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UBC9 Regulates the Stability of XBP1, a Key Transcription Factor Controlling the ER Stress Response

Aya Uemura[1*], Mai Taniguchi[2*], Yusaku Matsuo[2], Masaya Oku[1], Sadao Wakabayashi[2], and Hiderou Yoshida[1,2]**

[1]*Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan,*
[2]*Department of Molecular Biochemistry, Graduate School of Life Science, University of Hyogo, Hyogo 678-1297, Japan*

ABSTRACT. **XBP1 is a key transcription factor regulating the mammalian endoplasmic reticulum (ER) stress response, which is a cytoprotective mechanism for dealing with an accumulation of unfolded proteins in the ER (ER stress). The expression of XBP1 is regulated by two different mechanisms: mRNA splicing and protein stability. When ER stress occurs, unspliced XBP1 mRNA is converted to mature mRNA, from which an active transcription factor, pXBP1(S), is translated and activates the transcription of ER-related genes to dispose of unfolded proteins. In the absence of ER stress, pXBP1(U) is translated from unspliced XBP1 mRNA and enhances the degradation of pXBP1(S). Here, we analyzed the regulatory mechanism of pXBP1(S) stability, and found that a SUMO-conjugase, UBC9, specifically bound to the leucine zipper motif of pXBP1(S) and increased the stability of pXBP1(S). Suppression of UBC9 expression by RNA interference reduced both the expression of pXBP1(S) and ER stress-induced transcription by pXBP1(S). Interestingly, overexpression of a UBC9 mutant deficient in SUMO-conjugating activity was able to increase pXBP1(S) expression as well as wild-type UBC9, indicating that UBC9 stabilizes pXBP1(S) without conjugating SUMO moieties. From these observations, we concluded that UBC9 is a novel regulator of the mammalian ER stress response.**

Key words: ER stress response, unfolded protein response, chaperone, XBP1, UBC9

Introduction

The endoplasmic reticulum (ER) is an organelle where secretory proteins are synthesized and folded with the assistance of ER-localized chaperones and folding enzymes (collectively called ER chaperones), including BiP, GRP94 and protein disulfide isomerases (Gething, 1997). When nascent proteins are malfolded or unfolded in the ER, they are disposed of by a mechanism called ER-associated degradation (ERAD) (Bernasconi and Molinari, 2011; Hampton, 2002; Hebert *et al.*, 2010). When the synthesis of secretory proteins is increased and overwhelms the capacity of ER chaperones and ERAD components, or when environmental stresses such as ultraviolet irradiation hamper proper folding of secretory proteins (Komori *et al.*, 2012; Mera *et al.*, 2010; Wu *et al.*, 2002), unfolded proteins accumulate in the ER (ER stress) and induce apoptotic cell death. To cope with ER stress, eukaryotic cells activate a cytoprotective mechanism called the ER stress response (also called the unfolded protein response) to upregulate expression of ER chaperones as well as ERAD components; this process is well conserved from yeast to mammals (Kimata and Kohno, 2011; Mori, 2009; Tabas and Ron, 2011; Walter and Ron, 2011; Wang and Kaufman, 2012; Yoshida, 2009).

Mammalian cells have three pathways in the ER stress response: the PERK, ATF6 and IRE1 pathways. IRE1 is a type I transmembrane protein localized in the ER, of which the cytoplasmic portion contains a kinase domain and RNase domain (Cox *et al.*, 1993; Mori *et al.*, 1993). Upon sensing the accumulation of unfolded proteins, IRE1 forms homo-oligomers, is autophosphorylated and then activated. IRE1 cleaves unspliced *XBP1* mRNA (*XBP1(U)* mRNA), and an unidentified RNA ligase ligates its exons, resulting in the production of spliced *XBP1* mRNA (*XBP1(S)* mRNA) (Calfon *et al.*, 2002; Shen *et al.*, 2001; Yoshida *et*

*These authors equally contributed to this work.
**To whom correspondence should be addressed: Hiderou Yoshida, Department of Molecular Biochemistry, Graduate School of Life Science, University of Hyogo, Harima Science Garden City, Hyogo 678-1297, Japan.
Fax: +81–791–58–0212, Tel: +81–791–58–0219
E-mail: hide@sci.u-hyogo.ac.jp
Web site: http://www.sci.u-hyogo.ac.jp/life/biochem2/index-j.html

al., 2001a). Because this splicing mechanism of *XBP1* mRNA is independent of conventional mRNA splicing machinery, and its reaction occurs in the cytoplasm, it is called cytoplasmic splicing or frame switch splicing (Mori, 2003; Ruegsegger *et al.*, 2001; Uemura *et al.*, 2009). The splicing reaction removes a 26 nt-long intron from *XBP1(U)* mRNA, resulting in a frame shift in the *XBP1(S)* mRNA. Thus, proteins translated from unspliced and spliced *XBP1* mRNA (pXBP1(U) and pXBP1(S)) all contain the basic leucine zipper motif responsible for DNA-binding and dimerization in the N-terminal region, but their C-terminal regions are quite different. pXBP1(S) contains a transcriptional activation domain in the C-terminal region, whereas pXBP1(U) has a domain enhancing the degradation and nuclear exclusion of pXBP1(S) (Yoshida *et al.*, 2006b, 2009) as well as the domains responsible for association with the ER membrane and the ribosomal tunnel (Yanagitani *et al.*, 2009, 2011). pXBP1(S) forms a heterodimer with pATF6(N) (a key transcription factor of the ATF6 pathway), binds to a cis-acting element UPRE, and activates the transcription of genes involved in ERAD (Adachi *et al.*, 2008; Yamamoto *et al.*, 2007; Yoshida *et al.*, 2003), while pXBP1(U) enhances degradation of pXBP1(S) (Yoshida *et al.*, 2006b), anchors *XBP1(U)* mRNA to the ER membrane (Yanagitani *et al.*, 2009) and stalls translation transiently (Yanagitani *et al.*, 2011) (see upper part of Fig. 11).

Although the unconventional splicing mechanism of *XBP1* mRNA is relatively well characterized, the mechanism by which the stability of pXBP1(S) is regulated by pXBP1(U) remains to be fully elucidated. pXBP1(S) is rapidly degraded in cells by the proteasome (Calfon *et al.*, 2002; Yoshida *et al.*, 2001a), although the factors regulating its stability, including ubiquitin ligases, remain unidentified. Interestingly, Tirosh and colleagues reported that pXBP1(U) is degraded by the proteasome without ubiquitination (Navon *et al.*, 2010). In the present study, we identified UBC9 as a novel binding protein of pXBP1(S) and characterized its regulatory function on pXBP1(S) during the mammalian ER stress response.

Results

Isolation of XBP1-binding proteins by yeast two hybrid screening

To isolate proteins binding to XBP1 proteins by yeast two hybrid screening, we expressed pXBP1(U) fused with the DNA binding domain of yeast Gal4p transcription factor (Gal4p-DBD-pXBP1(U)), and human pancreatic proteins fused with the activation domain of Gal4p (Gal4p-AD-cDNA) in *his3Δ* mutant yeast cells (AH109) containing the *HIS3* reporter gene under the control of a Gal4p-binding site. From 20 million transformants, 4 positive clones that were able to form colonies on growth plates lacking histidine (SD-H plates) in an XBP1-dependent fashion were obtained. All four positive clones contained a plasmid expressing a SUMO conjugase, UBC9. To confirm the association between pXBP1(U) and UBC9, AH109 cells expressing both Gal4p-DBD-pXBP1(U) and Gal4p-AD-UBC9 were plated on SD-H plates (Fig. 1A). AH109 cells expressing both Gal4p-DBD-pXBP1(U) and Gal4p-AD-UBC9 grew on SD-H plates, whereas those expressing either Gal4p-DBD-pXBP1(U) or Gal4p-AD-UBC9 alone did not, suggesting that Gal4p-DBD-pXBP1(U) associates with Gal4p-AD-UBC9 in AH109 cells.

To determine which domain of pXBP1(U) binds to UBC9, AH109 cells expressing deletion mutants of pXBP1(U) fused with Gal4p-DBD as well as Gal4p-AD-UBC9 were plated on SD-H plates (Fig. 1B, 1C and 1D). Deletion mutants expressing the leucine zipper motif of pXBP1(U) formed colonies on SD-H plates (Fig. 1D, lanes 1–4, 6 and 7), whereas those lacking it did not (lanes 5 and 8), indicating that the leucine zipper motif of pXBP1 proteins is essential for the association with UBC9. We also determined which domain of UBC9 binds to pXBP1(U), using AH109 cells expressing Gal4p-DBD-pXBP1(U) (1–133) and deletion mutants of UBC9 fused with Gal4p-AD (Fig. 2). AH109 cells expressing UBC9 (1–129) or UBC9 (35–158) formed colonies on SD-H plates (Fig. 2C, lanes 2 and 9), whereas those expressing further deletion mutants did not, suggesting that the (35–129) region of UBC9 is essential for association with pXBP1(U).

To confirm that UBC9 binds to pXBP1(U) in mammalian cells, Gal4p-DBD-UBC9 and deletion mutants of pXBP1(U) fused with the activation domain of viral transcription factor VP16 (VP16AD) were transiently expressed in HeLa cells together with the firefly luciferase reporter gene under the control of a Gal4p-binding site (Fig. 3A). Deletion mutants of pXBP1(U) containing the leucine zipper motif increased luciferase activity (lanes 2–4), whereas those lacking it did not (lanes 5 and 6), indicating that pXBP1(U) associates with UBC9 in HeLa cells, and that the leucine zipper motif of pXBP1(U) is again essential for association. Expression of deletion mutants of pXBP1(U) was almost similar, though degradation materials were observed (Fig. 3B).

We also confirmed the association between XBP1 and UBC9 using an immunoprecipitation assay (Fig. 4). When whole cell lysates prepared from HeLa cells transiently expressing pXBP1(S) and myc-UBC9 were immunoprecipitated with an anti-myc antibody, pXBP1(S) was co-immunoprecipitated with myc-UBC9 (lane 11), whereas pXBP1(S) was not immunoprecipitated when myc-UBC9 was not co-expressed (lane 8). pXBP1(U) was also co-immunoprecipitated with myc-UBC9 (lane 12), although the efficiency of co-immunoprecipitation of pXBP1(U) was considerably lower compared with that of pXBP1(S), indicating that UBC9 binds to pXBP1(S) more efficiently. Thus, we changed the direction of the study from analysis of an XBP1-binding factor to that of a pXBP1(S)-binding

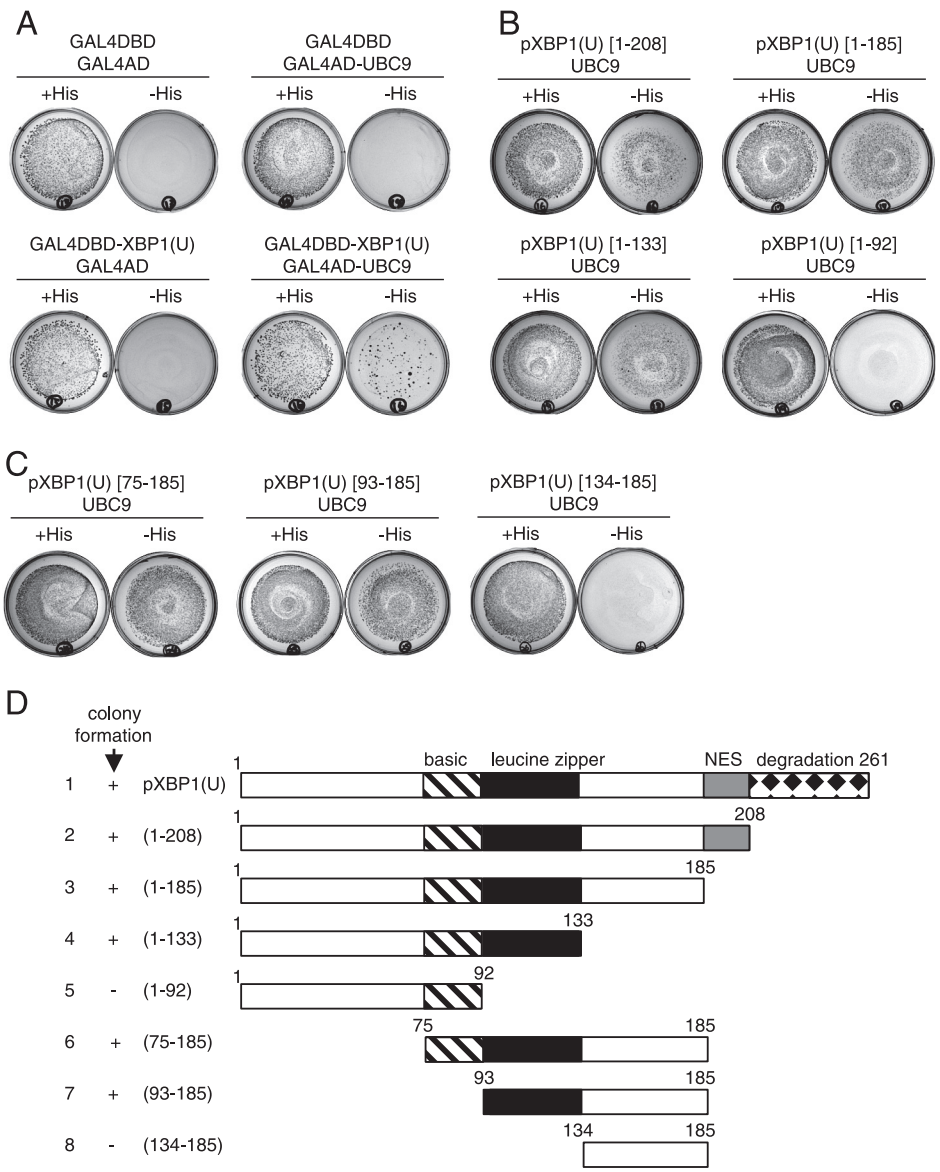

Fig. 1. Interaction between UBC9 and deletion mutants of pXBP1(U). Interaction between (A) UBC9 and full length pXBP1(U), (B) UBC9 and C-terminal deletion mutants of pXBP1(U) and (C) UBC9 and N-terminal deletion mutants of pXBP1(U). AH109 cells transfected with the indicated plasmids were plated onto SD plates containing (left) or lacking (right) histidine. (D) Schematic drawing of each of the deletion mutants of pXBP1(U).

factor. We considered that pXBP1(S) has higher affinity for UBC9 than pXBP1(U) owing to the difference in their molecular structure. That is, the C-terminal portion of pXBP1(U) might inhibit the interaction with UBC9. Notably, the expression of pXBP1(S) and pXBP1(U) was increased when UBC9 was co-expressed (lanes 2, 3, 5 and 6), suggesting that UBC9 stabilizes XBP1 proteins or enhances their transcription.

Overexpression of UBC9 increased expression of XBP1 proteins

The expression of exogenously expressed XBP1 proteins was increased when UBC9 was overexpressed (Fig. 4). Thus, we examined whether the expression of endogenous XBP1 proteins was also enhanced by UBC9 overexpression (Fig. 5A). In normally growing HeLa cells, a small amount of pXBP1(U) was expressed (lane 1), whereas the expression of pXBP1(U) was increased upon overexpression of

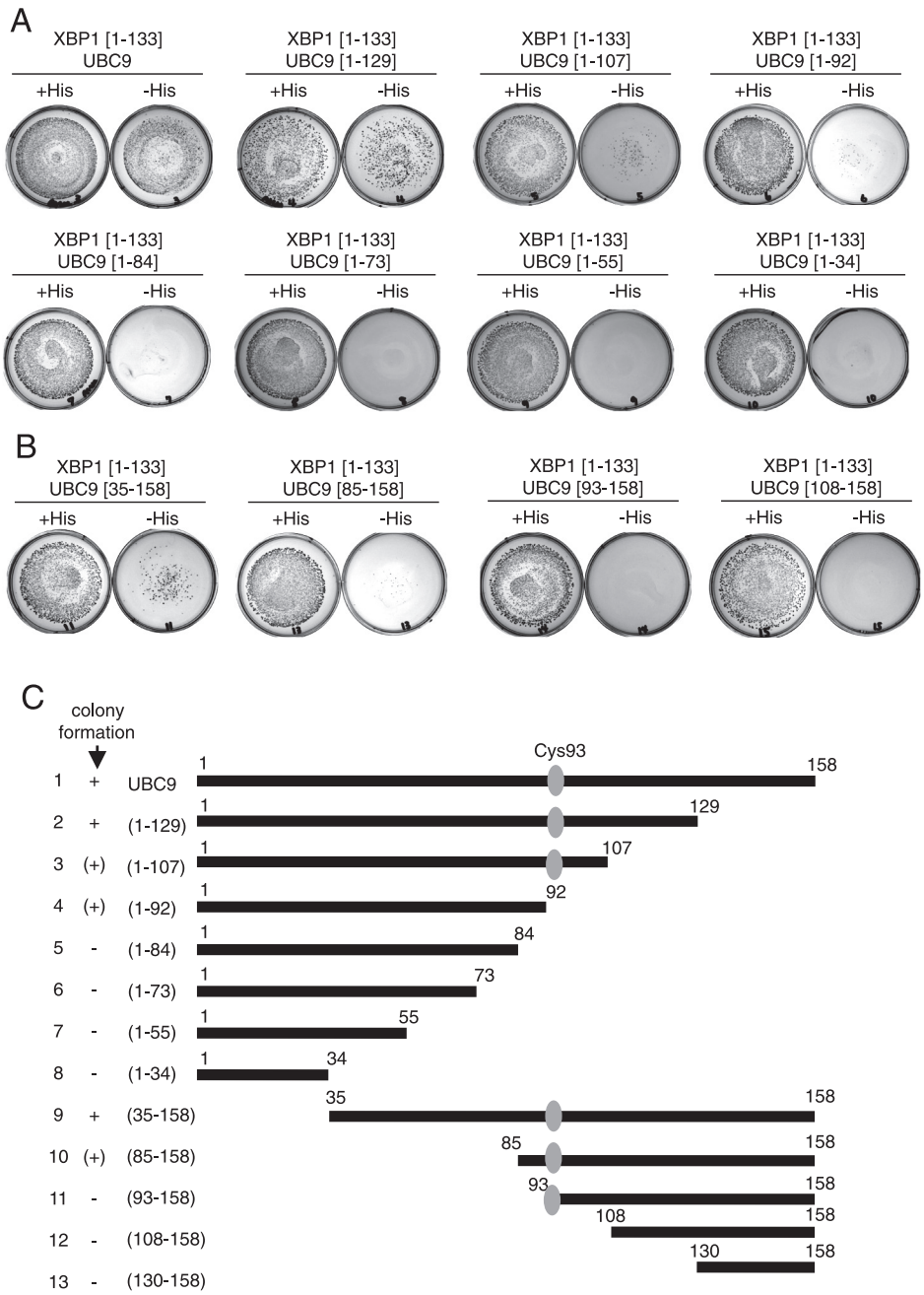

Fig. 2. Interaction between XBP1 and deletion mutants of UBC9. Interaction between (A) pXBP1 (1–133) and C-terminal deletion mutants of UBC9, or (B) between pXBP1 (1–133) and N-terminal deletion mutants of UBC9. AH109 cells were processed as described in Fig. 1. Data of UBC9 (130–158) was omitted. (C) Schematic drawing of each deletion mutant of UBC9. The position of the cysteine residue (Cys93) that is important for SUMO-conjugase activity of UBC9 is indicated.

UBC9 (lane 2). When cells were treated with an inducer of ER stress, such as thapsigargin, the expression of pXBP1(U) was reduced while that of pXBP1(S) was induced (lane 3), because most *XBP1(U)* mRNAs were converted to *XBP1(S)* mRNAs. When UBC9 was overexpressed, the expression of both pXBP1(S) and pXBP1(U) was enhanced (lane 4). These findings suggest that UBC9 overexpression increased the expression of endogenous XBP1 proteins. The bands indicated by double asterisks seem to be non-specific bands, because we usually do not detect such bands. It was also possible that the bands corresponded to degradation products of pXBP1(S). Overexpression of UBC9 did not increase

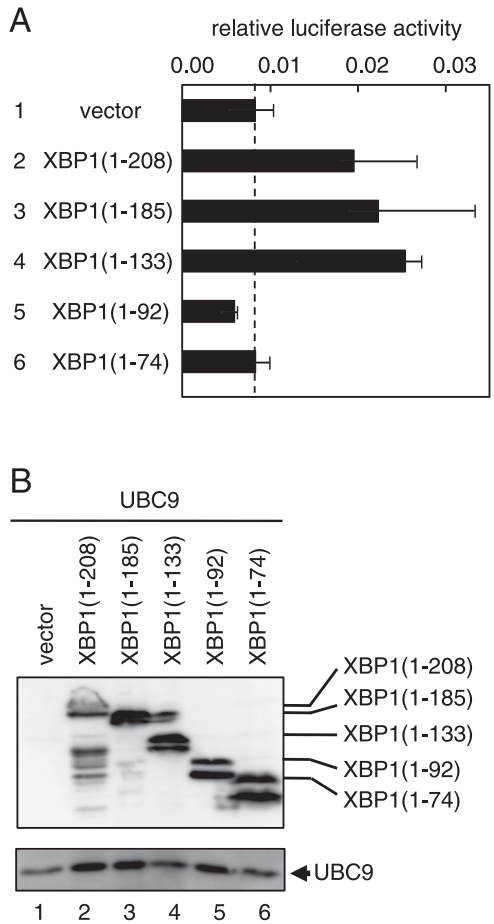

Fig. 3. Two hybrid assay between XBP1 and UBC9 in mammalian cells. (A) HeLa cells were transfected with Gal4p-DBD-UBC9, VP16-XBP1 deletion mutants and a Gal4p site-LUC reporter, and luciferase activity was measured. (B) Expression of XBP1 deletion mutants and UBC9 in a mammalian two hybrid assay. Whole cell lysates prepared from HeLa cells processed as in (A) were subjected to immunoblotting analysis using anti-VP16AD (upper panel) or anti-UBC9 (lower panel) antisera.

the amount of the bands (lanes 3 and 4), suggesting that truncated pXBP1(S) corresponding to the bands lacks a domain necessary for interaction with UBC9.

To examine whether transcriptional induction by pXBP1(S) was also enhanced by UBC9 overexpression, we transiently overexpressed UBC9 in HeLa cells with a luciferase reporter gene under the control of a UPRE enhancer (UPRE-LUC reporter) (Fig. 5B). In control cells, luciferase activity was increased upon treatment with thapsigargin (lanes 1 and 2). When UBC9 was overexpressed, transcriptional induction from UPRE in response to thapsigargin was increased remarkably (lanes 3 and 4). These suggest that UBC9 enhances pXBP1(S)-mediated transcriptional induction.

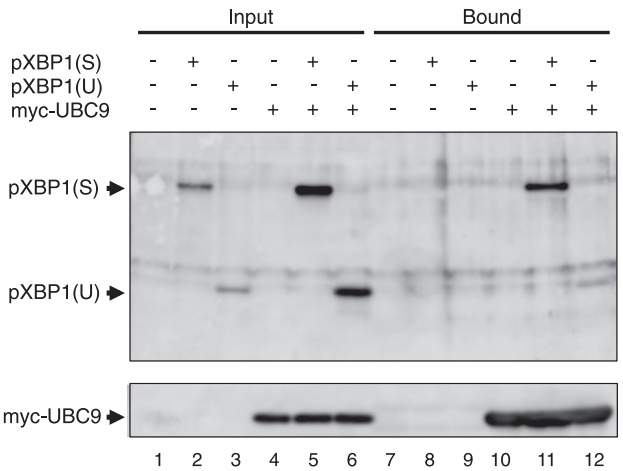

Fig. 4. Pull-down experiment between XBP1 and UBC9. Whole cell lysates were prepared from HeLa cells transfected with the indicated plasmids, and myc-UBC9 was immunoprecipitated using an anti-myc antibody. XBP1 proteins co-immunoprecipitated with myc-UBC9 were detected by immunoblotting analysis using anti-XBP1 antiserum (upper panel). Immunoprecipitated UBC9 was also visualized using an anti-myc antibody (lower panel).

Suppression of UBC9 expression decreased XBP1 proteins

To examine whether UBC9 is involved in transcriptional induction by pXBP1(S), we overexpressed shRNAs for UBC9 (shRNA-UBC9-A and shRNA-UBC9-B) in HeLa cells, and found that they effectively decreased the expression of UBC9 (Fig. 6A, lower panel, lanes 3 and 4). Expression of pXBP1(S) was induced by thapsigargin treatment (upper panel, lanes 1 and 2), whereas pXBP1(S) expression was considerably reduced when UBC9 expression was suppressed by shRNA-UBC9-A or B (lanes 3 and 4), suggesting that UBC9 is important for pXBP1(S) induction upon ER stress. We also examined whether UBC9 is required for transcriptional induction from UPRE in response to ER stress (Fig. 6B). When a UPRE-LUC reporter plasmid was transiently transfected into HeLa cells, transcription from UPRE was increased by thapsigargin treatment (lanes 1 and 2). In contrast, transcriptional induction from UPRE was considerably reduced when UBC9 expression was suppressed by shRNA-UBC9-A (lane 3 and 4). From the above results, we concluded that UBC9 is an important factor for transcriptional induction by pXBP1(S) during mammalian ER stress response. We also examined whether expression or subcellular localization of UBC9 changes upon ER stress by Northern and Western blotting analyses (Fig. 7) as well as immunocytochemistry (data not shown), and found that it hardly changed during normal growth conditions or upon ER stress, although *UBC9* mRNA levels were slightly increased during the early phase of ER stress response (2 to 4 h after

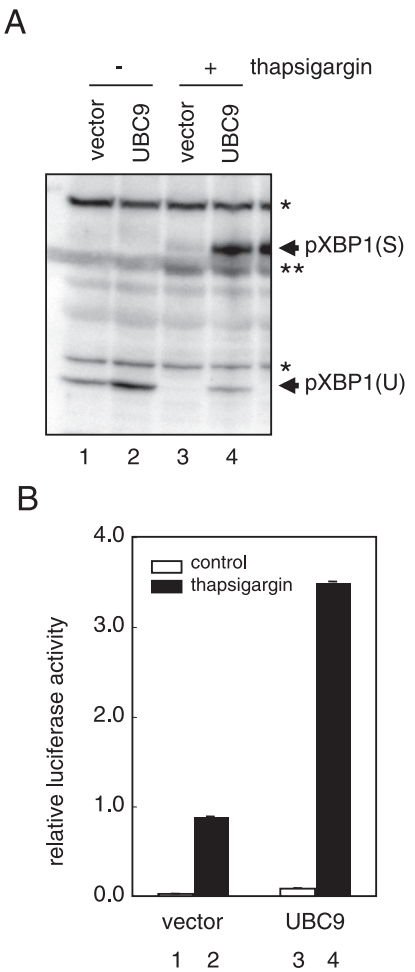

Fig. 5. Effect of UBC9 overexpression on XBP1 expression and XBP1-mediated transcription. (A) Whole cell lysates were prepared from HeLa cells that were transfected with a UBC9 expression plasmid and treated with or without thapsigargin, and were subjected to immunoblotting analysis using anti-XBP1 antiserum. Asterisks indicate the position of non-specific bands. (B) HeLa cells transfected with a UBC9 expression plasmid and a UPRE-LUC reporter plasmid were treated with thapsigargin for 16 h, and luciferase activity was measured using whole cell lysates.

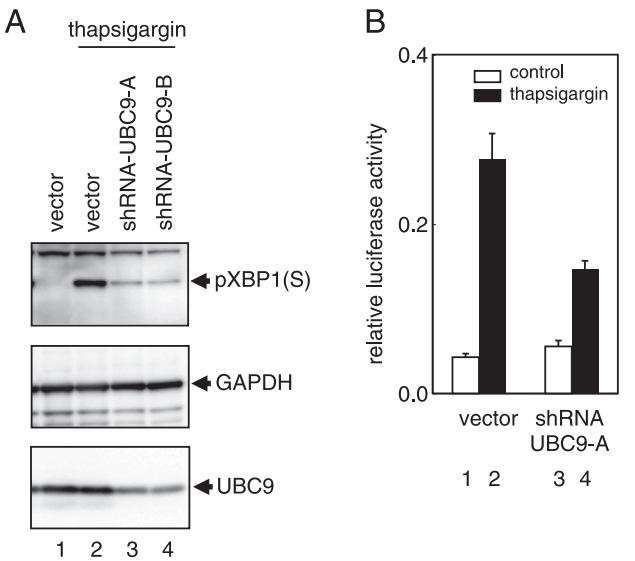

Fig. 6. Effect of UBC9 knockdown on XBP1 expression and XBP1-mediated transcription. (A) Whole cell lysates prepared from HeLa cells that were transfected with an expression plasmid for UBC9-shRNA were processed as in Fig. 5A. (B) HeLa cells transfected with an expression plasmid for UBC9-shRNA and a UPRE-LUC reporter plasmid were processed as in Fig. 5B.

thapsigargin treatment), which might contribute to slight increase of UBC9 protein level (4 to 8 h after thapsigargin treatment). It is possible that degradation of UBC9 is increased upon ER stress, and transcriptional induction of *UBC9* compensates for it. In Fig. 7A, the electromobility of *UBC9*, *BiP* and *GAPDH* mRNAs in lanes 5–7 was retarded slightly, possibly as a result of excessive salt concentrations in the samples. We did not observe such a shift usually.

UBC9 protects pXBP1(S) from degradation by the proteasome

Next we examined the mechanism of how UBC9 increases the expression of pXBP1(S). Because pXBP1(S) is a very unstable protein and is degraded rapidly by the proteasome (Calfon *et al.*, 2002; Lee *et al.*, 2003; Tirosh *et al.*, 2006; Yoshida *et al.*, 2001a), it is possible that UBC9 increases pXBP1(S) expression by protecting it from degradation or by enhancing transcription of the *XBP1* gene. To discriminate between these possibilities, we examined pXBP1(S) expression in the presence of MG132, an inhibitor of the proteasome (Fig. 8A). In the absence of MG132, a small amount of pXBP1(S) was expressed from an expression plasmid (lane 1). When UBC9 was overexpressed, the expression of pXBP1(S) was increased remarkably (lane 2). When cells overexpressing UBC9 were treated with MG132, no further increase in pXBP1(S) expression was observed (lane 3), suggesting that an increase in pXBP1(S) expression by UBC9 is mainly derived from the stabilization of pXBP1(S) proteins.

To confirm that UBC9 does not increase XBP1 transcription, we performed Northern blotting analysis (Fig. 8B). When HeLa cells were treated with ER stress inducers, such as thapsigargin and tunicamycin, the expression of *XBP1* mRNA was increased by transcriptional induction (lanes 1–3). When UBC9 was overexpressed, the expression of *XBP1* mRNA was not further increased (lanes 4–6). From these results, we concluded that UBC9 functions as a stabilizing factor of pXBP1(S), not as a transcriptional activator.

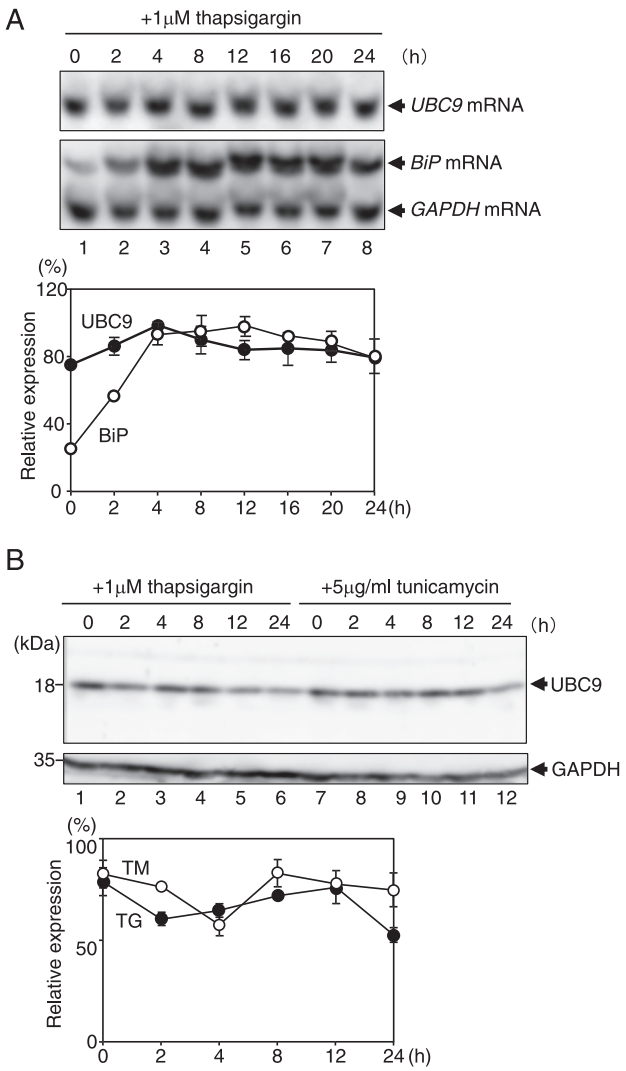

Fig. 7. Expression of UBC9 during ER stress. (A) Total RNA extracted from HeLa cells treated with the indicated ER stress inducers was subjected to Northern blotting analysis with the indicated cDNA probes. Data were quantified and shown in the lower panel. Closed and open circles indicate UBC9 and BiP mRNAs, respectively. (B) Whole cell lysates prepared from HeLa cells treated with tunicamycin (open circles) or thapsigargin (closed circles) were subjected to immunoblotting analysis using the indicated anti-sera. Data were quantified and shown in the lower panel. Closed and open circles indicate thapsigargin and tunicamycin, respectively.

SUMO conjugase activity is dispensable for enhancement of pXBP1(S) expression by UBC9

Because UBC9 is a well characterized SUMO conjugase, it seems likely that UBC9 enhances the expression of pXBP1(S) by conjugating SUMO moieties to pXBP1(S) or other substrate proteins. To verify this, we overexpressed a mutant UBC9 (UBC9-C93S), which dominant negatively suppresses the SUMO-conjugating activity of endogenous UBC9 owing to mutation of the cysteine residue critical for SUMO conjugating activity (Fig. 9). Surprisingly, the overexpression of UBC9-C93S in HeLa cells effectively increased the expression of pXBP1(S) and pXBP1(U) as well as wild-type UBC9 (Fig. 9A, lanes 3 and 4). Transcriptional induction from UPRE in response to ER stress (Fig. 9B, lanes 1 and 2) was also enhanced by UBC9-C93S (lanes 5 and 6) as well as wild-type UBC9 (lanes 3 and 4).

We also investigated whether UBC9-C93S binds to XBP1 in mammalian cells (Fig. 10A). When myc-UBC9-C93S was co-expressed with pXBP1(S), pXBP1(S) was co-immunoprecipitated with myc-UBC9-C93S (lane 17) as well as with myc-UBC9 (lane 14). pXBP1(U) was also co-immunoprecipitated with myc-UBC9 and myc-UBC9-C93S (lanes 15 and 18). Moreover, yeast AH109 cells expressing UBC9-C93S and XBP1 (1–133) were able to grow on SD-H plates as well as those expressing UBC9 and XBP1 (1–133) (Fig. 10B and 10C). These results indicate that UBC9 enhances the expression of pXBP1(S) without conjugating SUMO moieties.

Discussion

The active transcription factor pXBP1(S) is a key regulator of the mammalian ER stress response, controlling transcriptional induction of ER-related genes encoding ERAD factors. In this paper, we demonstrated that UBC9 specifically binds to pXBP1(S), enhances its expression by suppressing degradation by the proteasome, and increases transcription from UPRE upon ER stress. Interestingly, these activities of UBC9 toward pXBP1(S) are independent of its SUMO-conjugase activity. Our observations not only contribute to clarification of the regulatory mechanism of the mammalian ER stress response but also clarify a novel role of a SUMO-conjugating E2 enzyme, UBC9. UBC9 functions as a stabilization factor of substrate proteins without conjugating SUMO moieties.

Fig. 11 illustrates our current working hypothesis of how XBP1 and UBC9 function in the ER stress response. In normal growth conditions, pXBP1(U) is translated from unspliced *XBP1* mRNA, binds to pXBP1(S), translocates pXBP1(S) from the nucleus to the cytosol through the nuclear exclusion signal (NES) and enhances its degradation by the proteasome through the degradation domain (DEG). Nascent pXBP1(U) on the ribosome causes translational pausing through the C-terminal region (CTR) during translation, and anchors *XBP1(U)* mRNA to the ER membrane through hydrophobic region 2 (HR2). Upon ER stress, IRE1 is activated through oligomerization and splices *XBP1* mRNA, resulting in the production of pXBP1(S). UBC9 protects pXBP1(S) from degradation by pXBP1(U) and the proteasome, while pXBP1(S) forms a heterodimer with pATF6(N), binds to UPRE through the

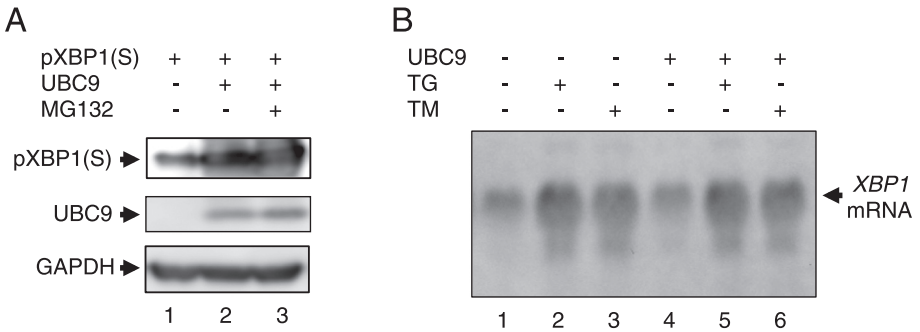

Fig. 8. Effect of UBC9 overexpression on degradation of XBP1 proteins and transcription of the *XBP1* gene. (A) HeLa cells transfected with indicated expression plasmids were treated or not treated with MG132, and whole cell lysates were subjected to immunoblotting analysis using anti-XBP1 (upper panel) and anti-UBC9 (lower panel) antisera. (B) HeLa cells transfected with an expression plasmid of UBC9 were treated with thapsigargin (TG) or tunicamycin (TM), and subjected to Northern blotting analysis with human XBP1 cDNA probe.

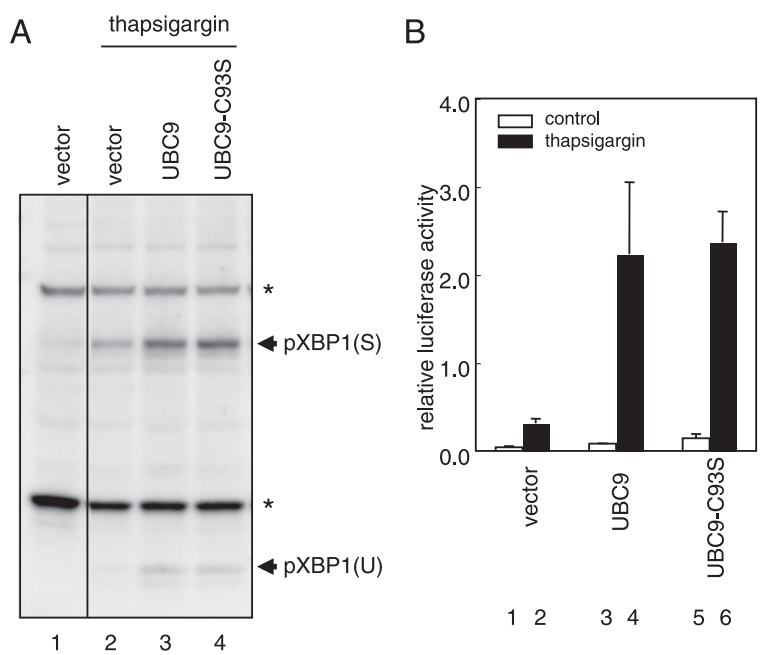

Fig. 9. Effect of mutation of SUMO-conjugase activity on the degradation of XBP1 proteins and transcriptional induction from UPRE. (A) Whole cell lysates prepared from HeLa cells expressing a UBC9-C93S mutant and treated with thapsigargin were subjected to immunoblotting analysis using anti-XBP1 antiserum. (B) HeLa cells were transfected with an expression plasmid for UBC9-C93S and a UPRE-LUC reporter and treated with thapsigargin, and luciferase activity was measured.

DNA binding domain (DBD) and activate transcription of ERAD genes through the transcriptional activation domain (AD).

How UBC9 stabilizes pXBP1(S)?

We revealed that UBC9 binds to and stabilizes pXBP1(S), although the mechanism of how UBC9 stabilizes pXBP1(S) still remains unclear. pXBP1(S) is a very unstable protein and is degraded rapidly by the proteasome, and treatment with an inhibitor of the proteasome such as MG132 greatly increased pXBP1(S) expression (Yoshida *et al.*, 2001a). pXBP1(U) is also a very unstable protein and contains a domain required for its rapid degradation at its C-terminal region (Yoshida *et al.*, 2006b). Interestingly, pXBP1(U) negatively regulates the expression of pXBP1(S), i.e. pXBP1(U) binds to pXBP1(S), relocates it from the nucleus to the cytoplasm, and enhances its degradation by the proteasome (Yoshida *et al.*, 2006b). It is possible that UBC9 prevents pXBP1(U) from binding to pXBP1(S), in order to

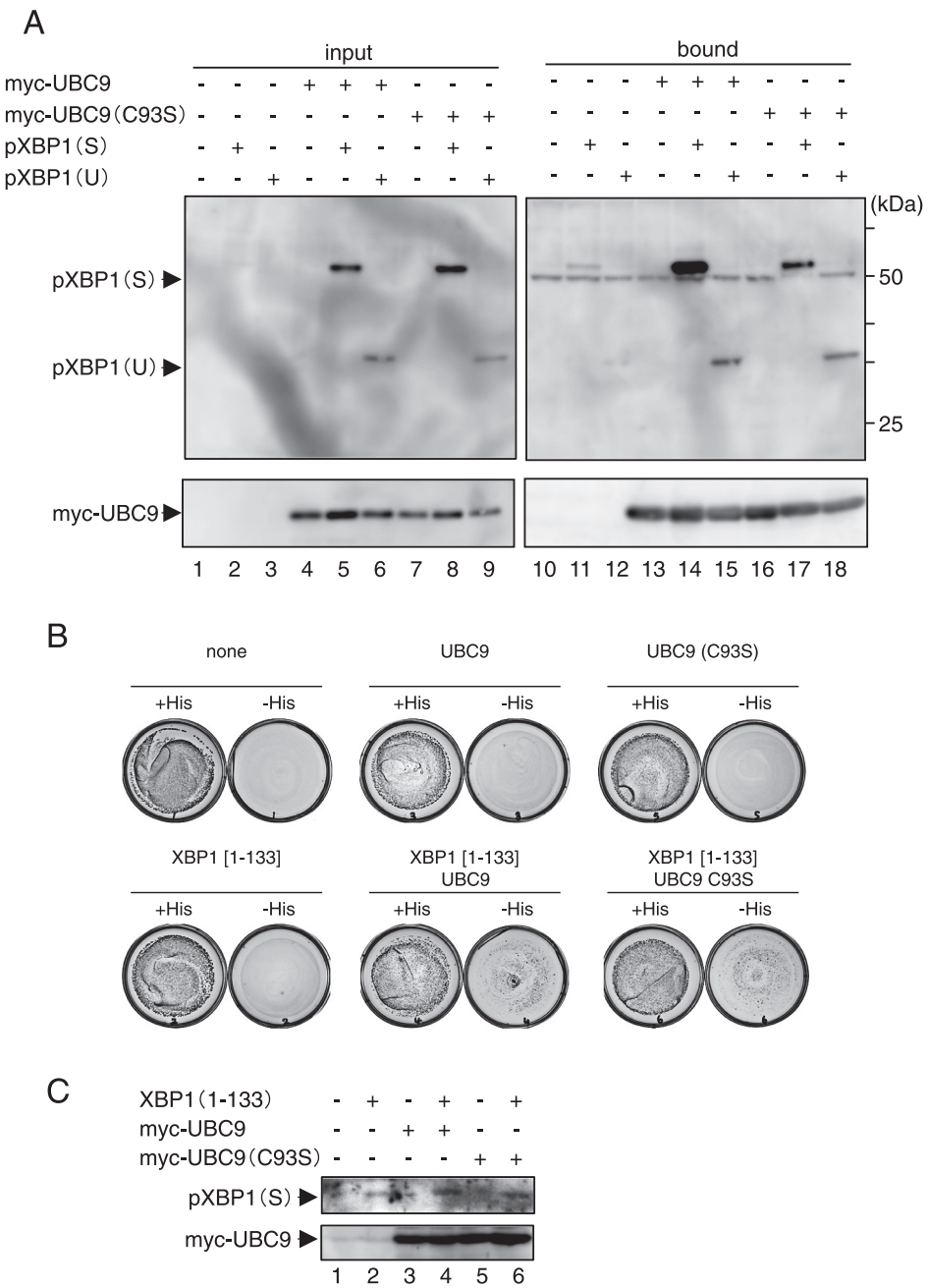

Fig. 10. Association between UBC9-C93S and XBP1. (A) Whole cell lysates prepared from HeLa cells expressing myc-UBC9-C93S and pXBP1(S) were subjected to immunoprecipitation with an anti-myc antibody, as shown in Fig. 4. (B) AH109 cells expressing the indicated proteins were inoculated in SD-H plates, as shown in Fig. 1. (C) Whole cell lysates were prepared from cells shown in Fig. 10B, and subjected to immunoblotting analysis with anti-myc (upper panel) or anti-XBP1 antisera.

block pXBP1(U)-mediated degradation of pXBP1(S). It is also possible that UBC9 may suppress the function of the degradation domain of pXBP1(U), because UBC9 also increased the expression of pXBP1(U) (Fig. 4). Identification of the unidentified ubiquitin ligase responsible for ubiquitination of pXBP1(S) would be required for clarification of the stabilization mechanism, which is an interesting subject for future research.

UBC9 as a multifunctional adaptor protein

UBC9 is a well-characterized conjugase of the ubiquitin-

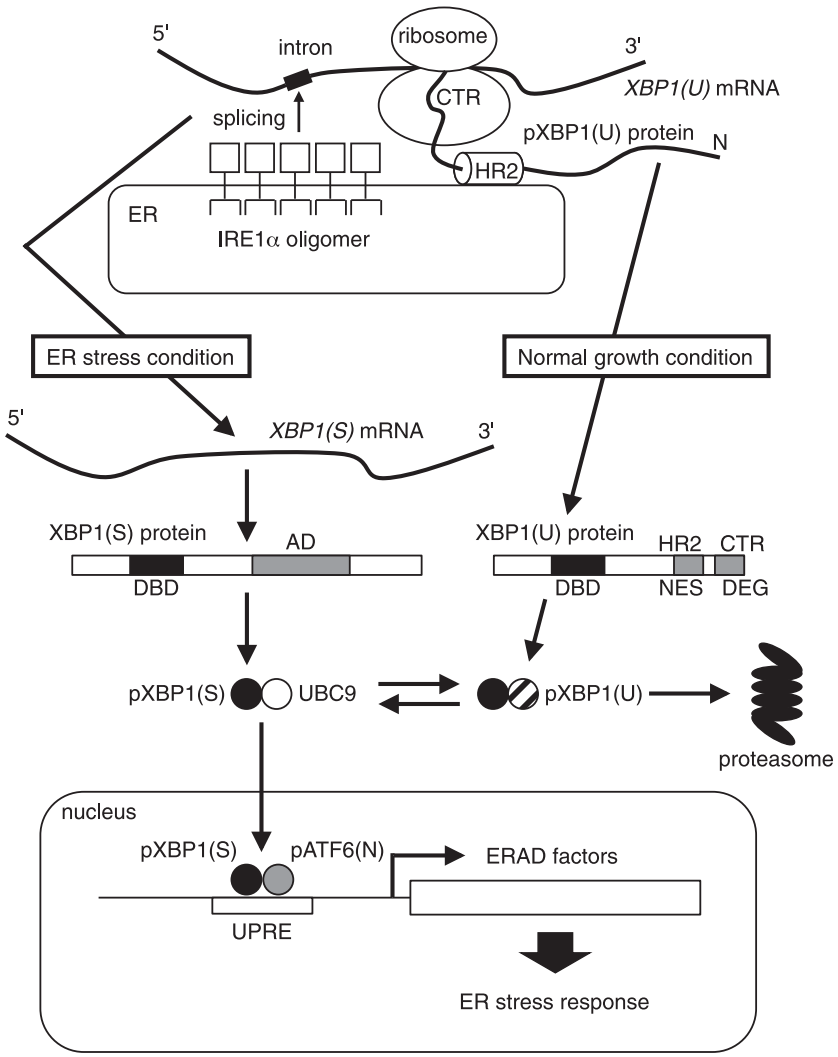

Fig. 11. Working hypothesis. Details are described in the text. DEG, degradation domain; NES, nuclear exclusion signal; HR2, hydrophobic region 2; CTR, C-terminal region; DBD, DNA binding domain (DBD); AD, transcriptional activation domain.

like modifier SUMO, although there are many reports that UBC9 has various other functions. Chen and colleagues reported that UBC9 binds to RAD52, a key factor involved in DNA repair and recombination, through the self-association domain of RAD52 (Shen *et al.*, 1996), and Wasylyk and colleagues revealed that UBC9 binds to a transcriptional activator ETS1 through the helix-loop-helix (HLH) domain, which is responsible for the dimerization of ETS1 (Hahn *et al.*, 1997). Nucifora and colleagues found that UBC9 binds to the transcriptional repressor TEL, an ETS family protein, through the HLH domain, and that UBC9 suppressed the repressor activity of TEL without conjugating a SUMO moiety (Chakrabarti *et al.*, 1999). Itoh and colleagues also reported that UBC9 binds to the nuclear receptor SF-1 and functions as a transcriptional co-activator of SF-1 in a SUMO conjugase activity-independent manner (Suda *et al.*, 2011). Mo and colleagues showed that UBC9 promotes tumor cell invasion and metastasis without conjugating a SUMO moiety (Zhu *et al.*, 2010). There is no established idea explaining this multi-functionality of UBC9, and we speculate that UBC9 is a multi-functional adaptor protein that links its target proteins with other functional complexes.

SUMOylation of pXBP1(S)

Previously, it was reported that pXBP1(S) is SUMOylated (Chen and Qi, 2010; Golebiowski *et al.*, 2009). Ling and colleagues revealed that two lysine residues in the transcriptional activation domain of pXBP1(S) (K276 and K297)

became SUMOylated when SUMO conjugase UBC9 and a SUMO ligase PIAS2 were overexpressed, and that the transcriptional activity of pXBP1(S) was increased when these SUMOylation events were ablated (Chen and Qi, 2010), suggesting that SUMOylation negatively regulates the transcriptional activity of pXBP1(S). However, endogenous pXBP1(S) was hardly SUMOylated upon ER stress, indicating that the contribution of SUMOylation of pXBP1(S) to the mammalian ER stress response may not be especially great. The stabilizing effect of UBC9 to pXBP1(U) observed here is SUMOylation-independent, because pXBP1(U) does not have the SUMOylation sites (K276 and K297), and was actually not SUMOylated (Chen and Qi, 2010).

Materials and Methods

Cell culture and luciferase assay

Cells of yeast *Saccharomyces cerevisiae* were cultured as described previously (Yoshida *et al.*, 1998). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM: 4.5 g/l glucose) supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics: 100 units/ml penicillin, 100 μg/ml streptomycin (Yoshida *et al.*, 2001b). Cells were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

Transient transfection of cultured cells

Transient transfection of HeLa cells was carried out by the standard calcium phosphate method (Sambrook *et al.*, 1989; Yoshida *et al.*, 2006a) and lipofection using FuGene 6 (Roche). For transfection using the calcium phosphate method, HeLa cells cultured in 24-well or 60 mm dishes were incubated with precipitates of calcium phosphate containing plasmids for 16 h at 37°C. Transfection by lipofection was carried out in accordance with the manufacturer's protocol. To induce ER stress, cells were treated with 1 μM thapsigargin or 2 μg/ml tunicamycin for 16 h and harvested for analysis. A UPRE-LUC reporter plasmid was kindly provided by Dr. Ron Prywes (Columbia University).

Luciferase assay

Transient transfection of HeLa cells was carried out as described previously (Oku *et al.*, 2011). In brief, HeLa cells cultured in a 24-well plate were incubated with precipitates of calcium phosphate containing plasmids for 12 h at 37°C. After washing with PBS to remove $CaPO_4$-DNA precipitates, the cells were incubated in fresh medium for 24 h, treated with ER stress-inducing chemicals for 16 h and harvested for luciferase assays.

Construction of plasmids

The construction of the UPRE reporter plasmid was described previously (Bruhat *et al.*, 2000; Wang *et al.*, 2000; Yoshida *et al.*, 1998; Yoshida *et al.*, 2000). To construct an yeast expression plasmid of Gal4p-DBD-XBP1(U), a full length XBP1(U) cDNA was inserted into the BamHI-SalI sites of a pBridge vector (Clontech). As for an expression plasmid of Gal4p-AD-UBC9, a full length UBC9 cDNA was cloned into the BamHI-SalI sites of a pACT2 vector (Clontech). Deletion mutants of XBP1(U) and UBC9 were constructed in a similar manner to the full length clone. To construct human expression plasmids of XBP1 and UBC9, a cDNA fragment of XBP1 was inserted into the XhoI and BamHI sites of a pcDNA3.1 vector (Invitrogen). A human expression plasmid of Gal4p-DBD-UBC9 and VP16AD-XBP1 was constructed using pBIND and pACT vectors (Promega), respectively, while that of myc-UBC9 was constructed using a pCMV-myc vector (Clontech). Expression plasmids of shRNAs for UBC9 were constructed by inserting oligonucleotides (GATCCCCAGCAGAGGCCTACACGATTTTCAAGAGAAATCGTGTAGGCCTCTGCTTTTTTA and GATCCCCGGGTCCGAGCACAAGCCAATTCAAGAGATTGGCTTGTGCTCGGACCCTTTTTA) into pSuper vector (Oligo-Engine). Point mutations of cysteine 93 to alanine of human UBC9 were introduced by site directed mutagenesis.

Two hybrid screening

Two hybrid screening was carried out essentially in accordance with the manufacturer's protocol. In brief, yeast AH109 cells (*MATa*, *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3*, *GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2*, *URA3 ::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ*) were transfected with pBridge-Gal4p-DBD-XBP1(U) and pACT-Gal4p-AD-cDNA libraries from human pancreas (Clontech) were plated onto histidine-lacking SD plates containing 0.2 mM 3-aminotriazole. Clones that grew on these plates lacking histidine were picked up, and plasmids containing the human cDNA were recovered from them.

Immunoblotting

Cells grown in a 60 mm culture dish were harvested using a cell scraper and pelleted by centrifugation. The pellet was suspended in 20 μl of ice-cold PBS containing protease inhibitors (100 μM AEBSF, 80 μM aprotinin, 1.5 μM E-64, 2 μM leupeptin, 5 μM bestatin and 1 μM pepstatin A, 10 μM MG132), mixed with 20 μl of 4×SDS-sample buffer (200 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, and 40% glycerol), and immediately boiled at 100°C for 10 min (Komori *et al.*, 2012). Portions of samples (10 μl) were subjected to SDS-polyacrylamide gel electrophoresis using 4–20% gradient gels, transferred onto a Hybond-P membrane (GE), and incubated with various antisera, in accordance with the standard protocol (Sambrook *et al.*, 1989). Anti-XBP1 (Santacruz, sc-7160), anti-UBC9 (IMGENEX, IMG-3286), anti-GAPDH (Trevigen, 2275-PC-100), anti-myc antisera (Wako, 017-21871), an ECL Western blotting detection kit (GE) and a LAS-3000 lumino-image analyzer (Fuji Film) were used to detect antigens.

Northern blotting hybridization analysis

Total RNA extracted from cells using guanidine-phenol was separated by electrophoresis on a 2% or 3% agarose gel containing 2.2 M formaldehyde, blotted onto a Hybond-N+ membrane (GE), hybridized with DIG-conjugated cDNA probes, and detected using a LAS-3000 lumino-image analyzer with a Gene Images AlkPhos Direct Labeling and Detection System (GE).

Immunoprecipitation experiments

Cell lysates were prepared from HeLa cells transfected with pcDNA-XBP1 and myc-UBC9 by three cycles of freeze-thawing, and myc-UBC9 was immunoprecipitated using agarose beads conjugated with anti-myc antibody. Immunoprecipitated materials were subjected to immunoblotting analysis.

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