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A highly sensitive assay of IRE1 activity using the small luciferase NanoLuc: Evaluation of ALS-related genetic and pathological factors

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ABSTRACT

Activation of inositol-requiring enzyme 1 (IRE1) due to abnormal conditions of the endoplasmic reticulum (ER) is responsible for the cleavage of an unspliced form of X-box binding protein 1 (uXBP1), producing its spliced form (sXBP1). To estimate IRE1 activation, several analytical procedures using green fluorescence protein and firefly luciferase have been developed and applied to clarify the roles of IRE1-XBP1 signaling pathways during development and disease progression. In this study, we established a highly sensitive assay of IRE1 activity using a small luciferase, NanoLuc, which has approximately 100-fold higher activity than firefly luciferase. The NanoLuc reporter, which contained a portion of the spliced region of XBP1 upstream of NanoLuc, was highly sensitive and compatible with several types of cell lines. We found that NanoLuc was secreted into the extracellular space independent of the ER-Golgi pathway. The NanoLuc activity of an aliquot of culture medium from the neuroblastoma-spinal neuron hybrid cell line NSC-34 reflected the toxic stimuli-induced elevation of intracellular activity well. Using this technique, we evaluated the effects of several genetic and pathological factors associated with the onset and progression of amyotrophic lateral sclerosis (ALS) on NanoLuc reporter activity. Under our experimental conditions, inhibition of ER-Golgi transport by the overexpression of mutant Sar1 activated luciferase activity, whereas the co-expression of mutant SOD1 or the C-terminal fragment of TDP-43 (TDP-25) did not. The addition of homocysteine elevated the reporter activity; however, we did not observe any synergistic effect due to the overexpression of the mutant genes described above. Taken together, these data show that our analytical procedure is highly sensitive and convenient for screening useful compounds that modulate IRE1-XBP1 signaling pathways as well as for estimating IRE1 activation in several pathophysiological diseases.

1. Introduction

The endoplasmic reticulum (ER) is responsible for folding and modifying newly synthesized transmembrane and secretory proteins [1,2]. A number of pathophysiological conditions disrupt ER function and cause the accumulation of unfolded and/or misfolded proteins in the ER. These phenomena, referred to as ER stress, activate various stress responses that are mediated by three major ER-resident stress sensors: PERK [3], IRE1 [4] and ATF6 [5,6]. A variety of genes have been identified as downstream targets of these three sensors, some of which, including those related to ER-resident chaperones and ER-associated degradation, control the quality of newly synthesized proteins in the ER and alleviate the damage caused by ER stress [7]; others, such as GADD153, have been demonstrated to promote cell death in various types of cells [8]. Among the three sensors, IRE1 is the most conserved signal transducer and possesses both Ser/Thr protein kinase and endoribonuclease activity, which enables it to splice unspliced XBP1

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(uXBP1) mRNA, converting it into spliced XBP1 (sXBP1), a potent transcription factor. To date, many types of genes that regulate protein quality control, protein glycosylation, and ER and Golgi biogenesis have been reported to be downstream targets of sXBP1 [9,10]. sXBP1 has been demonstrated to play crucial roles in the regulation of cellular functions in cells with well-developed ER, such as plasma B cells [11], the exocrine cells of the pancreas and salivary glands [12] and hepatocytes for liver lipogenesis [13]. Moreover, aberrant activation of the IRE-XBP1 pathway has been suggested to participate in the onset and progression of various types of diseases. Therefore, it is necessary to develop a procedure that enables more precise estimation of the activation of the IRE1-XBP1 pathway. Iwawaki et al. have reported unique reporter constructs that consist of a region around the splice site of XBP1, followed by firefly luciferase or the fluorescence protein Venus [14,15]. Very recently, we established a highly sensitive assay for the post-translational regulation of an ER stress-inducible trophic factor, mesencephalic astrocyte-derived neurotrophic factor (MANF), based on the application of a small luciferase, NanoLuc [16,17]. In the current study, we utilized NanoLuc to establish a highly sensitive assay for IRE1 activity. ER stress responses have been reported to be associated with the onset and progression of several neurodegenerative diseases [18]. Amyotrophic lateral sclerosis (ALS) is a common adult-onset motor neuron disease [19] and is characterized by the selective loss of motor neurons. Several humoral and genetic factors related to ALS [20–22] have been reported to be associated with ER stress responses [21,23]; however, the precise mechanisms underlying ALS are not fully understood. In the present study, we evaluated the effects of several ALS-related genetic and pathological factors on IRE1 activity using our NanoLuc-based assay system.

2. Materials and methods

2.1. Materials

Thapsigargin (Tg), tunicamycin (Tm), homocysteine (Hcy), N-acetylcysteine (NAC), and buthionine sulphoximine (BSO) were obtained from Sigma–Aldrich. Antibodies against Flag-, Myc-epitope, GADD153, actin and EGFP were purchased from Sigma–Aldrich, Santa Cruz Biotech, Calbiochem and Roche Life Sci, respectively.

2.2. Plasmid construction

The NanoLuc gene, which contained a Myc/His-epitope (NanoLuc-MH) at its C-terminus, or the Firefly luciferase gene were inserted into the pcDNA3.1 vector. A portion of the mouse XBP1 splice region (118 aa – 185 aa in mouse XBP1) from the unspliced XBP1 [14,15] was fused with NanoLuc-Myc/His, and the gene was inserted into the pFlag CMV vector (dXBP1-NL) (Fig. 1A). To prepare the human SOD1 constructs, the wild-type (wt) SOD1 and mutant SOD1 genes [G85R and G93A] were also amplified by PCR and inserted into the pFlag CMV vector. For the human TDP-25, the C-terminal region was amplified from the wild-type (wt) and mutant [M337V] full-length TDP-43 gene (from Dr. Leonard Petrucelli) by PCR and inserted into the pFlag CMV vector. An HA-tagged mutant Sar1 construct (Sar1[H79G]) was kindly provided by Dr. Wei Liu and Dr. Jennifer Lippincott-Schwartz [24]. A mutant Rab1α[S25N] construct generated by Dr. Terry Hébert was obtained from Addgene (#46777) [25].

2.3. Cell culture and treatments

NSC-34 [26], Neuro2a and HT-29 cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 8% fetal bovine serum. NIH3T3 cells were cultured in DMEM containing 10% bovine serum. Transfection of the indicated constructs was performed using Lipofectamine-Plus (Life Technologies) and PEI-Max reagents (Polysciences) as previously described, with a slight modification [27]. To establish NSC-34 cells that stably expressed dXBP1-NL, cells transfected with the dXBP1-NL reporter construct were selected with the appropriate amount of G418. To detect both dXBP1-NL protein and luciferase activities, cells were seeded into 6- or 12-well plates. For the analysis of luciferase activity, the indicated cells or NSC-34 cells that stably expressed an empty vector (mock) or dXBP1-NL were seeded into 48- or 96-well plates, grown to semi-confluence and used for subsequent experiments. The treatments used in this study were as follows: BSO, hydrogen peroxide (H_2O_2), homocysteine (Hcy), NAC, Tg and Tm, at the indicated concentrations.

2.4. Measurement of luciferase activity

After cells that transiently or stably expressed each of the indicated luciferase constructs were treated with the indicated reagents, the culture medium and cell lysates prepared with Passive Lysis buffer (Promega) were collected. After a brief centrifugation, the luciferase activity in each culture medium and lysate was measured using the Single Luciferase and NanoLuc assay systems (Promega), respectively [17]. By calculating the total luciferase activity in both the culture medium and the cell lysates, the percentage of secreted luciferase in each well was estimated. In some cases, Passive Lysis buffer was directly added to each culture well, and the total lysates were collected to measure the total luciferase activity, including both the culture medium and the cells.

2.5. Reverse-transcription polymerase chain reaction

To estimate the expression level of each gene by RT-PCR, total RNA was extracted from cells lysed with TRIzol reagent (Life Technologies) and converted to cDNA by reverse transcription using random nine-mers as primers for Superscript III RNase$^-$ reverse transcriptase (RT) (Life Technologies) [17,27]. Specific cDNAs were mixed and amplified with a PCR reaction mixture (Taq PCR Kit, Takara). The RT-PCR primers used in this study were as follows: *XBP1* sense primer, 5′-ACGCTTGGGAATGGACACG-3′; *XBP1* antisense primer, 5′-ACTTGTCCAGAATGCCCAAAAG-3′; *GADD153* sense primer, 5′-GAATAACAGCCGGAACCTGA-3′; *GADD153* antisense primer, 5′-GGACGCAGGGTCAAGAGTAG-3′; *GRP78* sense primer, 5′-ACCAATGACCAAAACCGCCT-3′; *GRP78* antisense primer, 5′- GAGTTTGCTGATAATTGGCTGAAC-3′; *GADD45α* sense primer, 5′-AGAGCAGAAGATCGAAAGGA-3′; *GADD45α* antisense primer, 5′-GATGTTGATGTCGTTCTCGC-3′; *p21* sense primer, 5′-GAGAACGGTGGAACTTTGAC-3′; *p21* antisense primer, 5′-GTGCAAGACAGCGACAAGG-3′; *GAPDH* sense primer, 5′-ACCACAGTCCATGCCATCAC-3′; and *GAPDH* antisense primer, 5′-TCCACCACCCTGTTGCTGTA-3′. The typical reaction cycling conditions were 30 s at 96 °C, 30 s at 58 °C and 30 s at 72 °C. The results represent 20–29 cycles of amplification. The cDNAs were separated by electrophoresis through 2.0% agarose gels and visualized using ethidium bromide.

2.6. Western blotting analysis

We detected the amount of spliced dXBP1-NL protein in the cell lysates as previously described, with a slight modification [17,27]. The cells were lysed with homogenate buffer [20 mM Tris–HCl (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% TritonX-100, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A]. After the protein concentration was determined, each cell lysate was dissolved with an equal amount of sodium dodecyl sulfate

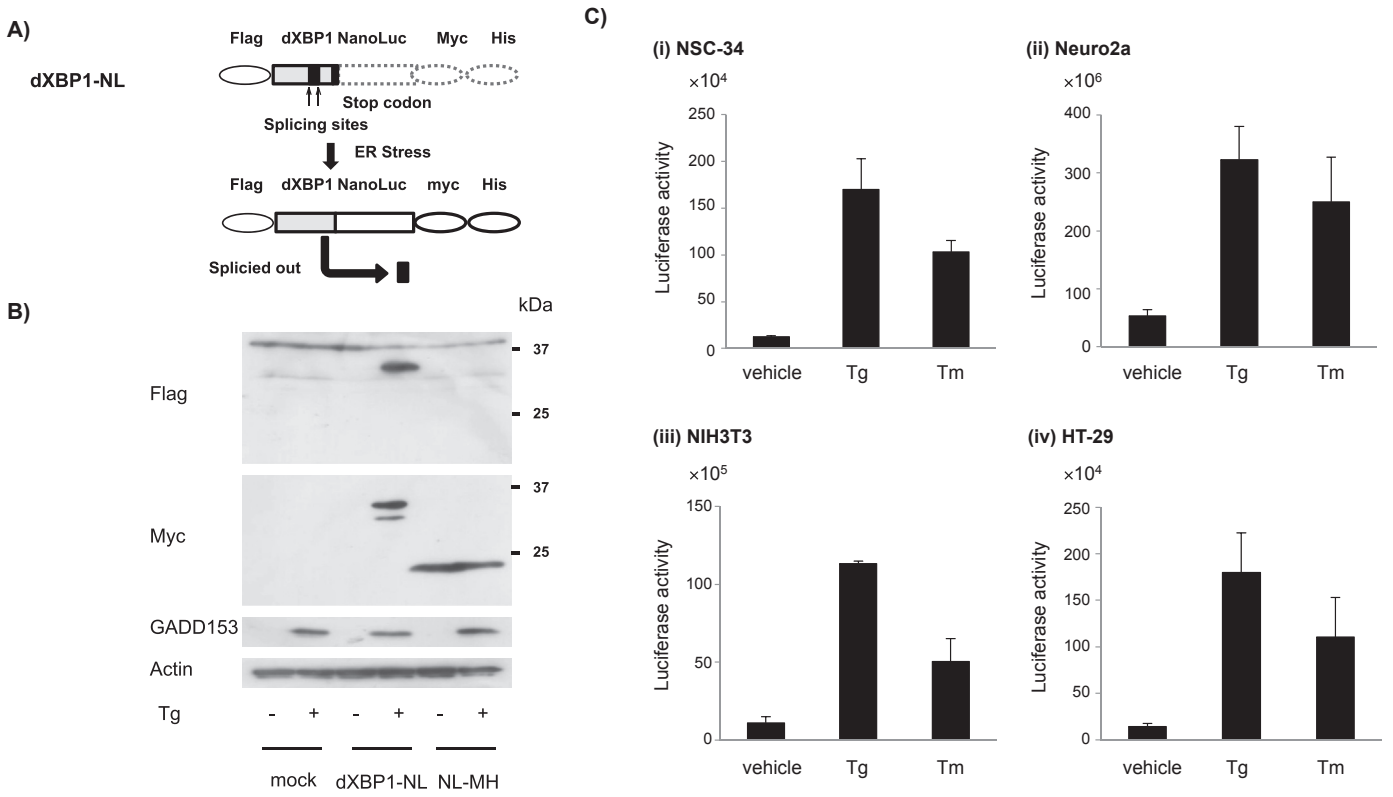

Fig. 1. Establishment of a highly sensitive assay of IRE1 activity using NanoLuc. A) Schematic of the NanoLuc-based IRE1 assay system used in this study. B) Twenty-four hours after transfection of the indicated gene into wild-type NSC-34 cells in a 6-well plate, the cells were treated with thapsigargin (Tg, 0.1 μM) for an additional 18 h. Expression of the indicated proteins in the cell lysates were detected by western blot using the indicated antibodies. C) Twenty-four hours after transfection of the dXBP1-NL reporter into NSC-34 (i), Neuro2a (ii) NIH3T3 (iii) and HT-29 (iv) cells in a 48-well plate, each cell line was treated with Tg (0.1 μM), tunicamycin (Tm, 2 μg/ml) or vehicle for an additional 18 h. After incubation, the cells were lysed and the luciferase activity was measured. The values represent the mean ± SD from 3 independent cultures.

(SDS)-Laemmli sample buffer. Equal amounts of cell lysate were separated on 15% SDS-polyacrylamide gels, immunoblotted onto polyvinylidene difluoride membranes (GE Healthcare) and identified by enhanced chemiluminescence (GE Healthcare) using antibodies against the Flag-, the Myc-epitope, GADD153, actin or EGFP.

2.7. Statistical analysis

The results are expressed as the mean ± SD of the indicated number. Statistical analyses were carried out using one-way ANOVA followed by the Fisher PLSD method. $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

Recently, we utilized NanoLuc to characterize the intra- and extracellular behavior of MANF in a post-translational manner [17]. Based on our knowledge, we employed NanoLuc to establish a highly sensitive assay for IRE1 activity [14,15,17] (Fig. 1A). We constructed a NanoLuc reporter construct that contained the mouse XBP1-splice region upstream. As shown in Fig. 1B, treatment with thapsigargin (Tg) remarkably induced the expression of dXBP1-NL protein. To assess the usefulness of the dXBP1-NL reporter, we transiently transfected it into four different types of cell lines in 48-well culture plates and treated them with Tg or tunicamycin (Tm). As shown in Fig. 1C, the NanoLuc activity in the lysates of NSC-34, Neuro2a, NIH3T3 and HT-29 cells cultured in 48-well plates increased similarly as a result of the treatment with the two different ER stress inducers. On the other hand, NSC-34 cells that expressed EGFP fused to the corresponding XBP1 region also responded to Tg by expressing the chimeric protein (Supplementary Fig. 1), but the NanoLuc reporter, dXBP1-NL, seemed to be more sensitive and convenient. These results suggest that this reporter system can be used in various types of cells.

In addition to the much higher activity of NanoLuc compared with the activity of a well-known firefly luciferase (Supplementary Fig. 2A), we found that the NanoLuc protein, which is approximately 19 kDa, was spontaneously secreted into the extracellular space. Overexpression of the Sar1[H79G] mutant did not interfere with its secretion (Supplementary Fig. 2B(i)). Because the secretion of the NanoLuc construct that contained the signal sequence (SP-NL-MH) was almost completely inhibited by the Sar1[H79G] mutant, the secretion of NanoLuc is independent of the canonical ER-Golgi pathway (Supplementary Fig. 2B). We next chose the motoneuron-like cell line NSC-34 and established NSC-34 cells that stably expressed the dXBP1-NL gene (dXBP1-NL-NSC34) to measure the luciferase activity inside or outside of the cells (Fig. 2). In addition to transient overexpression, the expression of dXBP1-NL protein was induced by treatment with Tg (Fig. 2B), and the increase in luciferase activity could be detected in both the cell lysates and the culture medium obtained from dXBP1-NL-NSC34 cells in a 96-well culture plate (Fig. 2C). In addition, this increase was also observed when the dXBP1-NL-NSC34 cells were treated with Tm and homocysteine but not when they were treated with H_2O_2. These data almost parallel the sXBP1 mRNA expression data analyzed by RT-PCR when the cells were treated with different neurotoxic stimuli (Fig. 2D and E) [28–30]. Among the stimuli used in this study, homocysteine has been suggested to affect the development of neurodegenerative diseases, including ALS [20]. In general, XBP1 is considered to alleviate ER stress via the induction

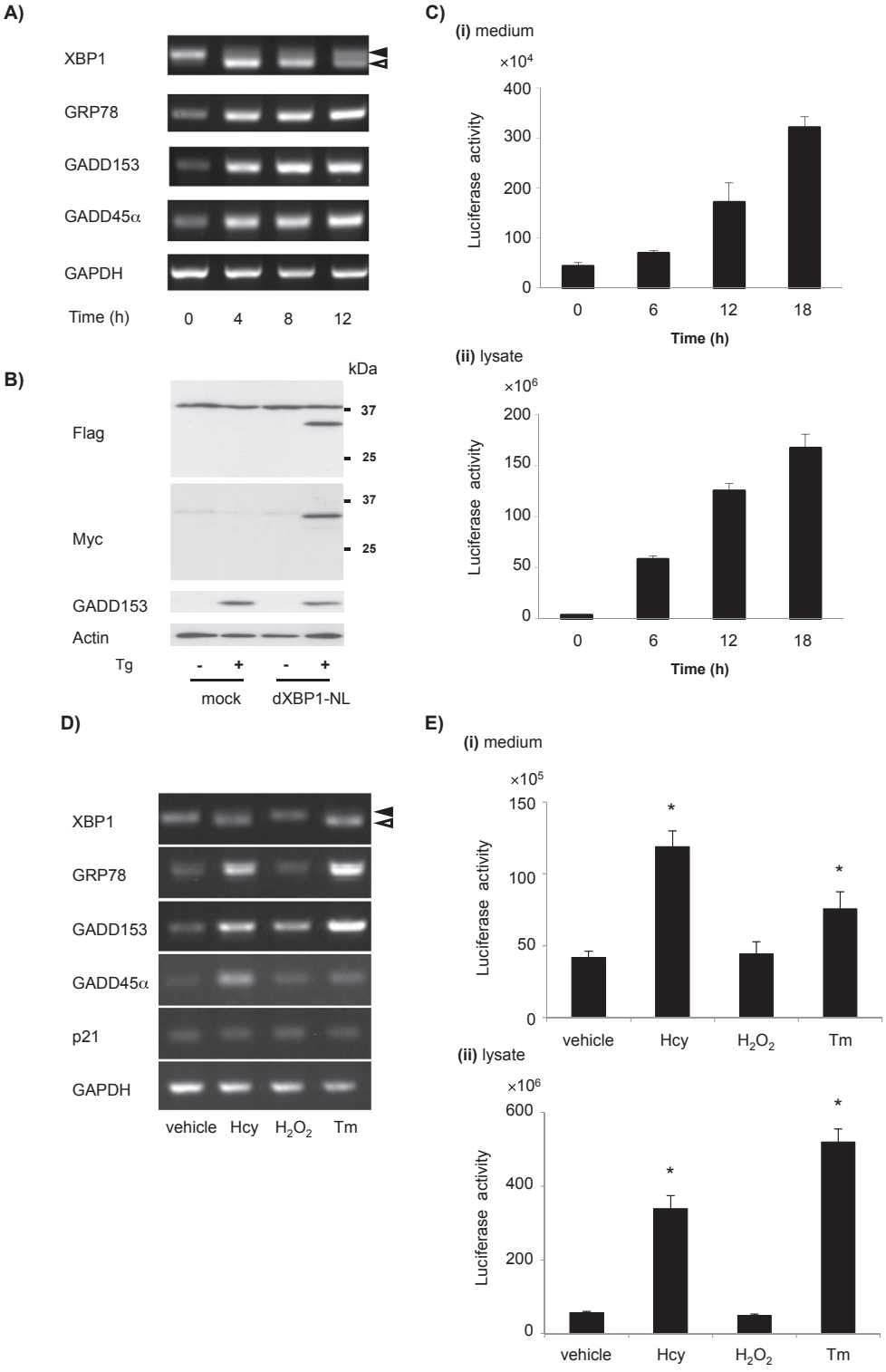

Fig. 2. Establishment of NSC-34 cells that stably express dXBP1-NL. A) Wild-type NSC-34 cells in 6-well plates were treated with Tg (0.1 μM) or vehicle for the indicated time. The expression levels of each gene were determined by RT-PCR as described in the materials and methods. Open and closed arrow-heads respectively indicate sXBP1 and uXBP1. B) NSC-34 cells that stably expressed the dXBP1-NL gene or an empty vector (mock) in 6-well plates were treated with Tg (0.1 μM) or vehicle for 18 h. Expression of the indicated proteins in the cell lysates were detected as described in Fig. 1. C) NSC-34 cells that stably expressed the dXBP1-NL gene (dXBP1-NL) in 96-well plates were treated with Tg (0.1 μM) or vehicle for the indicated time. The luciferase activity in each culture medium (i) and lysate (ii) obtained from the dXBP1-NL-NSC-34 cells were evaluated. Wild-type NSC-34 cells in 6-well plates (D) or the dXBP1-NL-NSC-34 cells in 96-well plates (E) were treated for 8 h (D) or 18 h (E) as follows: homocysteine (Hcy, 5 mM), hydrogen peroxide (H_2O_2, 25 μM), Tm (2 μg/ml) or vehicle. The expression levels of each gene (D) and the NanoLuc activity in the culture medium (E-i) and cell lysates (E-ii) were detected as described in the materials and methods. The values represent the mean ± SD from more than 3 independent cultures. The values marked with an asterisk are significantly different from the value of the control cells (vehicle).

of several factors that control protein quality and degrade misfolded proteins in the ER [10]. However, Hetz et al. reported that XBP1 deficiency is more resistant to the progression of mutant SOD1-induced ALS [23]. Then, we investigated the effects of several factors, including homocysteine, on IRE1 activity using the dXBP1-NL-NSC34 cells. As shown in Fig. 3, homocysteine treatment increased luciferase activity in a time- and dose-dependent manner. The neurotoxic action of homocysteine has been reported to be caused by oxidative stress [31,32]. Therefore, we tested the effects of supplementation with N-acetylcysteine (NAC), a GSH precursor, and of the BSO-induced depletion of GSH on luciferase activity in the dXBP1-NL-NSC34 cells treated with homocysteine. Unexpectedly, supplementation with NAC hardly down-regulated the homocysteine-induced luciferase activity. Meanwhile, 3 mM NAC upregulated luciferase activity to a small extent. On the other hand, BSO slightly but significantly enhanced the homocysteine-induced luciferase activity (Fig. 3C). A large part of ALS is sporadic, and the precise mechanisms underlying its onset and progression are not fully understood. On the other hand, several genetic mutations in ALS patients have been identified, but the roles of these mutations in triggering ALS remain to be determined. Among them, many point mutations in the SOD1 gene have been reported in ALS patients and have been suggested to relate to ER stress responses [19,21]. Therefore, we transiently transfected wild-type (wt) or mutant SOD1 [G85R and G93A] together with the dXBP1-NL reporter into NSC-34 cells and measured luciferase activity after a 66-h incubation. As shown in Fig. 4A, SOD1-overexpression did not increase luciferase activity. In addition, synergistic or additive effects of homocysteine treatment on the dXBP1-NL reporter activity were not observed in NSC-34 cells overexpressed either of the SOD1 mutants. We also examined whether another genetic factor, cleaved TDP-43 (TDP-25) [22], influences dXBP1-NL reporter activity in the NSC-34 cells. Although the GFP-tagged TDP-25 aggregated intracellularly (data not shown), TDP-25 overexpression also did not affect dXBP1-NL reporter activity in the absence and presence of homocysteine (Fig. 4B). Recently, Atkin et al. have reported that mutant SOD1 including SOD1[A4V and G85R] disrupts ER-Golgi transport due to the interaction of the mutant SOD1 with COPII components [33]. Because overexpression of neither of the SOD1 mutants showed an effect on dXBP1-NL reporter activity, we transiently transfected the Sar1[H79G] [24] or Rab1α[S25N] [25] mutants into NSC-34 cells together with the dXBP1-NL reporter (Fig. 4C). Interestingly, luciferase activity was slightly but significantly up-regulated by Sar1[H79G] overexpression. However, this overexpression did not show any synergistic effect on the homocysteine-induced luciferase activity. In contrast, mutant Rab1α (Rab1α[S25N]) overexpression had a negligible effect on luciferase activity in the absence and presence of homocysteine. When we examined the inhibitory effects of mutant Sar1 (Sar1[H79G]) and mutant Rab1α (Rab1α[S25N]) on the secretion of NanoLuc with the signal peptide at its N-terminal site (SP-NL-MH), the Sar1[H79G] mutant predominantly abolished

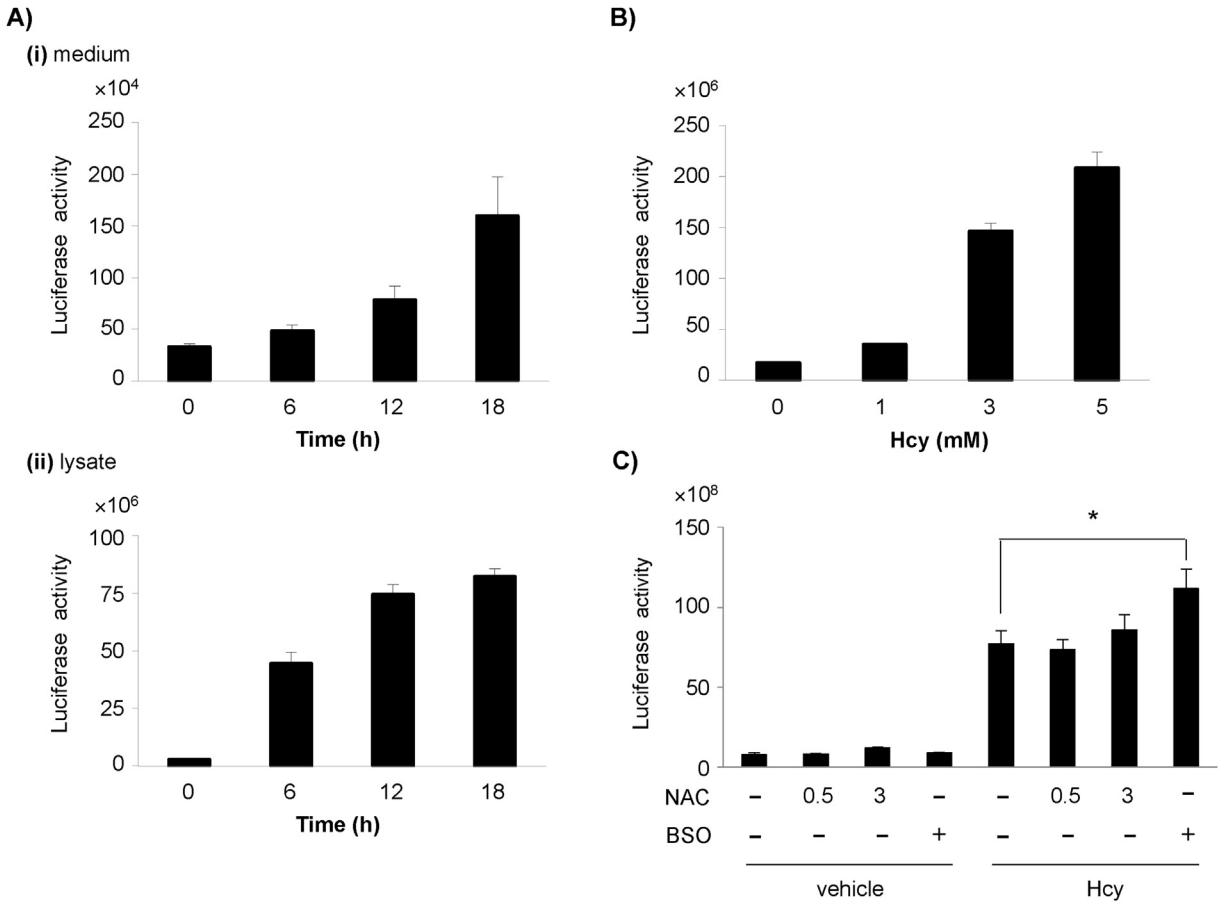

Fig. 3. Effect of homocysteine treatment on luciferase activity in dXBP1-NL-NSC-34 cells. A) dXBP1-NL-NSC-34 cells in 96-well plates were treated with homocysteine (Hcy) at 5 mM for the indicated time. The luciferase activity in each culture medium (i) and cell lysate (ii) obtained from the NSC-34 cells were evaluated as described in Fig. 1. B) dXBP1-NL-NSC-34 cells in 96-well plates were treated with homocysteine (Hcy) at the indicated concentration for 18 h. C) dXBP1-NL-NSC-34 cells in 96-well plates were treated with homocysteine (Hcy, 5 mM) in the absence and presence of N-acetylcysteine (NAC, 0.5 or 3 mM) or BSO (2 mM). After the addition of lysis buffer into each well, the total luciferase activity, consisting of the culture medium and cell lysates, was measured (B, C). The values represent the mean ± SD from more than 3 independent cultures.

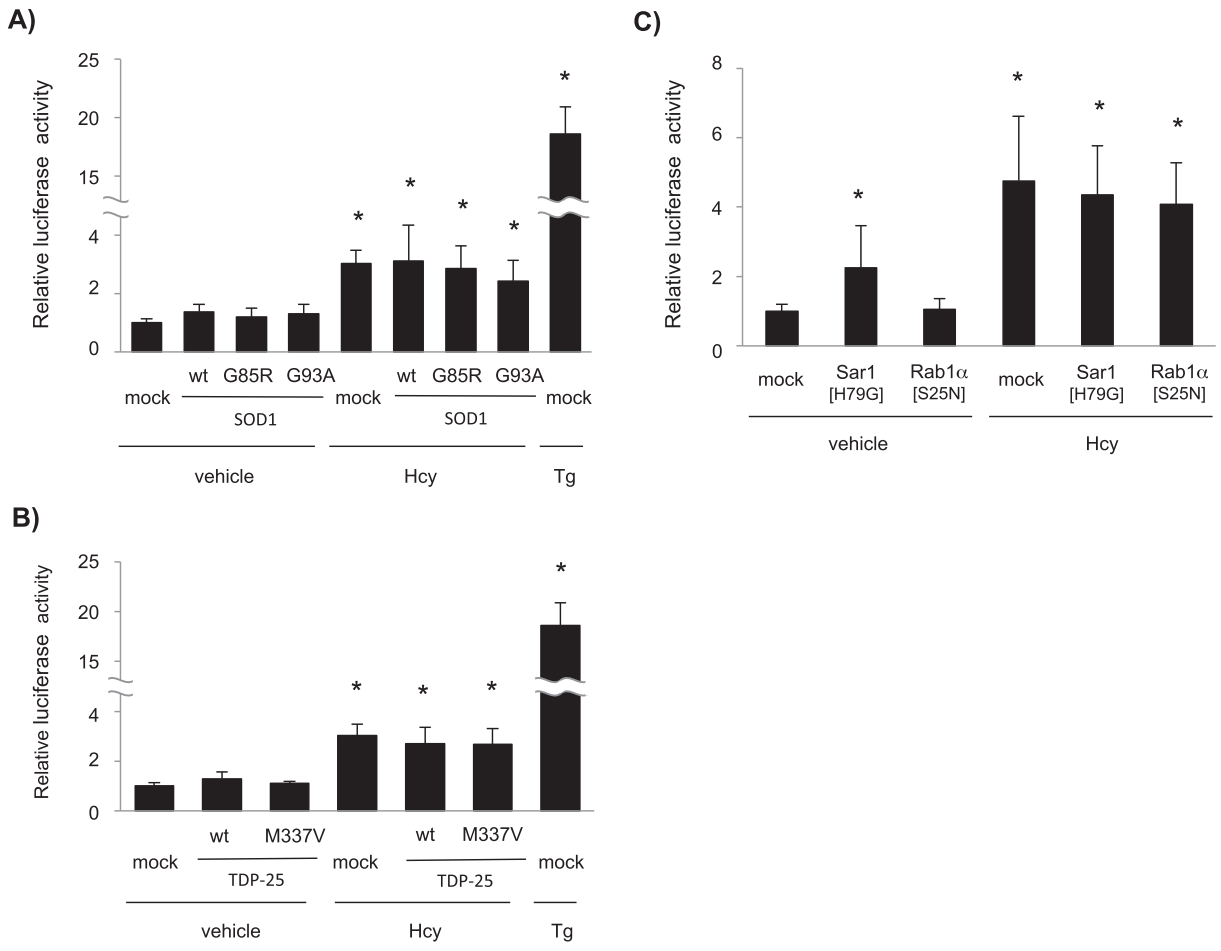

Fig. 4. Effect of mutant SOD1 and TDP-25 overexpression on dXBP1-NL activity in NSC-34 cells. Wild-type NSC-34 cells were transfected with SOD1 (A), TDP-25 (B) or Sar1[H79G] and Rab1α[S25N] (C) together with dXBP1-NL and cultured in 96-well plates. Forty-eight hours after transfection, the cells were treated with homocysteine (Hcy, 5 mM) or vehicle for an additional 18 h. After the addition of lysis buffer into each well, the total luciferase activity, consisting of the culture medium and cell lysates, was measured (A, B, C). The values represent the mean ± SD from more than 3 independent cultures. The values marked with an asterisk are significantly different from the value of the mock cells without stimuli.

secretion (data not shown). Therefore, the difference between the effects of Sar1[H79G] and Rab1α[S25N] on dXBP1-NL reporter activity seems to reflect the magnitude of their inhibitory actions on ER-Golgi transport. On the other hand, the disruption of zinc ion homeostasis is reported to relate to the pathogenesis of several neurodegenerative diseases including ALS. In particular, zinc depletion is known to trigger the conformational change of wtSOD1 and attenuate the elimination of misfolded proteins from the ER and causes ER stress [21,34]. However, treatment with TPEN, a zinc chelator, did not increase the expression level of sXBP1 mRNA in NSC-34 cells (Supplementary Fig. 3). Indeed, the ablation of intracellular zinc homeostasis is neurotoxic, but might not be a primary factor for the induction of IRE1 activity. In the current study, it is unclear why the transient overexpression of mutant SOD1 and TDP-25 did not increase IRE1 activity even though NSC-34 cells were incubated for 66 h after the transfection of each gene. In general, the degeneration of motoneurons progresses gradually, and it takes a longer period until the first symptom of ALS appears. Therefore, this transient overexpression is not long enough to trigger ER stress, including IRE1 activation in NSC-34 cells, or the ER, including ER-Golgi transport, might not be the primary target of mutant SOD1 and TDP-25. Our novel assay for IRE1 activity using NanoLuc is highly sensitive. Moreover, this assay requires only a small amount of cells, and in some cases, it is possible to continuously evaluate changes in luciferase activity in the culture medium in addition to analysis using the cellular lysates. Therefore, further analysis using this assay in more detail might provide new insights into the precise mechanisms of the onset and progression of ALS. In addition, this NanoLuc system will probably be useful in the characterization of several developmental and pathophysiological processes associated with the IRE1-XBP1 pathway.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.132.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.132.

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