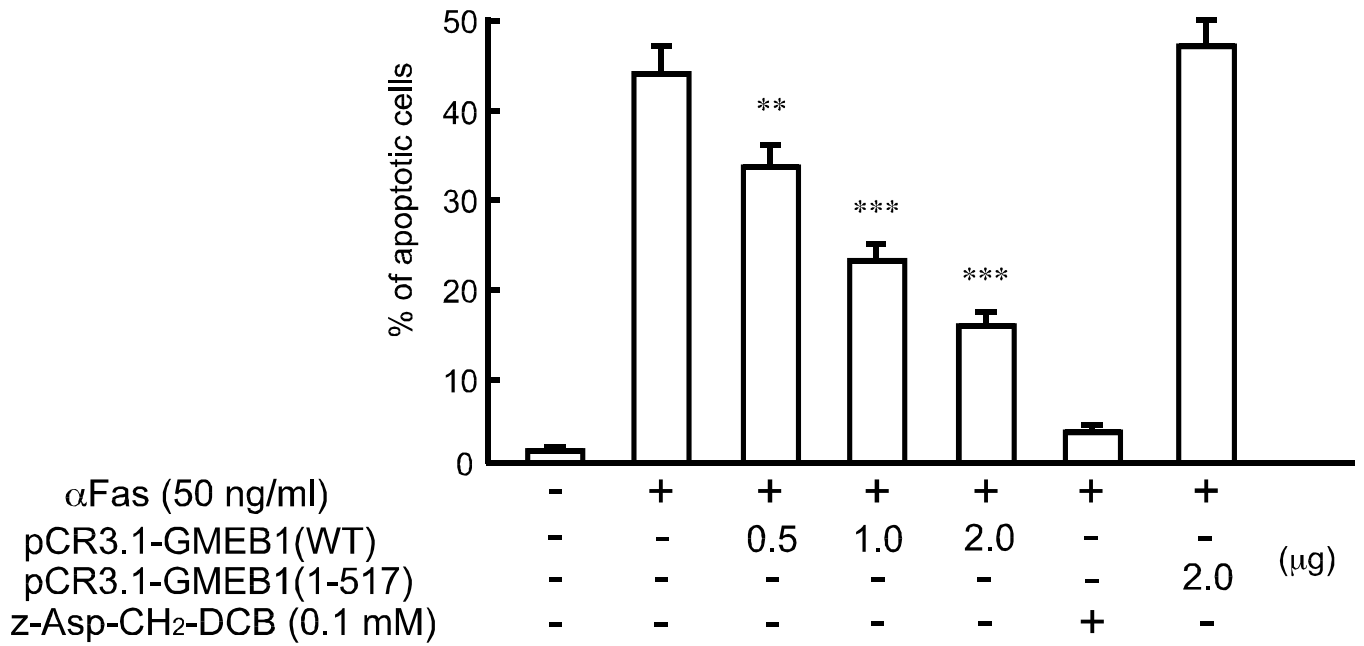


Figure 3. Tsuruma et al.

A



B



C

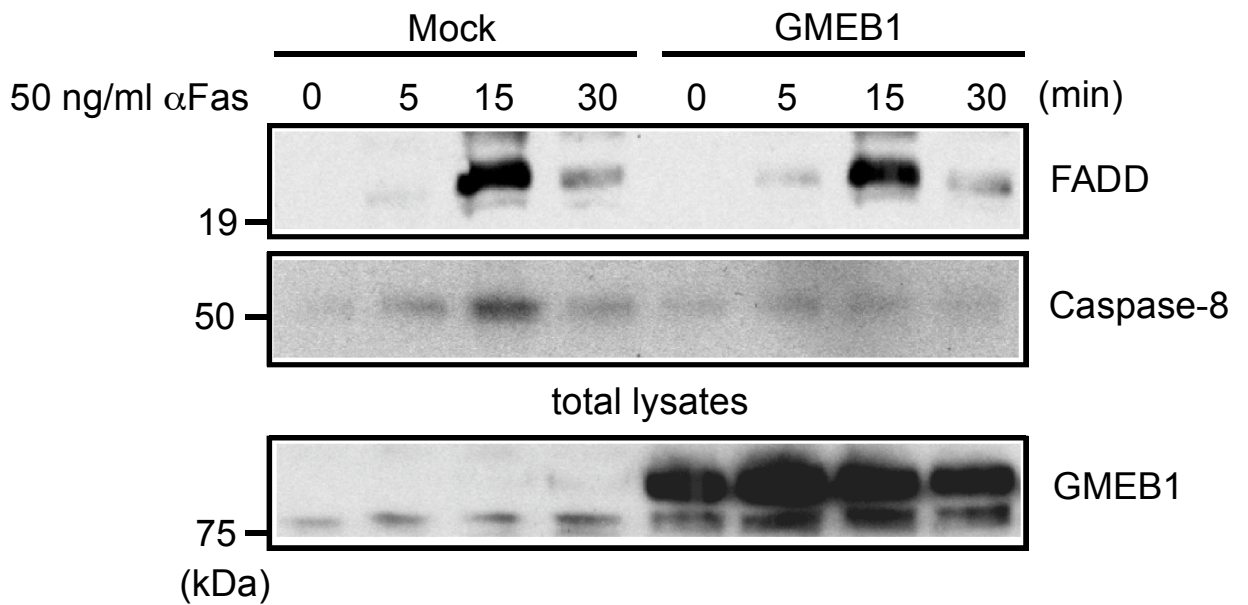


Figure 4. Tsuruma et al

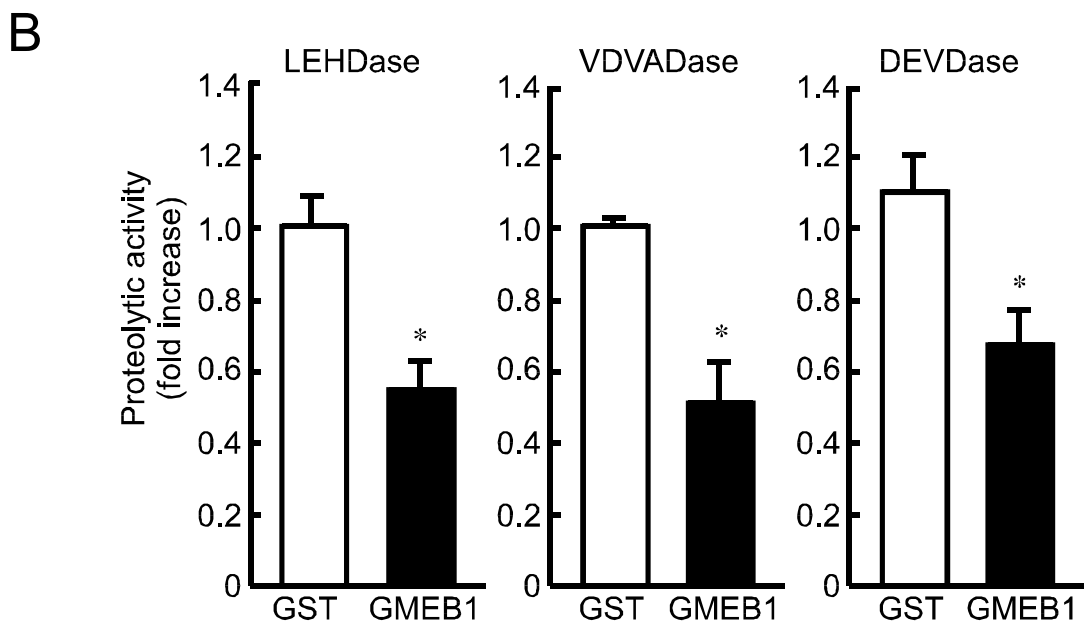
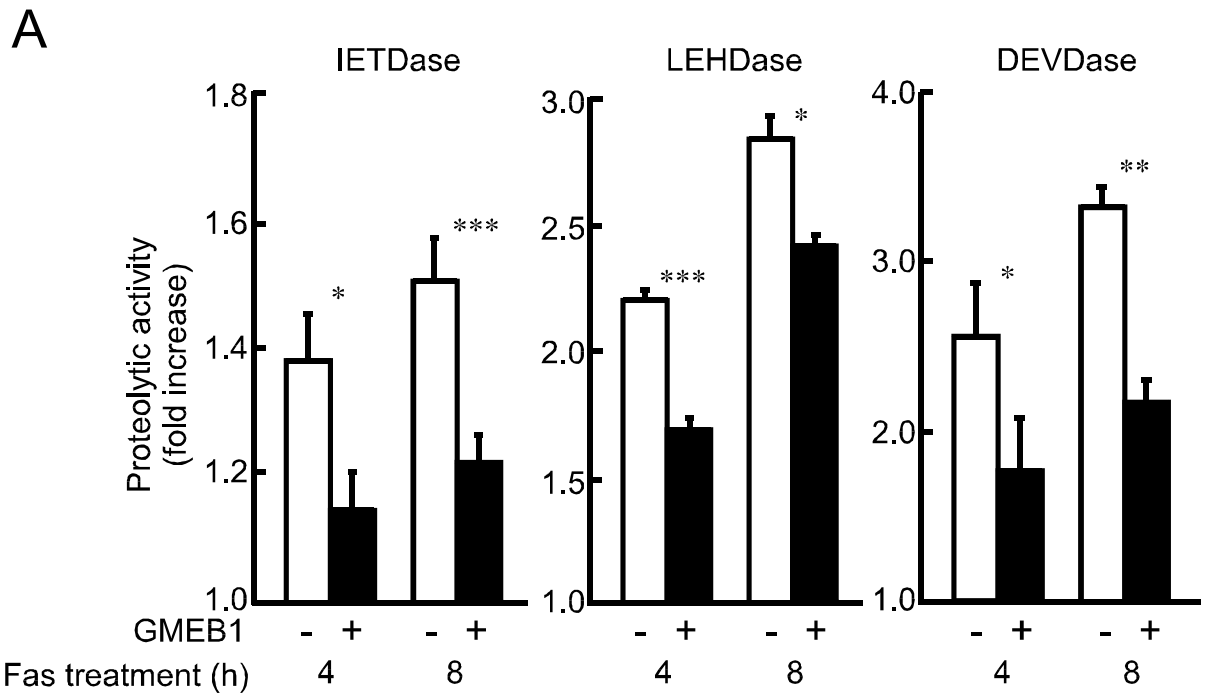


Figure 5. Tsuruma et al.

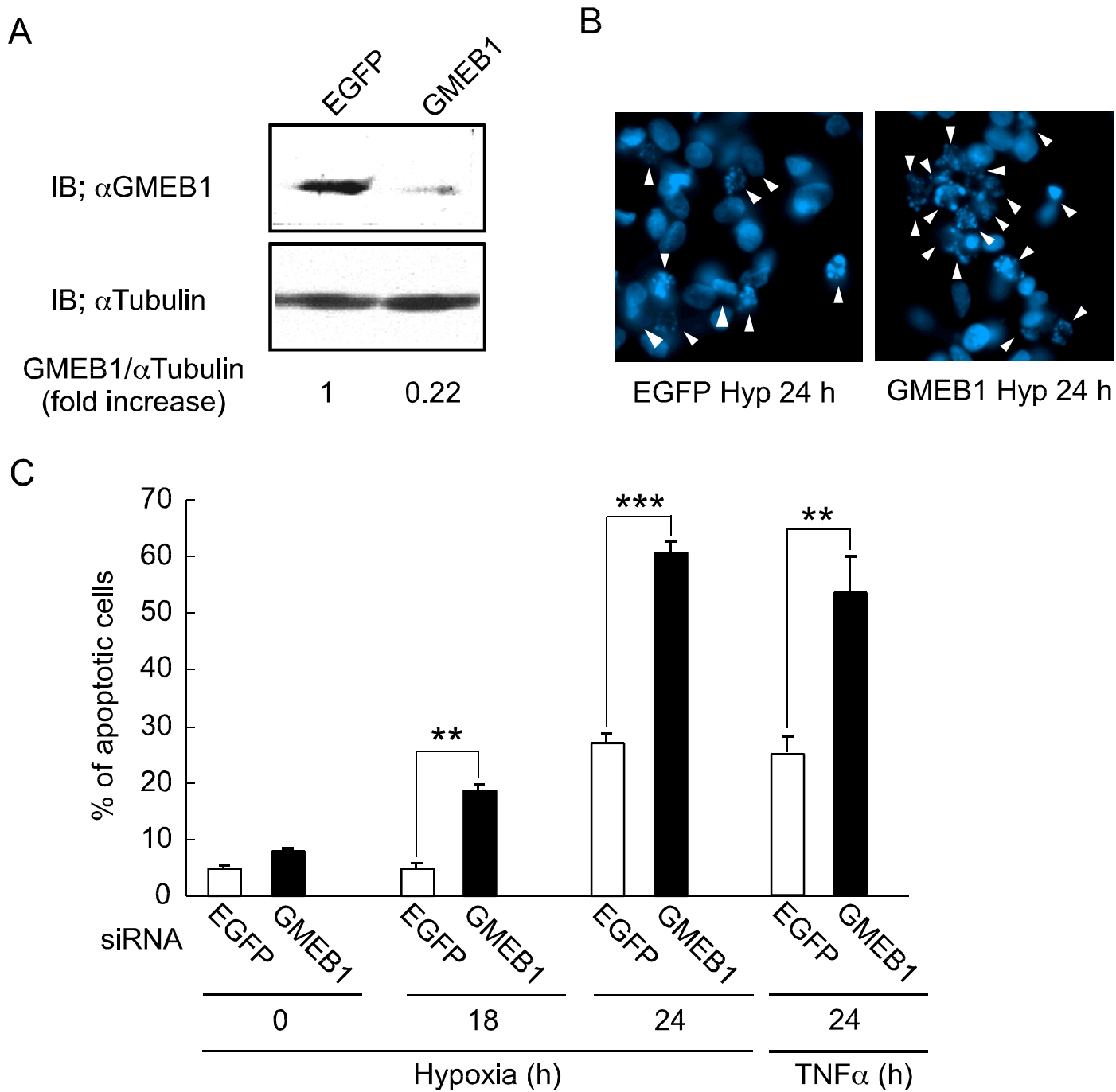
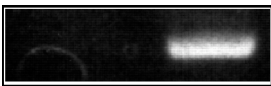


Figure 6. Tsuruma et al.

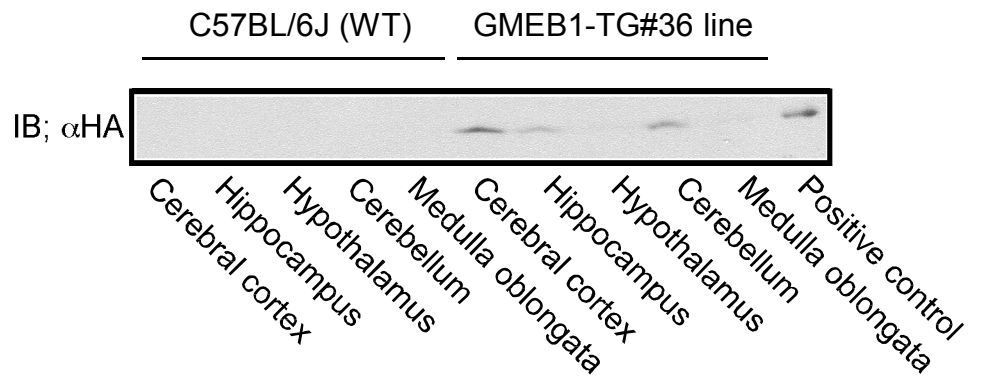
A



C57BL/6J (WT)

GMEB1-TG#36 line

B



IB; α HA

C57BL/6J (WT)

GMEB1-TG#36 line

Cerebral cortex

Hippocampus

Hypothalamus

Cerebellum

Medulla oblongata

Cerebral cortex

Hippocampus

Hypothalamus

Cerebellum

Medulla oblongata

Positive control

C

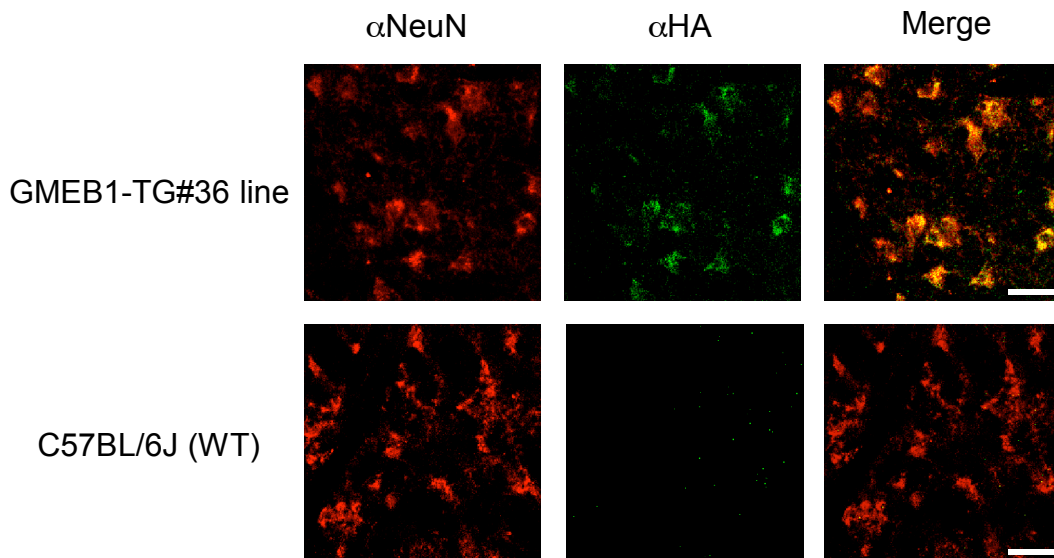


Figure 7. Tsuruma et al.

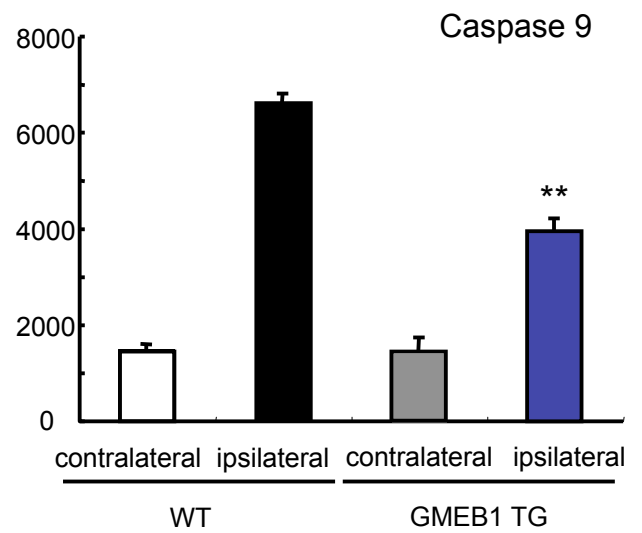
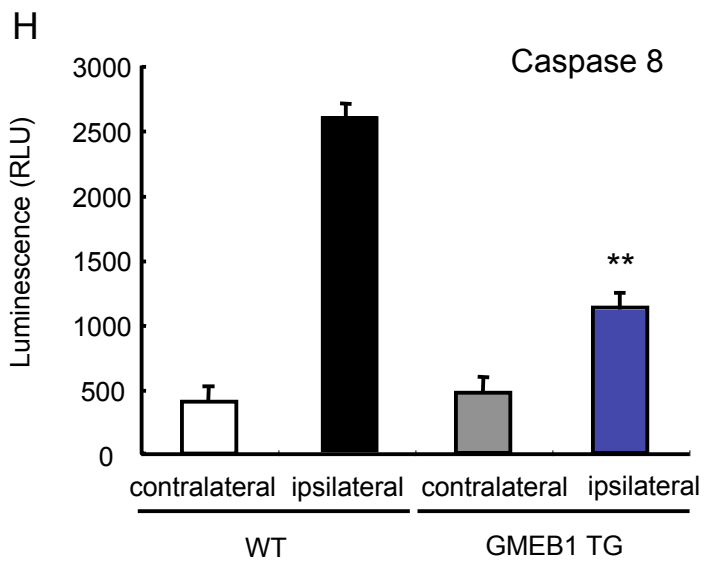
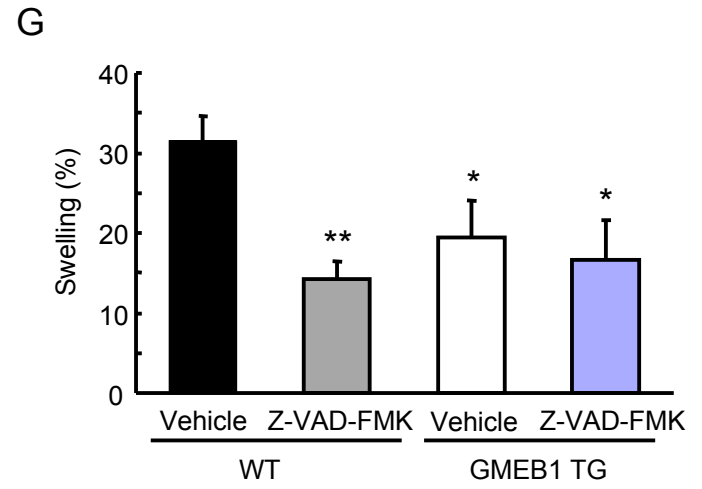
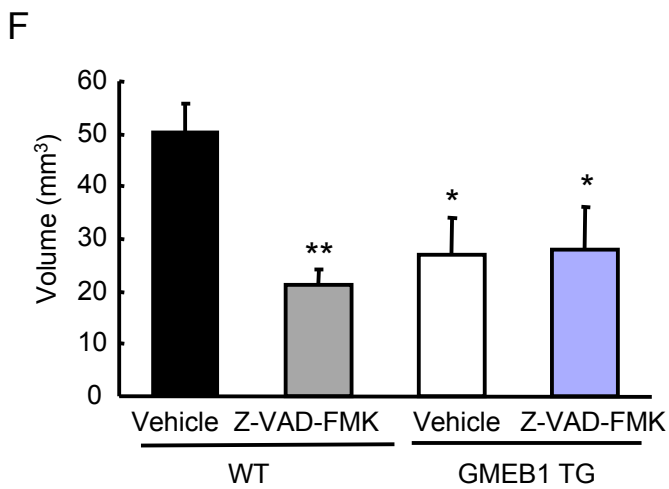
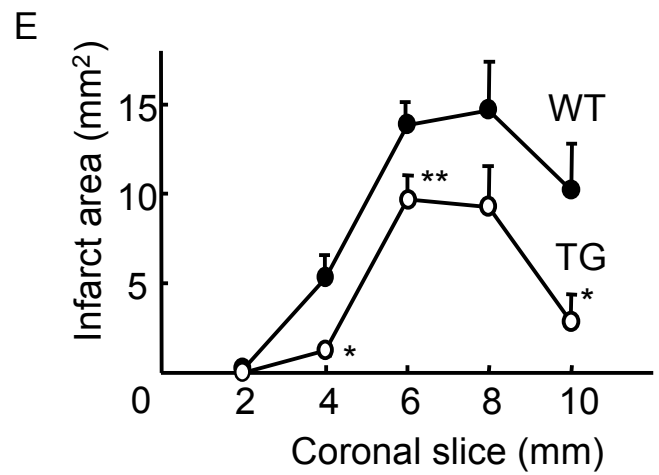
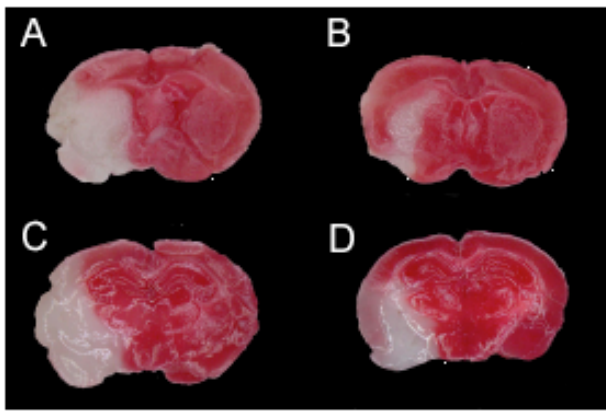


Figure 8. Tsuruma et al.