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p31, the mammalian orthologue of yeast Use1p, is an endoplasmic reticulum (ER)-localized soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE) that forms a complex with other SNAREs, particularly syntaxin 18. However, the role of p31 in ER function remains unknown. To determine the role of p31 in vivo, we generated p31 conditional knockout mice. We found that homozygous deletion of the p31 gene led to early embryonic lethality before embryonic day 8.5. Conditional knockout of p31 in brains and mouse embryonic fibroblasts (MEFs) caused massive apoptosis accompanied by upregulation of ER stress-associated genes. Microscopic analysis showed vesiculation and subsequent enlargement of the ER membrane in p31-deficient cells. This type of drastic disorganization in the ER tubules has not been demonstrated to date. This marked change in ER structure preceded nuclear translocation of the ER stress-related transcription factor C/EBP homologous protein (CHOP), suggesting that ER stress-induced apoptosis resulted from disruption of the ER membrane structure. Taken together, these results suggest that p31 is an essential molecule involved in the maintenance of ER morphology and that its deficiency leads to ER stress-induced apoptosis.

Several proteins have been implicated in the regulation of ER structure. The loss of function of molecules including BNIp1, p97, and p37 involved in ER morphology leads to the loss of three-way junctions; however, the tubular structure of the ER is relatively unaffected (20, 28, 29). Vedrenne and Hauri proposed the mechanisms underlying ER network formation as follows (31): ER membranes are pulled along MTs by MT plus end-directed kinesin-type motor kinesin-1 (8), and the resulting membrane extensions are stabilized by the cytoskeleton-linking ER membrane protein of 63 kDa (13, 30). If ER membranes get close to each other, they fuse in a p97-dependent manner (29). Fusion is further facilitated by the MT-associated p22 that binds to the ER, and thereby ER reticulation is promoted (2). However, the mechanism maintaining the tubular structure of the ER remains unclear.

p31, an ER-localized soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE), is a mammalian orthologue of yeast Use1p/Slt1p implicated in Golgi complex-to-ER transport (4, 5, 7). We previously reported that p31 forms a complex with two ER SNAREs, syntaxin 18 (an orthologue of yeast Ufe1p) (11) and BNIp1 (an orthologue of yeast Sec20p) which is involved in the formation of three-way junctions of the ER (20). In yeast, Ufe1p (syntaxin 18) was known to form a complex with Cdc48p, which participates in homotypic ER-ER membrane fusion (16, 23). p31 is a component of the syntaxin 18 complex, where other components are involved in various ER functions, suggesting that p31 also plays various ER functions. Thus, to clarify the biological functions of p31, we generated p31 knockout mice.

We found that p31 deficiency led to severe disorganiza-
All animals were bred in the Institute of Animal Experience Research of the Animal Care and Experimentation Committee of Gunma University, and animal procedures were performed in accordance with the guidelines of the protocols as previously described (24). Briefly, the p31 allele obtained by crossing with Flp transgenic mice (floxed), and null p31 allele (−−) obtained after Cre-mediated excision of exons 5 to 8. The wild-type p31 gene consists of eight coding exons (blue boxes) on chromosome 8. In the targeting vector, twoloxP sites (green triangles) were introduced into introns flanking exons 5 and 8, and an Flp recombination target (red triangles)-flanked SA-IREs-geo-poly(A) cassette was inserted between exon 4 and exon 5. In mice with β-geo cassettes in both alleles of the target gene (geo/geo), the transcription of the gene is expected to be interrupted by the strong bcl2 SA and terminated by the poly(A) signal at the end of the cassette. As a result, the expression of the gene is expected to be markedly attenuated. By crossing the geo/+ mice with transgenic mice that express Flp recombinase ubiquitously, the SA-IREs-β-geo-poly(A) cassette is expected to be excised (floxed/floxed), and the expression of the gene will be recovered. We use the resulting floxed/floxed mice to generate conditional knockouts by crossing them with Cre transgenic mice.

**MATERIALS AND METHODS**

**Construction of targeting vector and establishment of the knockout mouse.** All animal procedures were performed in accordance with the guidelines of the Animal Care and Experimentation Committee of Gunma University, and all animals were bred in the Institute of Animal Experience Research of Gunma University. p31 knockout mice were generated largely according to the protocols as previously described (24). Briefly, the p31 gene was isolated from a mouse genomic bacterial artificial chromosome library derived from the 129SvJ mouse strain (RPCI-22; Children’s Hospital Oakland Research Institute). In the targeting vector, an Flp recombination target (red triangles)-flanked SA-IREs-geo-poly(A) cassette was inserted into intron 8. This construct was used for the generation of p31 allele. To generate nullizygous mice, we crossed p31flox/flox mice with cytomegalovirus-Cre transgenic mice (Jackson Laboratory). For genotyping by PCR analysis, the primers used were as follows: Primer 1 (5′-TTAACCCTCACTGAGGGAAGCAG-3′), Primer 2 (5′-AGGCAAA GATGACCTGGGACCTC-3′), Primer 3 (5′-CCGTACAGTCCACAA AGGCAATCC-3′), Primer 4 (5′-CCCTTGTACTTGGGACCAACCTCTGC-3′), and Primer 5 (5′-ACTAGGGGTTGGAAACCTAGATGTGC-3′). Primers 1 and 2 detected the wild-type allele. Primers 1 and 3 detected the geo allele. Primers 4 and 5 detected the null allele.

**Cell culture.** p31floxed/flox mice generated by mating between p31flused/floxed mice and p31+/− mice were dissected on embryonic day 13.5 (E13.5), and mouse embryonic fibroblasts (MEFs) were isolated by trypsinization of embryos. MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO2/95% air atmosphere. Adenoviruses were prepared using an adenovirus Cre/loxP kit version 2.0 (Takara) according to the manufacturer’s instructions. Electroporation was carried out using MEF nucleofector kit 1 (Amaxa Biosystems) according to the manufacturer’s instructions.

**Western blot analyses and immunoprecipitation.** Western blot analyses were carried out as described previously (11). To prepare MEF lysates for immunoprecipitation, approximately 90% confluent cells grown on 60-mm dishes were lysed with lysis buffer (20 mM HEPES-KOH, pH 7.2, 150 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, and protease inhibitor cocktail [Nacalai Tesque]). The lysates were centrifuged in a microcentrifuge at 15,000 rpm for 10 min. The immunoprecipitation experiment was carried out as described previously (11). Western blot analyses for ATF6, IRE1α, and PERK were carried out as described previously (21).
Antibodies and probes. The following antibodies were used: anti-protein disulfide isomerase (anti-PDI) (Stressgen), anti-BiP (Affinity BioReagents), anti-green fluorescent protein (anti-GFP) (Nacalai Tesque), anti-CHOP (Santa Cruz Biotechnology), fluorescein isothiocyanate-labeled anti-a-tubulin (Sigma), anti-GS28 (BD Transduction Laboratories), anti-LAMP2 (clone Abl 93; Developmental Studies Hybridoma Bank), anti-Ire1α (Cell Signaling Technology), anti-PERK (Rockland), and anti-Sec13 (a gift from W. Hong, Institute of Molecular and Cell Biology, Singapore). Polyclonal antibodies against p31, BNIP1, ZW10, RINT-1, Bap31, and a monoclonal anti-syntaxin V(1871) on August 12, 2012 by OSAKA UNIV http://mcb.asm.org/ Downloaded from
FIG. 4. p31 deletion leads to ER stress. (A and B) RT-PCR analysis of ER stress-associated molecules in brains (A) and MEFs (B). Black and white arrowheads indicate spliced and unspliced forms of XBP-1, respectively. GAPDH was used as an internal control. (A) RT-PCR analysis of E17.5 brains from control mice (Ctrl) and CNS-specific knockout mice (cKO). (B) RT-PCR analysis of p31flox/ (Ctrl) or p31flox/ (cKO) MEFs infected with Ad-Cre. “dX” represents samples prepared on day X after adenovirus treatment. As a positive control, MEFs were treated with tunicamycin for 24 h (TM). (C) Quantitative RT-PCR to evaluate level of BiP mRNA showed increased expression in p31-deficient MEFs (days 2, 4, and 6) and embryonic brains (E17.5). The ratio of BiP mRNA to G6PDX (glucose 6-phosphate dehydrogenase X-linked) calculated from three independent samples, were normalized against data of control. P values were determined by Student’s t test. (D to F) Western blot analyses showed increased cleavage of ATF6α (D) and increased phosphorylation of PERK (E) and IREα (F). pATF6α(P), pATF6α(P)*, and pATF6α(N) indicate full-length ATF6α, nonglycosylated full-length ATF6α, and cleaved form of ATF6α, respectively.

RESULTS

p31 is an essential molecule for cell viability. To generate p31 conditional knockout mice, we used a revertible knockout system as described previously (24) (Fig. 1A and B). First, we analyzed conventional knockout mice that lack p31 in the whole body (p31 proc/proc and p31−/−). Conventional knockout mice showed early embryonic lethality before E8.5 (Fig. 1C). We therefore sought to generate tissue-specific knockout mice...
to determine the role of p31. To this end, p31<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> mice were generated by intercrossing between p31<sup>geo</sup>/H11001 mice that had been obtained by mating p31<sup>geo</sup>/H11001 mice with transgenic mice expressing Flp recombinase. p31<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> mice were born at Mendelian frequency and were indistinguishable from wild-type mice (data not shown). By breeding p31<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> or p31<sup>fl<sub>ox</sub>/+</sup> mice with transgenic mice expressing Cre recombinase under the control of the Pcp2 promoter (Pcp2-cre) and nestin promoter (Nestin-cre), we generated Purkinje cell-specific and central nervous system (CNS)-specific conditional knockout mice, respectively.

Mice lacking p31 in Purkinje cells (p31<sup>fl<sub>ox</sub>/+</sup>; Pcp2-cre) displayed cerebellar ataxia, such as abnormal gait, after 10 weeks postnatal. Hematoxylin and eosin staining of the cerebellum showed a significant decrease in the number of Purkinje cells (Fig. 2A). CNS-specific knockout mice (p31<sup>fl<sub>ox</sub>/fl<sub>ox</sub>; Nestin-cre) died within 1 day of birth, although the knockout was not lethal during embryogenesis (data not shown). Marked changes in

FIG. 5. p31 deletion causes vesiculation and fusion of the ER. (A) p31<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> (Ctrl) or p31<sup>fl<sub>ox</sub>/+</sup> (cKO) MEFs infected with Ad-Cre were stained with the antibody against PDI. The ER vesiculates at 1 day after Ad-Cre treatment. The vesiculated ER grows larger to form large vacuoles with time. (B) Cells with aberrant ER (vesiculation and vacuolation) among total cells were already observed 0 days after Ad-Cre treatment and increased with time. The error bars represent the mean ± the standard deviation (SD) (n = 3). (C) p31 deficiency caused ER vesiculation followed by ER vacuolation. The percentage of cells with vesiculated or vacuolated ER membranes (mean ± SD) was calculated from three independent samples. (D) p31<sup>fl<sub>ox</sub>/fl<sub>ox</sub></sup> (Ctrl) or p31<sup>fl<sub>ox</sub>/+</sup> (cKO) MEFs infected with Ad-Cre were double stained with antibodies against Bap31 (green) and PDI (red) 6 days after adenovirus treatment. Bar, 10 μm.
FIG. 6. ER aberration neither results from MT disruption nor results in apparent disruption of the Golgi complex and lysosome. $p31^{fllox/+}$ (Ctrl) or $p31^{fllox/-}$ (cKO) MEFs infected with Ad-Cre were double stained with antibodies against tubulin (green) and PDI (red) (A), GS28 (green) and BiP (red) (B), and LAMP2 (green) and BiP (red) (C) 6 days after adenovirus treatment. Bars, 10 μm.
brain architecture, such as dilated ventricles and a decrease in the thickness of the cerebral cortices, were observed in the embryonic brain (Fig. 2B). To clarify the cause of this phenotype, we performed TUNEL staining. As shown in Fig. 2C, a large number of apoptotic cells were observed in the brains of the p31\textsuperscript{flox/flox}; Nestin-\textsuperscript{cre} mice, suggesting that apoptosis is the cause of the abnormal brain morphology.

The p31 deficiency caused ER stress. To confirm the essentiality of the p31 gene, we deleted p31 in p31\textsuperscript{flox/−} MEFs using an adenovirus encoding Cre recombinase (Ad-Cre). Immunoblotting revealed that the expression of p31 remarkably decreased 2 days after Ad-Cre treatment (Fig. 3A). On the other hand, the expression of other syntaxin 18 complex components, such as syntaxin 18, BNIP1, ZW10, and RINT-1, were unchanged in Ad-Cre-treated cells (Fig. 3B).

We previously demonstrated that p31 forms a subcomplex with ZW10 and RINT-1 in a large syntaxin 18 complex as illustrated in Fig. 3C (11). Consistent with this, the p31 deficiency caused dissociation of ZW10 and partial dissociation of RINT-1 from syntaxin 18 (Fig. 3B). The incomplete dissociation of RINT-1 from syntaxin 18 may be attributed to the interaction of RINT-1 with BNIP1, another syntaxin 18-associated SNARE (20).

Similar to neurons in CNS-specific p31 knockout, the number of TUNEL-positive cells was larger in p31\textsuperscript{flox/−} MEFs infected with Ad-Cre than that in p31\textsuperscript{flox/−} MEFs infected with a control adenovirus encoding LacZ (Ad-LacZ), indicating the occurrence of apoptosis in these cells (Fig. 3D). To understand the mechanism by which apoptosis is induced in p31-deficient cells, we examined whether ER stress was induced by p31 depletion. We investigated the expression levels of ER stress-associated molecules (CHOP/GADD153, the spliced form of XBP-1, CA-VI [type B], BiP) (9, 12, 22). RT-PCR analysis revealed that the expression levels of these molecules were upregulated in p31-deficient brains (Fig. 4A and C) and MEFs (Fig. 4B and C).

Under ER stress, two ER stress sensors, PERK and IRE1, are known to be phosphorylated, and another ER stress sensor, ATF6, is cleaved. In p31-deficient MEFs, we observed increased cleavage of ATF6\textsuperscript{α} and increased phosphorylation of PERK and IRE1\textsuperscript{α} (Fig. 4D to F), further confirming that p31 deficiency caused ER stress.

The p31 regulates the ER morphology in MEFs. Next, we investigated the ER morphology in p31-deficient MEFs by immunofluorescence microscopy using an antibody against a luminal ER protein, PDI. At an early stage of p31 depletion, the majority of the cells showed vesiculated ER structures, and as time progressed, the percentage of the cells with vacuolated ER structures increased, whereas that of the cells with vesiculated ER structures decreased (Fig. 5A to C), suggesting the fusion of the ER vesicles. No change in ER morphology was observed in p31\textsuperscript{flox/−} MEFs infected with Ad-Cre or in p31\textsuperscript{flox/−} MEFs infected with Ad-LacZ (data not shown), ruling out the possibility that this morphological change of the ER was due to adenovirus infection.

To determine whether the aberrant staining pattern of PDI reflects aggregation of PDI within the ER or a change in the ER structure itself, we used Bap31 as a marker of the ER membrane. As shown in Fig. 5D, Bap31 was located on the membranes of vacuolated structures stained by anti-PDI antibody, suggesting that p31 deficiency caused the swelling of ER membrane.

FIG. 7. ER structure in p31-deficient MEFs. (A) Electron microscopic analysis of p31\textsuperscript{flox/−} MEFs infected with Ad-LacZ (Ctrl) or Ad-Cre (cKO) was performed 2 or 6 days after adenovirus treatment. The lumen of the ER markedly dilated 6 days after p31 depletion, whereas no change was observed in the control MEFs. Bar, 2 μm. (B) The same images as those shown in panel A, except that the ERs were labeled with pseudocolor.
The ER is linked to MTs with various proteins; thus, we sought to investigate MT organization and the relationship between MTs and the aberrant ER. The organization of MTs is similar between p31-deficient cells and control cells, indicating that ER aberration does not result from MT disruption (Fig. 6A).

To investigate the effects of p31 deficiency on the morphologies of the Golgi complex and lysosome, we stained MEFs with antibodies against GS28 or LAMP2. The staining patterns of GS28 and LAMP2 were relatively unchanged in cells with an abnormal ER (Fig. 6B and C).

Electron microscopic analysis showed an abnormal ER structure in p31-deficient MEFs and brains. To analyze the intracellular membrane structures in more detail, we carried out electron microscopy. We observed numerous small and round ERs at an early stage of p31 depletion (2 days after adenovirus treatment), and ER “vesicles” became significantly larger and fewer at the late stage of p31 depletion (6 days after adenovirus treatment).

FIG. 8. Characterization of abnormal ERs by electron microscopy. (A) Immunogold labeling of PDI was found in the vesiculated ER of p31-deficient MEFs. p31flox^/- MEFs infected with Ad-Cre were observed 6 days after adenovirus treatment. (B) The Golgi complex is not disrupted in p31-deficient MEFs. p31flox^/- MEFs infected with Ad-LacZ (Ctrl) or Ad-Cre (cKO) were observed 6 days after adenovirus treatment. (C) Enlargement of ERs (arrows) was also observed in neurons from the E16.5 brains of CNS-specific knockout mice (cKO) but not in neurons of control mice (Ctrl). Normal ERs are indicated by arrowheads. Bars, 500 nm.

FIG. 9. Disorganization of the ER caused by p31 deficiency led to ER stress response. (A) p31flox^/+ (Ctrl) or p31flox^/- (cKO) MEFs infected with Ad-Cre were double stained with antibodies against PDI (red) and CHOP (green). “day3” represents, e.g., samples prepared on day 3 after adenovirus treatment. The arrow indicates a cell with nuclear translocation of CHOP. (B) The ratio of the number of cells showing nuclear translocation of CHOP versus the number of cells showing the aberrant staining pattern of PDI increased in a time-dependent manner in p31-deficient cells. Note that no CHOP nuclear staining was observed at day 0 after Ad-Cre treatment when aberrant ER was already observed. The error bars represent the mean ± the standard error of the mean (n = 3). Bar, 10 μm (A).
adenovirus treatment) (Fig. 7). Immunoelectron microscopic analysis using an anti-PDI antibody unequivocally demonstrated that vesiculated membranes are derived from the ER (Fig. 8A). In contrast to the significant morphological change of the ER, the shape of the Golgi complex in p31-deficient cells was similar to that of control cells (Fig. 8B). To confirm whether p31 deficiency changes ER morphology in vivo, we next investigated ER morphology in neurons of CNS-specific knockout mice. We observed enlargement of the ER in p31-deficient neurons as observed in p31-deficient MEFs (Fig. 8C). These observations suggest that p31 is a molecule involved in the maintenance of the ER structure and that the aberrant ER

FIG. 10. ER-to-Golgi complex protein transport is impaired in p31-deficient MEFs. (A, C, and D) p31\textsuperscript{flox/−} MEFs were infected with Ad-LacZ (Ctrl) or Ad-Cre (cKO), followed by infection with another adenovirus encoding VSVG-GFP. Four days after the first adenovirus treatment, they were fixed 60 min after a temperature shift to a permissive temperature and double stained with anti-GFP and anti-BiP antibodies. The distribution of VSVG, which is detected by staining with the anti-GFP antibody, is shown in panel A. (C and D) Double-stained images of two cells in panel A (cKO) at higher magnification. (B) p31\textsuperscript{flox/+} (Ctrl) or p31\textsuperscript{flox/−} (cKO) MEFs infected with Ad-Cre were infected with adenovirus encoding MDR1-GFP and were stained using anti-GFP antibody. (A to D) Bars, 10 \mu m. (E and F) VSVG-expressing control MEFs (Ctrl) and p31-deficient MEFs (cKO) were treated as described above, except that the cells were lysed, subjected to Endo H treatment, and analyzed by immunoblotting with a polyclonal anti-VSVG antibody. A representative experiment is shown in panel E. R and S denote Endo H-resistant and Endo H-sensitive forms of VSVG, respectively. (F) Ratio of Endo H-resistant VSVG to total VSVG (mean ± standard error of the mean) calculated from three independent samples. P values were determined by Student’s t test.
morphology caused by the lack of p31 is likely to lead to neuronal death in CNS-specific knockout mice.

The disorganization of the ER caused by p31 deficiency led to ER stress response. Under ER stress, a transcription factor, CHOP, is upregulated and translocates to the nucleus to activate transcription of apoptosis-inducing machinery (33). CHOP accumulated in the nucleus in MEFs upon p31 depletion (Fig. 9A). To determine the temporal relationship between nuclear translocation of CHOP and the change in ER morphology, we double-stained MEFs with antibodies against CHOP and PDI at various times after p31 deletion. As shown in Fig. 9B and Fig. 5B, the percentage of cells with nuclear translocation of CHOP increased with time. This indicates that the morphological change of the ER preceded nuclear translocation of CHOP, suggesting that this change in ER morphology results in ER stress.

An abnormal morphology of the ER caused by p31 deficiency-impaired protein transport from the ER to the Golgi complex. Because of the marked morphological change of the ER in p31-deficient MEFs, protein transport from the ER to the Golgi complex may be affected. To test this possibility, we examined the transport of VSVG-GFP. In control cells, VSVG-GFP was transported from the ER to the Golgi complex and then to the plasma membrane, resulting in a broad staining of the plasma membrane 60 min after a temperature shift to a permissive temperature (Fig. 10A). In p31-deficient MEFs, a large fraction of VSVG-GFP was detected in the ER with an abnormal morphology, and it was detected only faintly on the plasma membrane, indicating that VSVGGFP transport from the ER to the Golgi complex was impaired in p31-deficient MEFs. A similar result was obtained when we examined the ER-to-Golgi complex transport of multidrug resistance protein 1 (MDR1-GFP) (Fig. 10B).

In p31-deficient MEFs, the amount of VSVG-GFP transported from the ER to the Golgi complex and plasma membrane appeared to roughly correlate with the extent of the intact ER; that is, the more disorganized the ER, the less VSVG-GFP was on the plasma membrane (compare Fig. 10C with D). Therefore, the disorganized ER structure may cause inefficient ER-to-Golgi complex protein translocation, which results in the delayed transport of VSVG-GFP from the ER to the plasma membrane.

To biochemically confirm that VSVG-GFP was retained in the ER, we examined the endoglycosidase H (Endo H) sensitivity of VSVG-GFP. If VSVGGFP is transported to the Golgi complex, it acquires Endo H resistance. The ratio of the Endo H-resistant form to total VSVG in p31-deficient MEFs was less than half that in p31^{flac/} MEFS infected with Ad-Cre (Fig. 10E and F).
One possible explanation for the partial impairment of VSVG-GFP transport in p31-deficient cells is that ER exit sites, which are responsible for protein export from the ER (15), are disorganized as a consequence of ER vesiculation. To explore this possibility, we stained MEFs using an antibody against Sec13, which is a marker of ER exit sites (27). As shown in Fig. 11A and B, punctate Sec13 localization, similar to that in control cells, was still observed in p31-deficient MEFs, ruling out the possibility that the disorganization of ER exit sites caused the impairment of VSVG-GFP transport in p31-deficient cells.

To further exclude the possibility that the impaired protein transport from the ER to the Golgi complex induces the disorganization of the ER in p31-deficient MEFs, we blocked protein transport from the ER using a dominant negative form of Sar1 (Sar1[T39N]) (14) in wild-type MEFs and observed the effect on ER structure. Overexpression of Flag-tagged Sar1[T39N] showed no obvious effect on the ER structure (Fig. 12A), suggesting that ER vesiculation was not due to impaired transport between the ER and the Golgi complex. Also, in cells treated with tunicamycin, we observed no effects on ER morphology, ruling out the possibility that ER stress was the cause of ER disruption (Fig. 12B). These results suggest that p31 has a direct role in maintaining the organization of ER tubules.

To rule out the possibility that ER stress causes the disorganization of ER structure in p31-deficient cells, we treated wild-type MEFs with tunicamycin, an inducer of ER stress. In cells with nuclear translocation of CHOP, we could not observe vesiculation and enlargement of the ER as observed in p31-deficient cells (Fig. 12B), suggesting that ER disorganization was not due to ER stress response.

**DISCUSSION**

The present study demonstrated that p31 is required for the maintenance of the ER tubular structure (Fig. 13). We also showed that p31 is essential for cell survival as in the case of its yeast orthologue Use1p/Stl1p (4, 5, 7); however, the role of Use1p/Stl1p in the regulation of ER morphology has not yet been shown.

**ER morphological changes induced by p31 deficiency.** We found that the lack of p31 induced marked morphological changes in the ER both in vivo (neurons) and in vitro (MEFs). Previous studies showed that the loss of function of molecules involved in ER morphological regulation, such as BNIP1, p97, and p37, leads to the loss of three-way junctions of ER tubules (20, 28, 29). However, the tubular structure of the ER was relatively unaffected. In contrast to these previous findings, our study showed that p31 deficiency disrupted the tubular structure itself, resulting in the formation of vesicles and large ER-derived vacuolar structures. This type of extensive structural changes in the ER tubules has not been demonstrated to date.

Although ER-to-Golgi complex protein transport was partly impaired in cells with an abnormal morphology of the ER (Fig. 10), this is not the reason for the disorganization of ER structure because blockade of ER-to-Golgi complex transport by a dominant-negative Sar1 did not affect the ER structure (Fig. 12A). Also, in cells treated with tunicamycin, we observed no effects on ER morphology, ruling out the possibility that ER stress was the cause of ER disruption (Fig. 12B). These results suggest that p31 has a direct role in maintaining the organization of ER tubules.

**Two steps are suggested: vesiculation and fusion.** We demonstrated that multiple steps were required for the formation or maintenance of the ER tubules, which has not been described so far. In the absence of p31, we found that the ER is disrupted in two steps: vesiculation and subsequent enlargement (probably by fusion). This suggests that p31 is required
for the maintenance of ER tubules as well as the prevention of homotypic fusion between ER structures.

p31 is a component of the syntaxin 18 complex, which mediates the homotypic fusion of the ER. We have previously shown that p31 forms a complex with syntaxin 18 and BNIP1 (11, 20). We showed that p31 deficiency caused no effects on the interaction between syntaxin 18 and BNIP1 in cells (Fig. 3B) and in an in vitro system (3). Taken together, these results show that another component(s) in the syntaxin 18 complex might be involved in the homotypic fusion of the ER and that p31 seems to be a negative regulator of homotypic fusion of the ER because p31 deficiency caused enlargement of the ER membrane following vesiculation of the ER. Considering the multiple putative functions of p31 regarding the morphology of the ER, identification of molecules that interact with p31 will greatly increase our knowledge of the molecular mechanisms underlying the formation of the ER in the future.

Disruption of ER morphology leads to apoptosis. In this present study, we showed a pathway of apoptosis that is induced by perturbation of ER morphology. We demonstrated that an extreme morphological change in the ER leads to a reduced efficiency in ER-to-Golgi complex transport. This reduced efficiency in ER-to-Golgi complex transport leads to the accumulation of proteins in the ER, which then leads to an ER stress response, subsequent accumulation of CHOP in the nucleus, and, finally, cell death.

ER stress has been suggested to be involved in some human neurodegenerative disorders, such as Parkinson’s disease and Alzheimer’s disease, as well as other disorders such as diabetes (12, 19, 36). However, the key mechanisms underlying these disorders have been thought to be aggregation of unfolded proteins within cells. The present report presented an additional mechanism of ER stress-induced cell death, suggesting the possibility that this pathway might be involved in cell death in other types of degenerative disease.

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