Biochemical and Biophysical Research Communications 609 (2022) 62–68

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Suppression of *RNF213*, a susceptibility gene for moyamoya disease, inhibits endoplasmic reticulum stress through SEL1L upregulation

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ARTICLE INFO

Article history:
Received 8 March 2022
Accepted 2 April 2022

Keywords:
RNF213
Endoplasmic reticulum stress
SEL1L
HRD1
Moyamoya disease

ABSTRACT

RNF213, a susceptibility gene for moyamoya disease, is associated with stress responses to various stressors. We previously reported that *Rnf213* knockout (KO) mitigated endoplasmic reticulum (ER) stress-induced diabetes in the Akita mouse model of diabetes. However, the role of *RNF213* in ER stress regulation remains unknown. In the present study, *RNF213* knockdown significantly inhibited the upregulation of ER stress markers (CHOP and spliced *XBP1*) by chemical ER stress-inducers in HeLa cells. Levels of SEL1L, a critical molecule in ER-associated degradation (ERAD), were increased by *RNF213* knockdown, and *SEL1L* knockdown prevented the inhibitory effect of *RNF213* suppression on ER stress in HeLa cells, indicating *SEL1L* involvement in this inhibition of ER stress. SEL1L upregulation was also confirmed in pancreatic islets of *Rnf213* KO/Akita mice and in *Rnf213* KO mouse embryonic fibroblasts. Additionally, *RNF213* suppression increased levels of HRD1, which forms a complex with SEL1L to degrade misfolded protein in cells under ER stress. In conclusion, we demonstrate that *RNF213* depletion inhibits ER stress possibly through elevation of the SEL1L-HRD1 complex, thereby promoting ERAD *in vitro* and *in vivo*.

1. Introduction

RNF213 (alias Mysterin) is a major susceptibility gene for moyamoya disease (MMD) [1,2], an idiopathic cerebrovascular disorder characterized by progressive stenosis of the internal carotid arteries with a hazy network-like structure that has the appearance of a “puff of cigarette smoke”, which is termed “moyamoya” in Japanese [3]. Many rare variants of *RNF213* are found in MMD patients worldwide [4] and a founder variant, p.R4810K (c.14429G > A, rs112735431), markedly increases the risk of MMD in East Asian patients [5]. This variant is also a risk factor for several vascular diseases other than MMD [6–8]. *RNF213* is an extremely large protein (591 kDa) of 5207 amino acids and functions both as an AAA + ATPase and an E3 ubiquitin ligase [1]. Recently, cryo-electron microscopy has revealed that *RNF213* consists of three structural components including an N-terminal structural motif, an AAA + ATPase domain with Walker A and B motifs, and a multidomain E3 module [9]. Although physiological and pathological roles of *RNF213* remain unclear, recent findings from ourselves and others indicate that *RNF213* is involved in regulating responses to various stressors, such as hypoxia/hypoperfusion [8,10,11], infection/inflammation [11–13], and lipotoxicity [14].

Endoplasmic reticulum (ER) stress, which is induced by accumulation of unfolded proteins in the ER, activates the unfolded protein response to reduce the unfolded protein burden through translational control, chaperon induction and ER-associated degradation (ERAD) [15]. Failure to remove misfolded ER proteins can result in their accumulation and aggregation, which may contribute to the pathogenesis of various diseases, such as type-1 diabetes [16]. We previously reported that *Rnf213* knockout (KO)

Abbreviations: KO, knockout; ER, endoplasmic reticulum; MMD, moyamoya disease; ERAD, ER-associated degradation; MEFs, mouse embryonic fibroblasts; Tm, tunicamycin; Tg, thapsigargin; WT, wild-type; DMEM, Dulbecco's modified Eagle's medium; SD, standard deviation; s-*XBP1*, spliced *XBP1*.

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https://doi.org/10.1016/j.bbrc.2022.04.007
0006-291X/

retarded the progression of diabetes in male Akita mice [17], which is a model for type 1 diabetes due to ER stress-associated pancreatic β-cell destruction [18,19]. *RNF213* depletion may therefore promote the unfolded protein degradation via ERAD. However, the role of *RNF213* in ER stress regulation remains unknown.

The present study was designed to evaluate the effect of *RNF213* supression on ER stress in *RNF213*-knockdown human cervical cancer cells (HeLa) and in mouse embryonic fibroblasts (MEFs) from *Rnf213* KO mice treated with tunicamycin (Tm) or thapsigargin (Tg), chemical ER stress inducers. Additionally, we explored SEL1L, a critical molecule for ERAD to reveal the mechanism by which *RNF213* depletion reduces ER stress *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals

Rnf213 KO and *Rnf213* KO/Akita mice were prepared as previously reported [17]. Akita and wild-type (WT) mice were purchased from Japan SLC. All experimental protocols of animal studies were approved by the Mie University Board Committee for Animal Care and Use (Approval number: 2019-6, Approval date: 2019/7/25).

2.2. Cell culture

HeLa cells were obtained from the Japanese Collection of Research Bio Resources Cell Bank (Osaka, Japan). MEFs were obtained from day 12.5–13.5 WT and *Rnf213* KO mouse embryos by standard procedures [20]. Pregnant mice, 12–13 days after observation of a vaginal plug, were killed and rinsed in PBS. Embryos were removed and their heads cut off with scissors. The heart and liver were also dissected and minced using scissors. The embryos were incubated in 0.25% trypsin-EDTA for 10 min and then disrupted by pipetting using a 10-mL pipette. The fragmented embryos were passed through a mesh filter and Dulbecco's modified Eagle's medium (DMEM) was added to inactivate the trypsin. Cells were pelleted by centrifugation and then plated onto dishes and cultured under standard culture conditions. The growing MEFs were split to a third every 4 days. After 10 passages, confluent cells were trypsinized, centrifuged, and the cell pellet re-suspended in a freezing medium. The cells were frozen and stored at −80 °C. HeLa cells and MEFs were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% (v/v) heat-inactivated FBS and 100 mg/l kanamycin. Cells were split to a fifth every 5 days for over 30 population doublings.

2.3. siRNA transfection and cell treatments

HeLa cells were transfected with 37 nM siRNA (Silencer Select) specific for *RNF213* (s33568), *SEL1L* (s12676) and negative control siRNA (AM4611) (Thermo Fisher Scientific, Waltham) using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham) according to the manufacturer's protocol. After incubation at 37 °C for 24 h, ER stress was induced by treating cells with 0.4 μM tunicamycin (Tm) (Merck, Kenilworth, NJ, USA) or 0.4 μM Thapsigargin (Tg) (Nacalai tesque, Kyoto, Japan) for 18 h. For MEFs, 24 h after cell seeding, Tm and Tg treatment was performed as above.

2.4. RNA extraction, cDNA synthesis and reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After extraction, RNA (2 μg) was reverse transcribed to obtain cDNA using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Waltham). RT-qPCR was performed with THUNDERBIRD SYBR qPCR mix (TOYOBO, Osaka, Japan) using cDNA template (10 ng) and specific primers (described in Table S1) on a StepOnePlus Real Time PCR System (Thermo Fisher Scientific, Waltham). Samples were analyzed in triplicate, and expression levels were normalized against *PPIA* mRNA.

2.5. Western blotting

Cells were lysed with ice cold RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride (Nacalai tesque, Kyoto) for 5 min. After centrifugation, the supernatant was collected. The protein concentration was measured with Coomasie Protein Assay Reagent Kit (ThermoFisher Scientific, Waltham). Equal amounts of protein were separated by 5–20% SDS-PAGE (SuperSep Ace, Fujifilm-Wako, Osaka, Japan), at 600 V, 40 mA for 40 min. Proteins in gels were transferred onto polyvinylidene difluoride membranes (Merck, Kenilworth) at 600 V, 136 mA for 40 min. Membranes were blocked with 5% (w/v) skimmed milk in Tris-buffered saline (pH 7.4) containing 0.1% (v/v) Tween 20 (TBS-T) for 1 h at room temperature, and then incubated with primary antibodies in TBS-T overnight at 4 °C. Then, the membranes were washed with TBS-T and incubated with conjugated secondary antibodies in TBS-T for 30 min at room temperature. The primary and secondary antibodies used are described in Table S2. After washing with TBS-T, the signals were visualized by enhanced chemiluminescence reagent (GE Healthcare, CA, United States) and detected using an Amersham ImageQuant 800 (Fujifilm, Tokyo, Japan). Signal intensity was quantified using ImageJ software (National Institutes of Health) and normalized against internal controls (GAPDH and β-actin).

2.6. Immunohistochemistry

Male *Rnf213* KO/Akita and Akita mice were killed under sevoflurane at 20 weeks of age. The pancreas was dissected and fixed in 10% formaldehyde, embedded in paraffin and sectioned. The paraffin-embedded pancreatic sections were rehydrated in xylene and an ethanol series. Tissue sections were then boiled for 5 min in 5% urea using a microwave at 500 W for antigen retrieval and then blocked with 1% skimmed milk in PBS pH 7.4 for 25 min. The sections were immunostained with anti-SEL1L rabbit polyclonal primary antibody (1:300, ab78298, Abcam, Cambridge, MA, USA) in a humid chamber. After washing, sections were treated with a specific biotinylated secondary antibody and avidin-biotin peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA). Immunoreactions were visualized by incubation with a DAB peroxidase substrate kit (Nacalai Tesque, Kyoto). The SEL1L-positive area was quantified in 15–20 islets per pancreas in three mice per genotype using ImageJ software.

2.7. Statistical analysis

Results are presented as the mean ± standard deviation (SD). Differences were analyzed using Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. RNF213 knockdown reduces ER stress in HeLa cells

To examine the effect of *RNF213* knockdown on ER stress, we knocked-down *RNF213* in HeLa cells using siRNA. We then treated them with Tm, which induces ER stress by causing protein misfolding in the ER with inhibiting protein glycosylation [21]. The

efficiency of *RNF213* knockdown was confirmed at mRNA and protein levels (Fig. 1A and B). Tm treatment significantly increased the levels of the ER stress markers, spliced *XBP1* (s-*XBP1*) [22] and CHOP [23] (Fig. 1C–E, *p < 0.05) compared with no treatment. Transfection of *RNF213* siRNA significantly reduced mRNA levels of s-*XBP1* and CHOP compared with negative control siRNA in Tm-treated HeLa cells (Fig. 1C and D). Western blotting also revealed that *RNF213* knockdown significantly inhibited Tm-induced CHOP upregulation (Fig. 1E). These results indicate that *RNF213* knockdown inhibits ER stress induced by a chemical stressor.

3.2. RNF213 knockdown increases SEL1L levels in HeLa cells

To elucidate the mechanism by which *RNF213* knockdown reduces ER stress in HeLa cells, we examined SEL1L, a key molecule for ERAD [24,25]. Western blotting showed both glycosylated

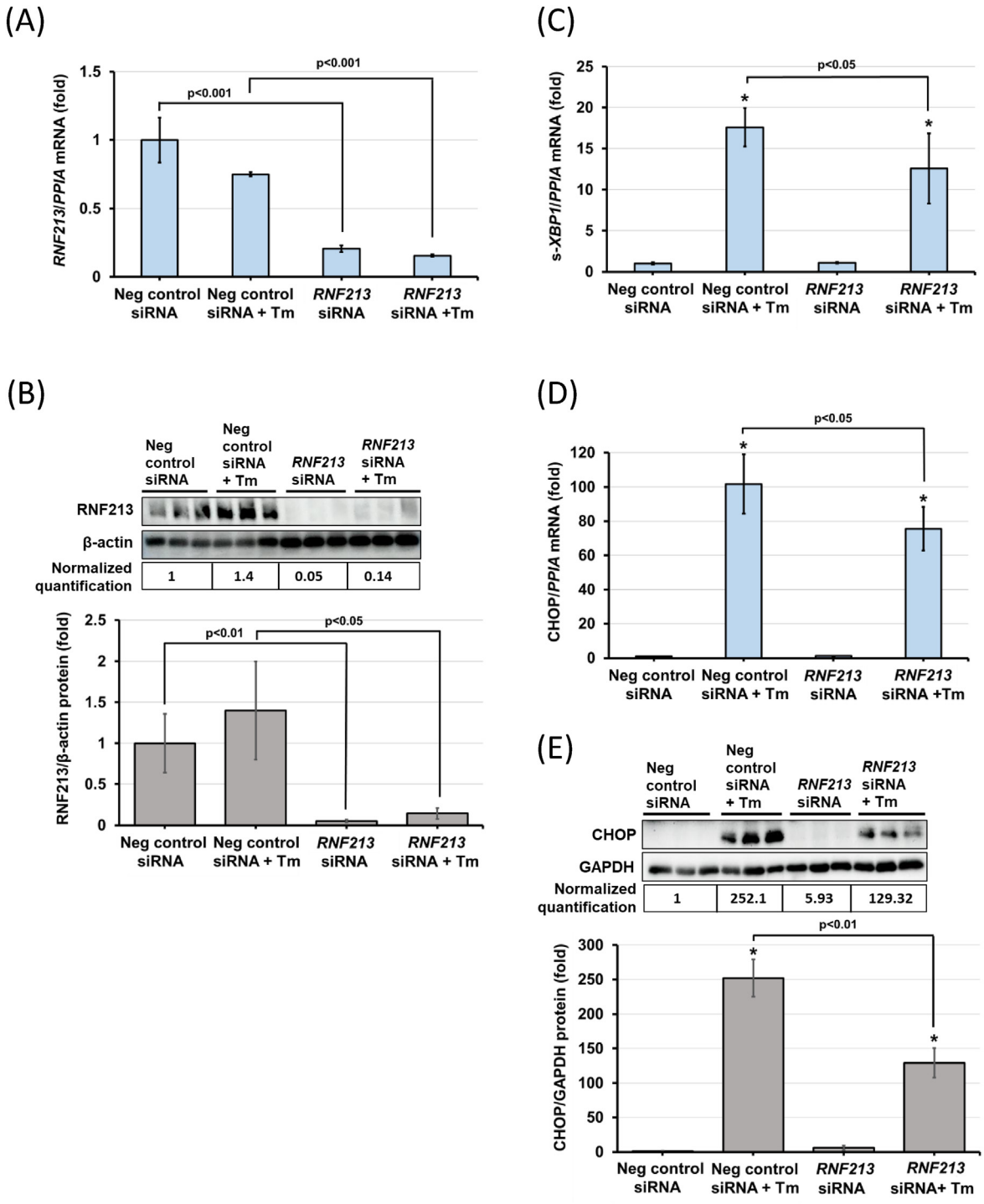

Fig. 1. ER stress marker expression in *RNF213* knockdown HeLa cells treated with Tm. Levels of (A) *RNF213* mRNA and (B) RNF213 protein, (C) s-*XBP1* mRNA, and (D) CHOP mRNA and (E) CHOP protein in HeLa cells transfected with *RNF213* siRNA followed by Tm exposure. *PPIA* (mRNA), β-actin and GAPDH (protein) were used as internal controls. The relative intensity of the control was set at 1. The data are expressed as the mean ± SD of triplicate samples. *p < 0.05 vs corresponding sample without Tm treatment.

mature SEL1L and unglycosylated immature SEL1L in HeLa cells treated with Tm (Fig. 2A) because Tm can inhibit glycosylation of SEL1L [26]. We analyzed total levels of SEL1L, and *RNF213* knockdown significantly increased SEL1L levels with or without Tm treatment (Fig. 2A). To clarify the role of *SEL1L* in ER stress, we knocked down *SEL1L* using siRNA (confirmed in Fig. 2A), which significantly upregulated CHOP in *RNF213* knockdown cells treated with Tm (Fig. 2B). This indicated that *SEL1L* is essential in the inhibition of ER stress by *RNF213* knockdown.

We then used another ER stress-inducing agent, thapsigargin (Tg), which induces ER stress by disrupting the homeostatic balance of Ca^{2+} in the ER [27] without influencing protein glycosylation (note, no unglycosylated immature band of SEL1L in Fig. 2C). Experiments using Tg exhibited similar results in SEL1L and CHOP as Tm treatment (Fig. 2C and D).

3.3. *SEL1L levels are elevated in pancreatic islets of Rnf213 KO/Akita mice, and SEL1L and HRD1 are increased in Rnf213 KO MEFs under ER stress*

To check SEL1L elevation *in vivo*, we examined SEL1L expression in pancreas tissue from *Rnf213* KO/Akita mice, in which ER stress-induced pancreatic β-cell destruction and diabetes are mitigated [17]. Immunohistochemical analysis revealed that the SEL1L-positive area in pancreatic islets was significantly greater in *Rnf213* KO/Akita mice than in Akita mice (Fig. 3A). To confirm the effect of *Rnf213* suppression in the KO model, we used MEFs derived from *Rnf213* KO mice. In *Rnf213* KO MEFs with or without Tm, levels of SEL1L protein were significantly higher than those in WT MEFs (Fig. 3B). *Rnf213* KO significantly inhibited Tm-induced elevated CHOP levels in MEFs (Fig. 3C). We also verified similar SEL1L

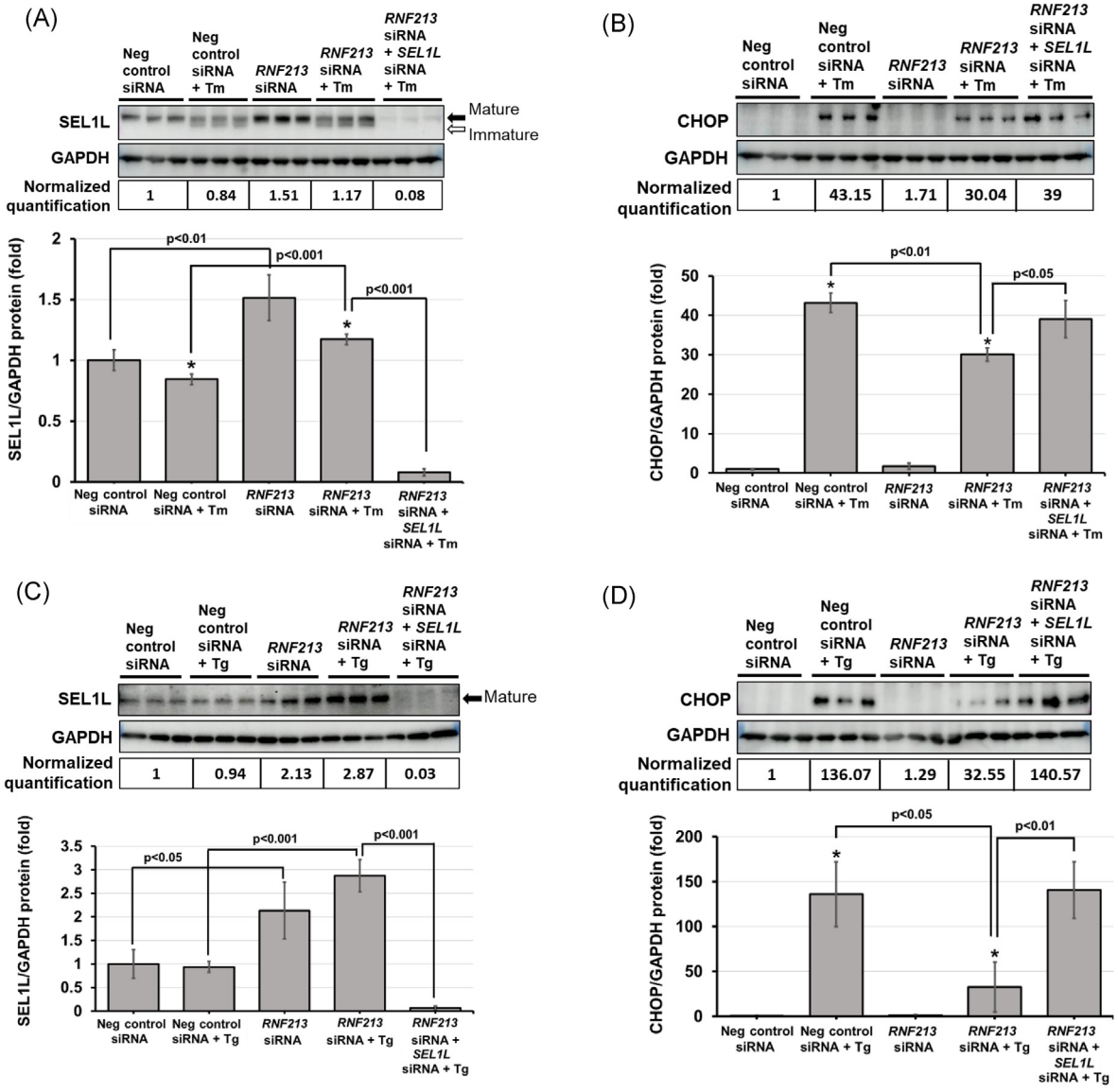

Fig. 2. Levels of SEL1L and CHOP in *RNF213* knockdown HeLa cells treated with Tm or Tg. Protein levels of (A, C) SEL1L and (B, D) CHOP in HeLa cells transfected with *RNF213* and *SEL1L* siRNA followed by Tm (A, B) and Tg (C, D) treatment. In the SEL1L western blot images, the black arrow represents glycosylated mature SEL1L protein, and the white arrow represents unglycosylated immature SEL1L protein. The relative intensity of the control was set at 1. The data are expressed as the mean ± SD of triplicate samples. *p < 0.05 vs corresponding sample without Tm or Tg treatment.

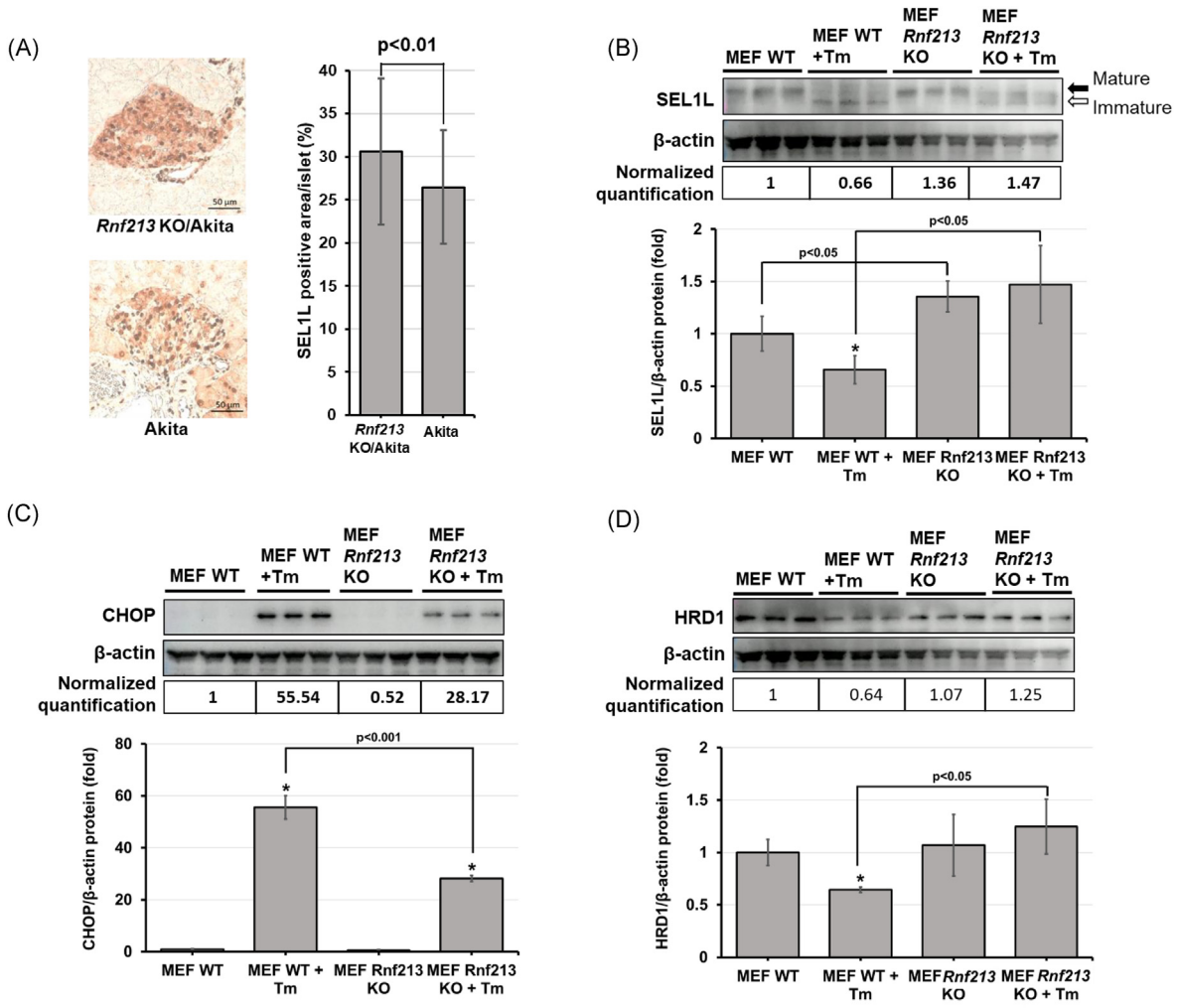

Fig. 3. Levels of SEL1L, CHOP and HRD1 in *Rnf213* KO models under ER stress. (A) SEL1L immunohistochemistry of pancreas from male *Rnf213* KO/Akita and Akita mice. Quantification of the immunohistochemistry data is expressed as the mean ± SD of 46 islets from three *Rnf213* KO/Akita mice and of 53 islets from three Akita mice. Protein levels of (B) SEL1L and (C) CHOP in *Rnf213* KO MEFs treated with Tm. In the SEL1L western blot image, the black arrow represents glycosylated mature SEL1L protein and the white arrow represents unglycosylated immature SEL1L protein. Protein levels of HRD1 in *Rnf213* KO MEFs treated with Tm (D). The relative intensity of the control was set at 1. The quantification of western blot data is expressed as the mean ± SD of triplicate samples. *p < 0.05 vs corresponding sample without Tm or Tg treatment.

upregulation and CHOP inhibition in Tg-exposed *Rnf213* KO MEFs (Supplementary Figs. S1A and S1B). These results indicate that the inhibitory effect of *RNF213* suppression on ER stress via SEL1L upregulation was observed even in endogenous and chronic *RNF213* deficiency.

SEL1L forms a complex with E3 ubiquitin ligase, HRD1, which plays an important role in ERAD [24,25]. We therefore examined HRD1 levels. HRD1 protein were significantly increased in *Rnf213* KO MEFs compared with WT MEFs after treatment with Tm (Fig. 3D). Significantly higher levels of HRD1 were observed after treatment with Tg (Supplementary Fig. S1C). Also, *RNF213* siRNA significantly increased HRD1 protein levels compared with negative control siRNA in HeLa cells treated with Tm and Tg (Supplementary Figs. S1D and S1E). Therefore, *RNF213* suppression upregulated HRD1, which might have resulted from SEL1L-mediated stabilization of the HRD1-SEL1L complex.

4. Discussion

In the present study, we demonstrated that *RNF213* knockdown inhibited ER stress induced by chemical ER stress-inducing agents using two different cell models. We focused on SEL1L as an essential molecule for ERAD [24,25]. *RNF213* suppression increased SEL1L expression, and the inhibitory effect of *RNF213* suppression on ER stress was prevented by *SEL1L* knockdown, showing that *SEL1L* is the major mediator of this ER stress regulation. *RNF213* suppression also elevated the level of HRD1 under ER stress. SEL1L and HRD1 are key molecules involved in ERAD, through which misfolded secretory proteins in the ER are translocated to the cytosol and ubiquitylated for proteasomal degradation [28,29]. HRD1, an ER-resident E3 ligase, forms a complex with SEL1L and plays a critical role in the degradation of a wide spectrum of misfolded proteins [24,25]. SEL1L is an indispensable adaptor of HRD1, which is essential for HRD1 stability and probably also substrate recruitment [24,25]. We previously reported that *Rnf213* KO reduced pancreatic β-cell destruction and improved diabetes in male Akita mice [17], in which a mutation in the insulin gene results in the misfolding of proinsulin, ER stress, and apoptosis of pancreatic β-cells [19]. In this study, SEL1L levels were elevated in pancreatic islets in male *Rnf213* KO/Akita mice compared with male Akita mice. Collectively, *RNF213* suppression increased levels of SEL1L and HRD1, thereby promoting ERAD and inhibiting ER stress.

ERAD is regulated by ubiquitin/proteasome-mediated degradation of ERAD-related molecules, including SEL1L [30]. Control of

ERAD by SEL1L degradation plays an important role in protein quality control in the steady-state as well as in the ER stress response [30], which is consistent with our results showing that SEL1L upregulation by *RNF213* suppression was observed in cells with or without ER stress-inducing reagents. UBC6e, a membrane-anchored E2 ubiquitin-conjugating enzyme localized to the ER, is a key molecule for the proteasomal degradation of SEL1L [30,31]. Considering that *RNF213*, an E3 ubiquitin ligase, mediates ubiquitination by collaborating with several E2 enzymes [9,12,13], *RNF213* may be involved in ubiquitination and proteasomal degradation of SEL1L.

In summary, *RNF213*, a major susceptibility gene for MMD, plays an important role in regulating ER stress by altering the levels of key ERAD molecules. ER stress is associated with a broad-range of diseases, such as cardiovascular diseases, diabetes and neurodegenerative diseases; therefore it is of great interest regarding the development of preventive or therapeutic agents [32]. Improvement of diabetes by *Rnf213* KO in Akita mice [17] indicates that *RNF213* is a potential therapeutic target of diabetes.

Funding

This work was supported by JSPS KAKENHI Grant Number 19K10608 (H.K.) and 17H06397 (A.K.).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Akio Koizumi has patent #RNF213 (P130009545) licensed to Akio Koizumi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.007.

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