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Autism spectrum disorder is related to endoplasmic reticulum stress induced by mutations in the synaptic cell adhesion molecule, CADM1

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder with an unknown molecular pathogenesis. A recent molecular focus has been the mutated neuroligin 3, neuroligin 3(R451C), in gain-of-function studies and for its role in induced impairment of synaptic function, but endoplasmic reticulum (ER) stress induced by mutated molecules also deserves investigation. We previously found two missense mutations, H246N and Y251S, in the gene-encoding synaptic cell adhesion molecule-1 (CADM1) in ASD patients, including cleavage of the mutated CADM1 and its intracellular accumulation. In this study, we found that the mutated CADM1 showed slightly reduced homophilic interactions *in vitro* but that most of its interactions persist. The mutated CADM1 also showed morphological abnormalities, including shorter dendrites, and impaired synaptogenesis in neurons. Wild-type CADM1 was partly localized to the ER of C2C5 cells, whereas mutated CADM1 mainly accumulated in the ER despite different sensitivities toward 4-phenyl butyric acid with chemical chaperone activity and rapamycin with promotion activity for degradation of the aggregated protein. Modeling analysis suggested a direct relationship between the mutations and the conformation alteration. Both mutated CADM1 and neuroligin 3(R451C) induced upregulation of C/EBP-homologous protein (CHOP), an ER stress marker, suggesting that in addition to the trafficking impairment, this CHOP upregulation may also be involved in ASD pathogenesis.
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Autism spectrum disorder (ASD) is the most common heritable neurodevelopmental disorder, characterized by impaired social interactions, communication impairments, and restricted and repetitive behaviors; over 15 susceptibility loci are currently estimated,[1] but the major mode of inheritance remains unknown. Genetic testing in individuals with ASD has identified mutations in the genes encoding several synaptic cell adhesion molecules, including neuroligin (NLGN) 3 and 4, cell adhesion molecule-1 (CADM1), and contactin-associated protein-like 2.[2–4] NLGNs are postsynaptic cell adhesion proteins that interact with neurexins on the presynaptic membrane,[5] and they are required for synapse maturation.[6] Neurexin–NLGN interactions induce differentiation of γ-aminobutyric acid (GABA) and glutamate postsynaptic specializations.[7] The ASD-related mutant, NLGN3(R451C), is retained intracellularly, which limits its pre–post synaptic interactions.[8] Some of the mutated NLGN4 also fails to transport to the cell surface and is instead retained in the endoplasmic reticulum (ER).[9]

CADM1 (also known as RA175/SynCAM1) is a membrane glycoprotein belonging to the immunoglobulin (Ig) superfamily and is localized to both sides of the synaptic cleft. Its extracellular domain displays calcium-independent homophilic *trans*-cell adhesion activity,[10,11] and its intracellular domain associates with calmodulin-associated serine/threonine kinase via individual PDZ-binding domains (PDZ: post synaptic density protein/Drosophila disc large tumor suppressor/zonula occludens-1 protein).[10] Recently, we identified two missense mutations, C739A (amino acid: H246N) and A755C(Y251S), in the *CADM1* gene of male Caucasian patients with ASD and their family members.[3] Both mutations are located in the third Ig (Ig3) domain of CADM1, which is essential for *trans*-active interactions. These ASD-related mutations stimulate the cleavage of CADM1 and induce defective trafficking to the cell surface.[3] These results suggest an association between impaired synaptogenesis and the pathogenesis of ASD.

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Keywords: autism; Cadm1; ER stress
Abbreviations: ASD, autism spectrum disorder; CADM1, cell adhesion molecule-1; Ig, immunoglobulin; NLGN, neuroligin; CHOP, upregulation of C/EBP-homologous protein; PDZ, post synaptic density protein/Drosophila disc large tumor suppressor/zonula occludens-1 protein; eIF2α, eukaryotic initiation factor-2 α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; UPR, unfolded protein response; TSC, tuberous sclerosis complex; GABA, aminobutyric acid; 4-PBA, phenyl butyric acid; Thap, thapsigargin
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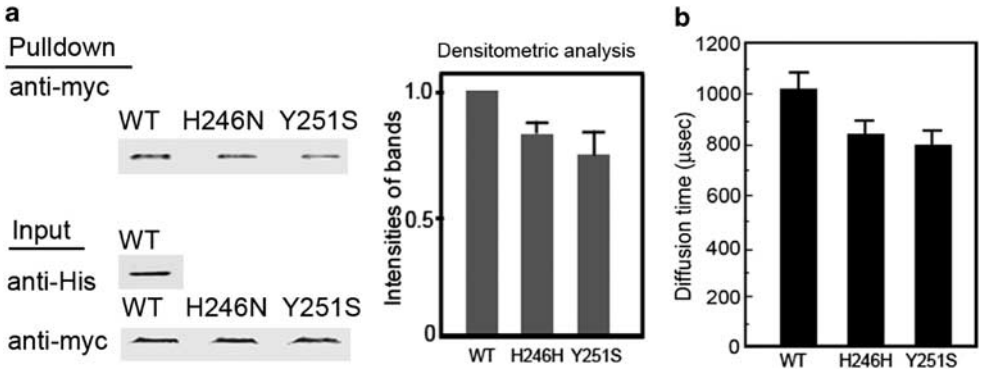

Figure 1 Effects of the mutations on the interaction activity. (**a**) Interaction between His-tagged wild-type (WT) CADM1 and myc-tagged WT or mutated CADM1. (left panel) Immunoblot analysis. Myc-WT and mutated (H246N- or Y251S-) CADM1 interacting with His-WT CADM1 were detected by western blot analysis using anti-myc antibody. Compared with the WT, the mutated CADM1 was not associated with His tagged-CADM1. (right panel) Densitometric analysis. Data from three experiments were scanned. Band intensities for the bound CADM1(H246N) and (Y251S) were normalized to the bound wild-type CADM1 and presented as the mean ± s.d. All experiments were performed three times, and typical data are shown. $P<0.05$ compared with wild-type. (**b**) FCS analysis of the protein–protein interaction between recombinant proteins of WT CADM1 or those of mutated CADM1. The interaction was detected by FCS using TAMRA-labeled molecules as probe. We examined the interaction between TAMRA-labeled and non-labeled WT-CADM1, TAMRA-labeled and non-labeled CADM1(H246N), and TAMRA-labeled and non-labeled CADM1(Y251S). All experiments were performed three times. Results are the mean ± s.d. of three different determinations. $P<0.05$ compared with wild-type

However, *nlgn3*-deficient mice do not show the core symptoms observed in patients with ASD.[6] In contrast, knock-in mice that express NLGN3(R451C), a mutation implicated in ASD,[12] do show behaviors analogous to the core symptoms of ASD, including impaired social behavior.[12,13] This finding suggests that both a loss-of-function and a gain-of-function mutation are involved in the pathogenesis of ASD. ER stress is a gain-of-function associated with the mutated protein.

The ER quality control system recognizes unfolded or misfolded proteins and activates stress-signaling pathways termed the unfolded protein response (UPR), via ER stress sensors.[14–16] The PKR-like ER kinase, one of the stress sensors in the ER, specifically upregulates the translation of the CAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP/Gadd153)[17] via phosphorylation of eukaryotic initiation factor-2 α (eIF2α). It is of interest that CHOP regulates synaptic function by regulating membrane trafficking[18] and that an eIF2α kinase, GCN2, controls synaptic plasticity, learning, and memory.[19,20] However, little is known regarding the association between ASD-related mutated molecules and ER stress.

In this study, we show that the ASD-related CADM1 mutations, H246N and Y251S, and the NLGN3 mutation, R451C, cause an UPR response with upregulation of CHOP as a gain of function.

Results

At first, we examined the *trans*-interaction between wild-type and mutated CADM1 molecules *in vitro* by pull-down and western blot analysis (Figure 1a). We compared the *trans*-interactions between bead-conjugated recombinant CADM1-His-tag proteins that lacked the transmembrane domain and either wild-type CADM1 or mutated CADM1 proteins (Figure 1a). Compared with the interaction between wild-type and wild-type, interactions between the mutant and wild-type proteins were slightly reduced and most of their interaction, 83.5% of CADM1(H246N) and 74.6% of CADM1(Y251S), remained. To evaluate this homophilic interaction quantitatively, we prepared recombinant extracellular domains of the wild-type and mutated CADM1 proteins and examined their interactions by fluorescence correlation spectroscopy (Figure 1b). Mutated CADM1 homophilic interaction activity was about 20 ± 5% weaker than that of wild-type CADM1.

We also modeled the chemical structure of CADM1 (Figure 2a) with a model in which the H246 and Y251 residues are located in the region that corresponds to the NH2-terminal strand of the Ig3 domain (amino acid sequence 160–324). The model showed that H246N and Y251S mutations represented reductions in the size of the side-chains of the NH2-terminal strand. Mutations also induced a conformational change (Figure 2b), and compared with H246N, the Y251S mutation induced a larger conformational change in the region.

To examine the relationship between a mutated CADM1 and the pathogenesis of ASD, we transfected myc-tagged wild-type CADM1 and H246N- or Y251S-mutated CADM1 sequences into neurons and examined their colocalization with synaptophysin, a marker of synapse. We excluded influences of the endogenous Cadm1 by using neurons isolated from Cadm1-deficient mouse embryos at embryonic day (E)16. Dendrites of the transfected neurons mainly showed three types of morphology (Figure 3, Table 1): most neurons that expressed wild-type CADM1 had long dendrites ($>100\,\mu$m) with synaptophysin-positive spines; neurons that expressed mutated CADM1 either had short dendrites ($<100\,\mu$m) without synaptophysin or no dendrites at all. Thus, neurons that expressed mutated CADM1 showed abnormal dendrites and impaired synaptogenesis.

Compared with the wild-type molecules, the mutated CADM1 more frequently showed intracellular accumulation. We examined the intracellular localization of wild-type CADM1 and CADM1(Y251S) proteins in transfected C2C5

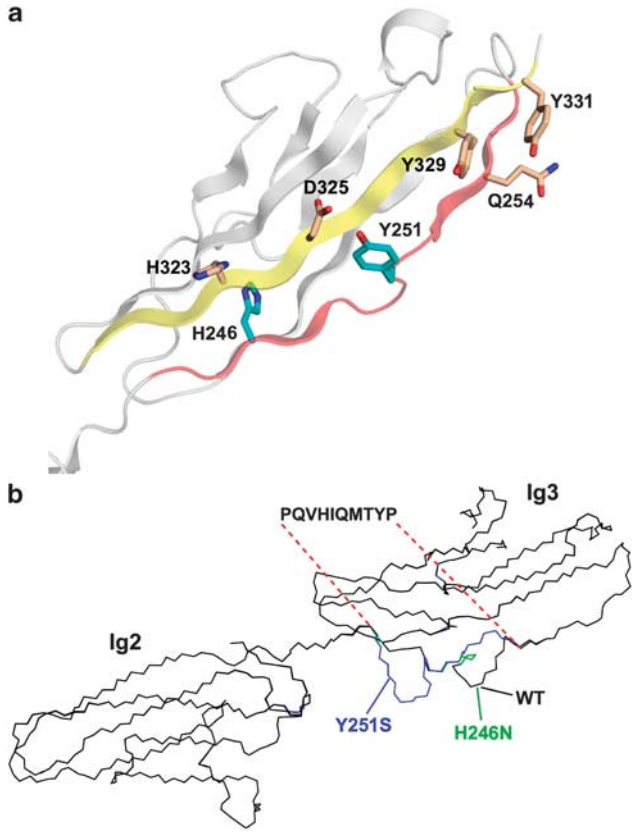

Figure 2 The modeled structure of mutated CADM1. (**a**) The Ig3 domain of CADM1, which was built using the Ig3 domain of NCAM as a template and registered in MODBASE.[39] The NH_2- and COOH-terminal strands of the model are red and yellow, respectively. The residues shown as sticks are possibly involved in the *trans*-interaction. The mutant residues, H246 and Y251, are in cyan. (**b**) The superimposed models of wild-type (black), H246N mutant (green), and Y251S mutant (blue) CADM1 using MOLMOL.[40] The amino acid sequence, PQVHIQMTYP, shows the altered region in the Y251S mutant compared with the wild-type

cells and neurons (Figure 4a). Wild-type CADM1 was partly colocalized with anti-KDEL, a marker for ER, whereas CADM1(Y251S) was predominantly accumulated in the ER. Wild-type CADM1 was also partly colocalized with beclin, which is localized on the various organelle including *trans*-Golgi, mitochondria, and ER,[21] whereas the accumulated CADM1(Y251S) was colocalized with ER-localized beclin (Figure 4b). The beclin was mainly localized on the ER under ER stress (Figure 4c), suggesting that the accumulation of CADM1(Y251S) in the ER caused the ER stress.

The cells expressing CADM1(Y251S) frequently showed an abnormal round cell morphology (Figures 4 and 5a). 4-phenyl butyric acid (4-PBA), a potential chemical chaperone in the ER[22] reduced the intracellular accumulation and the abnormal morphology of the cells expressing the mutated CADM1(Y251S) (Figure 5a and b). ER-localized beclin has a role in regulating autophagy.[21] Rapamycin, which stimulates the degradation of the accumulated protein via activation of the autophagosome,[23] inhibited the intracellular accumulation of CADM1(Y251S) (Figure 5a and b).

We also examined the mutated CADM1-induced CHOP upregulation downstream of the ER stress.[24,25] Both CADM1(H246N) and CADM1(Y251S) induced CHOP upregulation (Figure 6a), and CHOP-positive cells were more frequently detected in cells that expressed CADM1(H246) and (Y251S) (Figure 6b). In the cells expressing NLGN3(R451C), CHOP-positive cells are also more frequently detected compared with the cells expressing wild-type NLGN3 (Figure 6c). Thus, the mutated CADM1(H246) and (Y251S) proteins as well as the NLGN3(R451C) protein most likely stimulated ER stress, causing CHOP upregulation.

Discussion

ER stress and the mutated CADM1. The mutated CADM1 showed the most *trans*-interaction activity *in vitro* but accumulated in the ER and showed impaired trafficking, suggesting that the impaired synaptic function caused by defective trafficking of the mutated CADM1 could be related to the pathogenesis of ASD. However, *cadm1*-deficient mice[26] as well as *nlgn3*-deficient mice[6] did not show all of the core symptoms of ASD (Takayanagi *et al*., submitted elsewhere); in addition to infertility,[26] *cadm1*-deficient mice manifested abnormal anxieties and impaired ultrasonic vocalization but did not show the impaired social interaction and restricted stereotyped behaviors. In contrast with cadm1 in the testis, the loss of synaptic function of cadm1 in *cadm1*-deficient mice could be partly compensated by other members of the Cadm family. This possibility suggests that the loss of function of CADM1 or NLGN3 is one of the factors but not a sufficient factor alone to cause ASD. The gain of function of their mutated molecules could be another feature related to the pathogenesis of ASD.[27]

The ER stress is a gain of function associated with the mutated proteins.[25] The misfolded and unfolded proteins are checked under the surveillance of the ER quality control system in the ER, followed by processing by the ER-associated degradation (ERAD) system, but if degradation is not sufficient, they are accumulated and cause ER stress with UPR.[25] ER stress is the signal elicited by the quality control system in the initial stages of the UPR. Rapamycin inhibits ER stress by stimulating the activation of autophagosome formation,[25,28] which also contributes to the degradation of the accumulated mutated proteins on the ER membrane in cooperation with ubiquitin/proteasome-mediated ERAD.[23,29] The sensitivity of CADM1(Y251S) to the rapamycin suggests that ubiquitin/proteasome-mediated ERAD is not sufficient for its degradation. This inference is also supported by the altered conformation and the sensitivity to 4-PBA. Furthermore, the mutated CADM1 as well as NLGN3(R451C) caused CHOP upregulation, suggesting that the mutated CADM1 as well as NLGN3(R451C) were retained in the ER, causing ER stress with CHOP upregulation as the UPR. This will be one of the important issues in the future.

Relationship between ER stress and the pathogenesis of ASD. Chronic and excess ER stress leads to neuronal cell death and may be related to the pathogenesis of

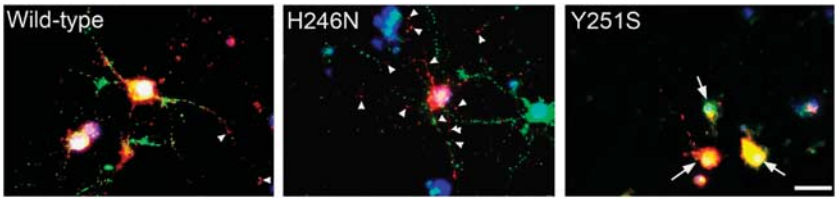

Figure 3 Localization of wild-type and mutated CADM1 in the neurons. Isolated neurons from *cadm1*-deficient mice embryos (E16) were transfected with wild-type and mutated CADM1 (H246N or Y251S). Their localization at the synapse was examined by immunostaining with anti-SynCAM (Cadm1; red), anti-synaptophysin (green), and Hoechst (blue). Closed arrowheads: CADM1 with no synaptophysin; arrows, neurons with short or no dendrites. Scale bars: 25 μm

Table 1 Percentages of the neurons expressing three types of dendrites in the neurons transfected with wild-type or mutated CADM1

Types[a] (8 DIV[b])	Wild-type	H246N	Y251S
Neurons with long dendrites, $>1000\,\mu$m	63.6 ± 6.0%	30.0 ± 2.9%	9.1 ± 1.8%
Neurons with short dendrites, $<1000\,\mu$m	27.3 ± 2.6%	40.0 ± 3.8%	36.4 ± 3.5%
Neuron with no dendrites, $<100\,\mu$m	9.1 ± 0.8%	30.0 ± 2.2%	54.5 ± 4.3%

The schematic illustration and evaluation of the length of the dendrites are described in Supplementary Figure S1. [a] Total dendritic length/cell. [b]DIV = days *in vitro*

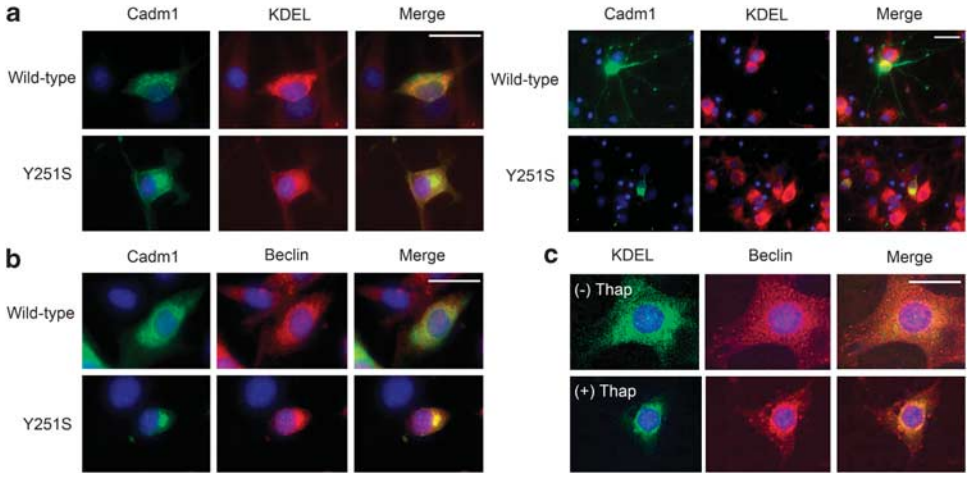

Figure 4 The localization of wild-type and mutated CADM1. (**a**) C2C5 cells and neurons were transfected with wild-type and mutated CADM1 (Y251S) for 26 h. After fixation, cells were immunostained with anti-SynCAM (Cadm1, green), anti-KDEL (red). (**b**) Transfected C2C5 cells were immunostained with anti-SynCAM (green), anti-beclin (red), and Hoechst (blue). (**c**) C2C5 cells were also treated with or without thapsigargin (Thap, 1 μM) for 24 h and were detected by anti-KDEL (green), anti-beclin (red), and Hoechst (blue). Scale bars: 20 μm

neurodegenerative diseases.[30] However, little is known about the relationship between ER stress and the pathogenesis of ASD. Other studies have shown that tuberous sclerosis complex (TSC), a neurogenetic disorder caused by a loss-of-function mutation in either the *TSC-1* or *TSC-2* genes, may also be related to ER stress. TSC frequently results in prominent central nervous system manifestations, including epilepsy, mental retardation, and ASD.[31,32] TSC-deficient cells have shown constitutive activation of mammalian target of rapamycin and proved to be highly susceptible to ER stress.[33] Thus, a wide variety of mutations that cause ER stress may be linked to the pathogenesis of ASD.

ASD may be the result of abnormal membrane trafficking of the synaptic functional molecules induced by ER stress. CHOP interacts with the heterodimeric receptors $GABA_{B1a}R$/$GABA_{B2}R$ and inhibits the formation of heterodimeric complexes; this results in intracellular accumulation and reduced cell surface expression of receptors.[18] In ASD patients, the $GABA_{B1}R$ level is significantly decreased in Brodmann area 9 and Brodmann area 40 of the cerebrum and cerebellum, whereas the $GABA_{B2}R$ level is significantly reduced in the cerebellum.[34] Therefore, it is possible that relatively low levels of ER stress may alter the intracellular transport of $GABA_BR$ to the cell surface by upregulation of CHOP without affecting the cell death of the neurons in the brain.

Abnormal morphology of neurons expressing mutated molecules may be due to the ER stress and ER stress-associated the abnormal membrane trafficking. At present, however, it is not clear whether the mutated molecules-mediated ER stress is linked with ER stress-mediated autophagosome activation in the pathogenesis of ASD.

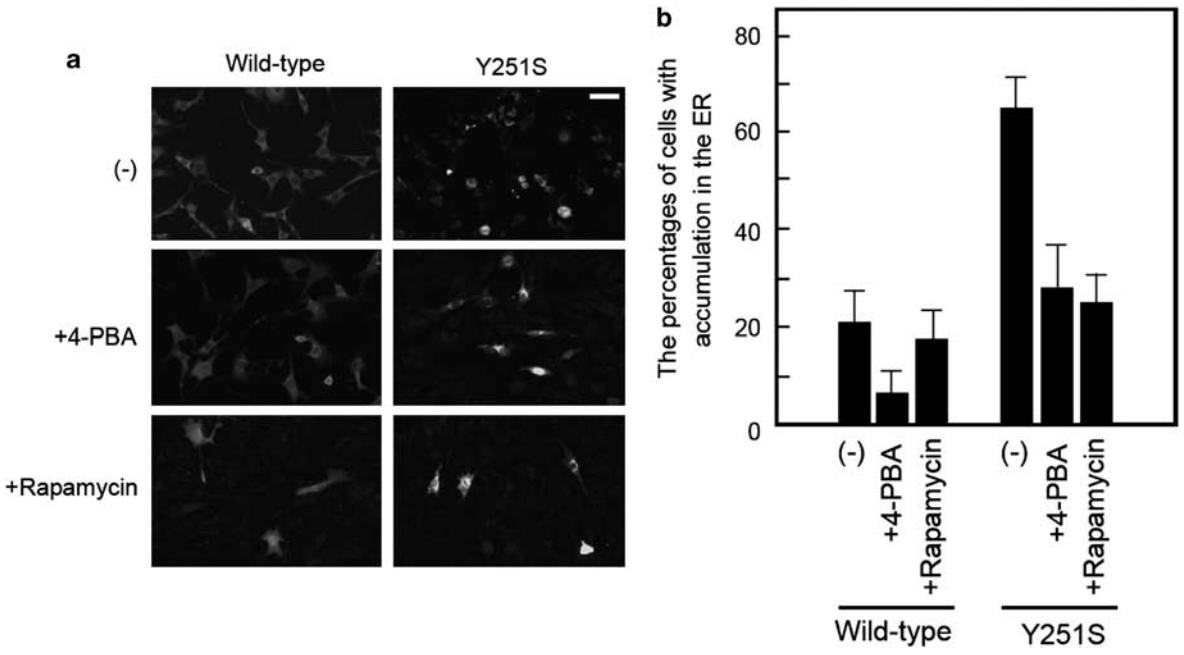

Figure 5 The effects of 4-phenyl butyric acid (4-PBA) or rapamycin on the intracellular accumulation of the mutated CADM1 in C2C5 cells. (**a**) The accumulation of the mutated CADM1 (Y251S) in the presence or absence of 4-PBA (7.5 mM) or rapamycin (10 μg/ml). C2C5 cells were transfected with wild-type and CADM1(Y251S) for 28 h and then fixed with paraformaldehyde and immunostained with anti-SynCAM (Cadm1). Scale bars: 50 μm. (**b**) The cell population showing accumulation of wild-type and mutated CADM1 (Y251S) in the presence or absence of 4-PBA or rapamycin. Three experiments were done. Bars indicate s.d.

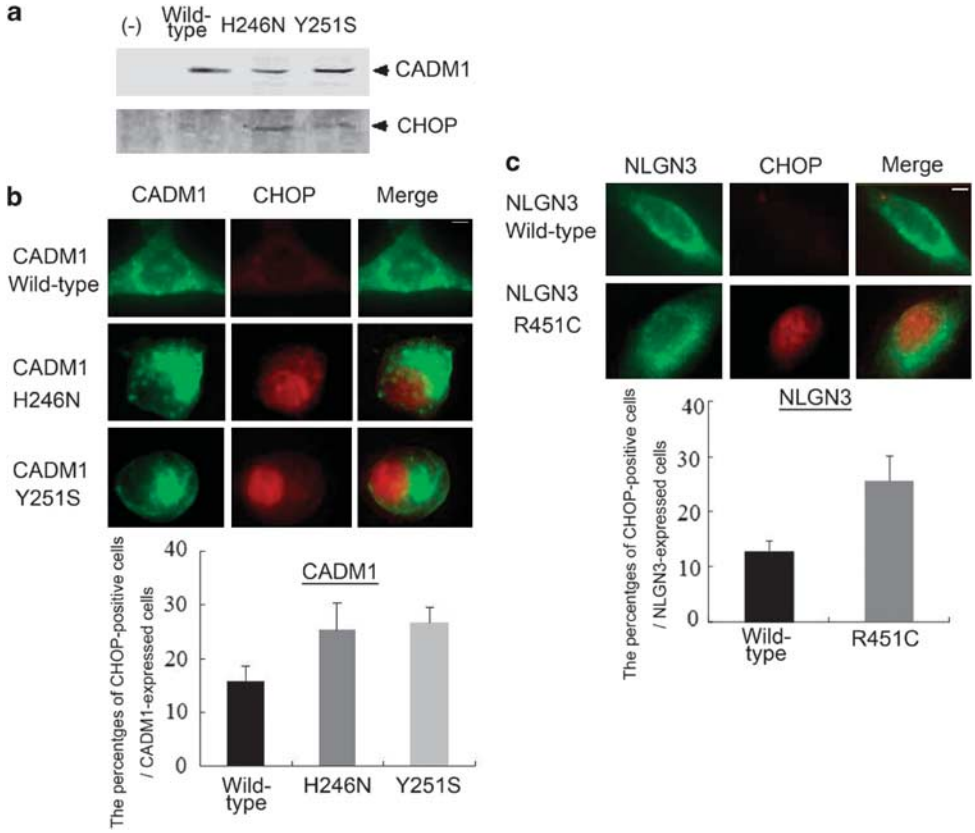

Figure 6 The expression of CHOP in the cells expressing the mutated CADM1 and the mutated neuroligin 3. (**a**) The mutated CADM1-induced upregulation of CHOP. C2C5 cells were transfected with wild-type, CADM1 (H246N), or (Y251S) cDNA and incubated for 24 h. The upregulation of CHOP was analyzed by immunoblot analysis using anti-CHOP. (**b**) The percentage of the CHOP-positive cells in the cells expressing wild-type or mutated CADM1s. (**c**) The percentage of the CHOP-positive cells in the cells expressing wild-type or R451C-mutated neuroligin (NLGN) 3. Scale bars: 5 μm. Bars indicate s.d.

Regulation of the mutated molecule-mediated ER stress will be another important issue in the future. Knock-in mice that express the mutated cadm1 related to the human CADM1 (H246N) or (Y251S) will provide more insight into the relationship between the ER stress and the pathogenesis of ASD.

Materials and Methods

Protein–protein interaction assay. His-tagged recombinant protein wild-type-CADM1 (48–334 a.a. including three Ig domains) lacking the transmembrane domain were prepared using silkworm cells (Katakura Industries Co., Tokyo, Japan).[35] His-tagged CADM1 (48–334 a.a.) was purified by Ni-column according to the manufacturer's protocol (Qiagen Science, Germantown, MD, USA).

Wild-type or mutated CADM1 in a pcDNA vector was transfected into COS cells using Lipofectamine 2000 (Invitrogen). After incubation for 28 h, the cells were lysed with PBS containing 1% Triton X-100, and then centrifuged at 12 000 r.p.m. for 20 min, COS-cell extracts. His-tagged recombinant Cadm1 (48–334) protein (2 μg protein) was incubated with the extracts (500 μg protein) from COS cells expressing wild-type, H246N-, or Y251S-mutated Cadm1 (48–334) -myc at 4°C overnight. The complexes were isolated from the incubation mixture by binding with the Ni-column and detected by immunoblot analysis using anti-myc (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and evaluated by densitometric analysis. Data from three experiments were scanned and analyzed for quantification with Image J software (National Institutes of Health).

Fluorescence correlation spectroscopy (FCS). TAMRA-labeled recombinant protein (mutated or wild-type Cadm1 (48–334 a.a. including three Ig domains)) lacking the transmembrane domain was prepared using the *in vitro* Pinpoint Fluorescence Labeling Kit 543).[36] TAMRA-labeled and non-labeled recombinant protein were purified using the RTS 100, *E. coli* HY Kit (Roche, Basel, Switzerland). FCS measurements were performed using an MF20 single molecule fluorescence detection system (Olympus, Tokyo, Japan). A helium–neon laser (543 nm) was used for the detection of TAMRA-labeled recombinant protein. TAMRA-labeled recombinant mutated or wild-type Cadm1 (4 nM) was mixed with non-labeled recombinant mutated or wild-type Cadm1 (0–40 nM) and added to the mixture in PBS with 0.05% Tween 20. After the mixtures were incubated at 37°C for 1 h, an aliquot (50 μl) of each sample was transferred to a microplate (24 $\times$ 16 wells, Olympus). A standard solution (MF-D543PX, Olympus) was used to derive the optical parameters necessary for a proper measurement. All measurements were carried out in more than duplicate and with 10 scans, each lasting 10 s at room temperature. The obtained data were fitted according to an autocorrelation function embodied in the accompanying software.

Modeling the structure of mutated CADM1. The structure of the Ig2 and Ig3 domains of wild-type and mutant CADM1 were built by SWISS-MODEL[37] using the crystal structure of MuSK (PDB entry: 2IEP) as a template and the amino acid sequence from 163–327 of CADM1. For the modeling of the H246N and Y251S mutants of CADM1, the input sequences were modified corresponding to their mutations.

Transfection of the wild-type and mutated CADM1-myc into neurons. Neurons were isolated from the brains of *cadm1*-deficient mice at embryonic day 16 as described.[38] Neurons were cultured using Neurobasal medium with 2% B27 supplement (Invitrogen, Carlsbad, CA, USA) and L-glutamine (0.5 mM). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 6 days *in vitro* (DIV), neurons were transfected with wild-type or mutated (H246N) or (Y251S) myc-tagged CADM1 using the calcium phosphate method and incubated for 2 DIV.

Immunostaining. For the immunostaining assay for intracellular localization of CADM1 and synaptophysin in the neurons and C2C5 cells, cells were transfected with wild-type and H246N- or Y251S-mutated pcDNA–CADM1 in the presence or absence of 4-PBA (7.5 mM) or rapamycin (10 μg/ml), and fixed in 4% paraformaldehyde, washed with PBS, and then incubated with mouse anti-synaptophysin (Sigma, St Louis, MO, USA), mouse anti-KDEL (Stressgen Biotechnologies Corp., Victoria, BC, Canada), rabbit anti-beclin (Cell Signaling Technology, Beverly, MA, USA), mouse anti-CHOP (Santa Cruz), or chicken anti-SynCAM1 (Cadm1) (MBL, Nagoya, Japan) overnight at 4°C. Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies against chicken and mouse or rabbit IgG were purchased from Molecular Probes (Eugene, OR, USA). Nuclei were detected by Hoechst 33342 staining (Molecular Probes). The reactivity was viewed using a confocal laser-scanning microscope (CSU-10, Yokogawa, Yokokawa Electric Co., Tokyo, Japan).

Conflict of interest

The authors declare no conflict of interest.

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