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RNase domains determine the functional difference between IRE1α and IRE1β

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Abstract **Endoplasmic reticulum (ER) stress is associated with the functional disorder of the ER. During conditions of ER stress, cells induce at least two responses to maintain ER function: transcriptional upregulation of ER quality control genes, and translational attenuation of protein synthesis. Induction of ER quality control proteins is mediated by IRE1α, which activates the transcription factor XBP1 via an unconventional splicing event, while a partial translational attenuation is mediated by IRE1β. Here, we show by both in vivo and in vitro analyses that the RNase domain of IRE1 determines the functional specificities of each of these isoforms.**

Keywords: ER stress; IRE1; Unfolded protein response

1. Introduction

Endoplasmic reticulum (ER) stress is caused by the accumulation of malfolded proteins in the ER and is fatal to living cells. To protect themselves, cells undergoing ER stress activate a pathway called the unfolded protein response (UPR) [1]. This response includes the transcriptional induction of ER quality control proteins, which promote protein folding and ER-associated protein degradation in the ER, and the global translational repression of protein synthesis, which reduces the influx of ER client proteins [2].

Mammalian cells have paralogous ER stress sensors, IRE1α and IRE1β. They consist of an N-terminal ER luminal sensor domain and a C-terminal cytosolic effector region that contains both kinase and endoribonuclease (RNase) domains. Comparing the amino acid (aa) sequences of human IRE1α and IRE1β, the sensor, kinase and RNase domains show 48%, 80% and 61% identity, respectively [3]. Although these two isoforms have structural similarity, they possess quite different functions [3–5]. Under conditions of ER stress, IRE1α catalyzes the spliceosome-independent processing of the pre-mRNA encoding transcription factor XBP1. This leads to the production of the mature form of XBP1 protein, which in turn induces UPR target genes [6–8]. On the other hand, as we have reported previously [3], human IRE1β mediates the site-specific cleavage of 28S rRNA and translational attenuation of protein synthesis. In this report, we examine the functions of the IRE1α and IRE1β RNase domains by generating chimeric IRE1 mutants and assaying their functions in vivo and in vitro.

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Abbreviations: aa, amino acid; ER, endoplasmic reticulum; hIRE1, human IRE1; rIRE1, recombinant hIRE1 protein; RNase, endoribonuclease; Tm, tunicamycin; UPR, unfolded protein response

2. Materials and methods

2.1. Plasmid construction

To generate human IRE1 (hIRE1) expression plasmids, each cDNA was inserted into the mammalian expression vector pCAGGS. Plasmids containing hIRE1α and hIRE1αK599A cDNA were gifts from Dr. R.J. Kaufman (University of Michigan). pGL3-hBiP (−132) was a gift from Dr. K. Mori (Kyoto University). The baculovirus vector expressing the cytosolic region of hIRE1α or hIRE1β was constructed by inserting a PCR fragment encoding (aas) 468–977 of hIRE1α or 451–925 of hIRE1β into pFastBacHTa (Invitrogen), respectively. The XBP1 in vitro transcription vector, including nucleotides 410–633 of human *XBP1* cDNA, was inserted into pBluescript II KS+ (Stratagene). IRE1 and XBP1 mutations were created using overlap PCR.

2.2. Cell culture and transfection

HeLa cells (HeLa Tet-Off, Clontech) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Sf9 insect cells (Invitrogen) were cultured in Sf-900II (Invitrogen) at 28 °C. Transient transfection of plasmids was performed as described previously [3].

2.3. Western blot analysis

After SDS–PAGE, the proteins were electrotransferred onto a nitrocellulose membrane, and immunodetected with polyclonal antiserum against the C-terminal 14 aa of hIRE1α or hIRE1β.

2.4. RT-PCR analysis of XBP1 mRNA splicing

Total RNA was prepared using Isogen (Nippon Gene). First-strand cDNA synthesis was performed with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). To amplify *XBP1* cDNA, PCR was carried out using 5′-GAACCAGGAGTTAAGACAGC-3′ and 5′-AGTCAATACCGCCAGAATCC-3′ primers.

2.5. Luciferase assay

HeLa cells (5×10^4 cells) were transfected with 0.5 μg of a hIRE1 expression plasmid, 0.25 μg of a reporter plasmid pGL3-hBiP (−132) and 10 ng of pRL-SV40. Measurement of BiP promoter activity was performed using the Dual-luciferase assay system (Promega).

2.6. Northern blot analysis of 28S rRNA cleavage

Northern blot analysis was performed as described previously [3]. 28S rRNA was detected using the 3′ end specific probe (5′-ACAAACCCTTGTGTCGAGGGCTGA-3′).

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2.7. Baculovirus expression and purification of hIRE1 proteins

hIRE1 proteins were expressed according to the manufacturer's protocol described in the Bac-to-Bac Baculovirus Expression Systems manual (Invitrogen). Baculovirus-infected Sf9 cells were lysed in 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40. hIRE1 proteins containing an N-terminal hexahistidine tag were purified using TALON His-Tag Purification Resin (Clontech).

2.8. In vitro autophosphorylation assay of IRE1

Recombinant IRE1 proteins (rIRE1s) (5 pmol) were incubated with 0.74 MBq [γ-^{32}P] ATP in 20 μl kinase buffer (20 mM HEPES, 1 mM DTT, 10 mM $Mg(OAc)_2$, 50 mM KOAc [pH 7.3 at 4 °C]) at 30 °C for 30 min. After incubation, the rIRE1s were precipitated with 10% (w/v) trichloroacetic acid, and the precipitate was resolved by 10% SDS–PAGE and visualized by autoradiography.

2.9. In vitro XBP1 mRNA cleavage assay

[^{32}P]-labeled *XBP1* mRNAs were transcribed in vitro using T7 RNA polymerase (Promega). The RNA (4 fmol) was incubated in 20 μl kinase buffer containing 2 mM ATP with the rIRE1s (5 pmol) at 30 °C. After incubation, the RNA was isolated using Isogen and resolved on a 7 M urea-denatured 6% polyacrylamide gel.

2.10. In vitro 28S rRNA cleavage assay

Polysomes were isolated from HeLa cells as described previously [9]. The polysome fraction (containing 5 μg RNA) was incubated in 20 μl kinase buffer containing 2 mM ATP with 5 pmol rIRE1s at 30 °C. After incubation, ribosomal RNA was isolated using Isogen and 28 S rRNA was detected by Northern blot analysis.

3. Results and discussion

3.1. Requirement of the hIRE1α RNase domain for XBP1 mRNA splicing

In previous studies, it has been shown that both murine IRE1α and IRE1β cleave *XBP1* mRNA [6–8]. On the other hand, we have reported that hIRE1β effected site-specific cleavage of 28S rRNA [3]. We therefore, speculated that IRE1α and IRE1β may have different functions and that this functional difference might be determined by their respective RNase domains. To test this idea, we examined the effects of various IRE1 mutants on the splicing of *XBP1* mRNA and cleavage of 28S rRNA.

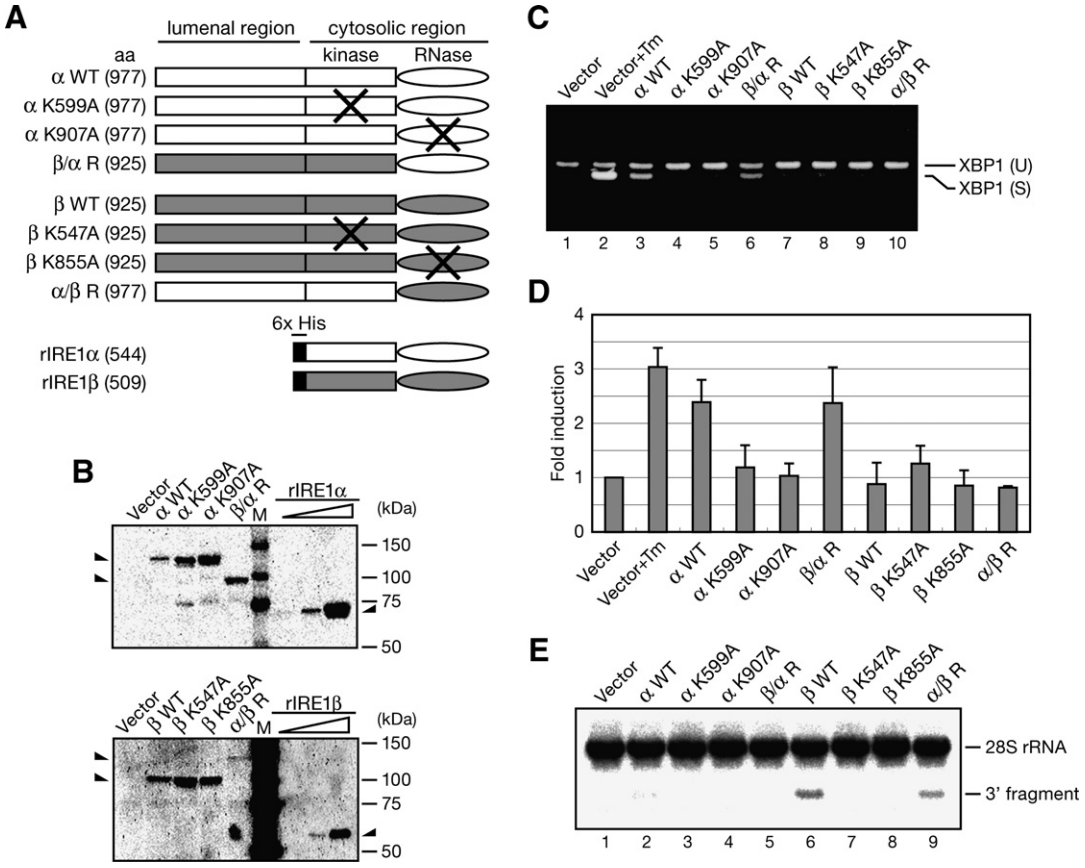

Fig. 1. Requirements of the hIRE1 RNase domain for *XBP1* mRNA splicing or 28S rRNA cleavage. (A) Schematic representations of the hIRE1 variants and recombinant hIRE1 (rIRE1) used in this study. αWT, αK599A, αK907A, βWT, βK547A, βK855A, β/αR and α/βR are described in the text. The numbers in parentheses indicate total amino acid residues of each molecule. X indicates the marked domain contains an amino acid substitution. (B) To assess the expression of each variant, Western blot analysis was performed. Each variant of hIRE1α (109.7 kDa) and hIRE1β/αR (102.6 kDa) (10 μg/lane) and hIRE1β (102.3 kDa) and hIRE1α/βR (109.4 kDa) (20 μg/lane) was immunodetected with anti-hIRE1α or anti-hIRE1β antiserum at 30 h post-transfection. rIRE1α and rIRE1β contain 0.5, 1, 2 pmol/lane and 1, 2, 4 pmol/lane of the respective recombinant proteins. The positions of IRE1 valiants are indicated by arrowheads, and the positions of molecular size standard (lane M: *Precision Plus protein molecular weight markers* [Bio-Rad]) are also shown. (C) *XBP1* mRNA splicing was detected by RT-PCR. At 30 h post-transfection, cells were treated with or without 2 μg/ml tunicamycin (Tm) for 6 h, and then total RNA was prepared. The unspliced and spliced forms of *XBP1* mRNA are referred to as XBP1(U) and XBP1(S), respectively. (D) To examine the transcriptional induction of the UPR genes, a reporter assay using the BiP promoter was performed. At 30 h post-transfection, cells were treated with or without 2 μg/ml Tm for 6 h, and then luciferase activity was measured. Error bars indicate the S.D. of triplicate experiments. (E) 28S rRNA cleavage was detected by Northern blot analysis using the 3′ end specific probe at 36 h post-transfection.

To determine which domain of hIRE1 is required for *XBP1* mRNA splicing, we performed RT-PCR analysis of *XBP1* mRNA splicing in HeLa cells overexpressing various IRE1s using the constructs shown in Fig. 1A. αWT, αK599A, αK907A, βWT, βK547A and βK855A indicate wild-type, a kinase-dead mutant, and RNase-dead mutant of hIRE1α and hIRE1β, respectively [10]. Overexpression of IRE1 results in its spontaneous activation, even in the absence of ER stress [10]. The expression levels of each variant were analyzed by immunoblotting (Fig. 1B). Splicing of *XBP1* mRNA was detected in cells overexpressing wild-type hIRE1α (αWT) or a chimeric hIRE1β (β/αR) consisting of the sensor and kinase domains of hIRE1β (aa: 1–776) and the RNase domain of hIRE1α (aa: 829–977) (Fig. 1C). It is noted that wild-type hIRE1α clearly showed high activity of *XBP1* mRNA splicing, even though it was expressed at lower amounts compared to wild-type hIRE1β (βWT). However, splicing was not detected in cells overexpressing any other variant including βWT (Fig. 1C). We also examined the transcriptional induction of UPR genes caused by the transcription factor XBP1 in a reporter assay using the ER chaperone BiP promoter, one of the target genes of XBP1 [6]. Consistent with the result of *XBP1* mRNA splicing, luciferase activity was induced in only αWT and β/αR-overexpressing cells (Fig. 1D). Thus, the RNase domain of hIRE1α is necessary for *XBP1* mRNA splicing.

3.2. Requirement of hIRE1β RNase domain for 28S rRNA cleavage

To examine which domain is required for the site-specific cleavage of 28S rRNA, we performed Northern blot analysis of 28S rRNA in cells overexpressing IRE1 variants. Cleavage was clearly detected in cells overexpressing βWT or a chimeric

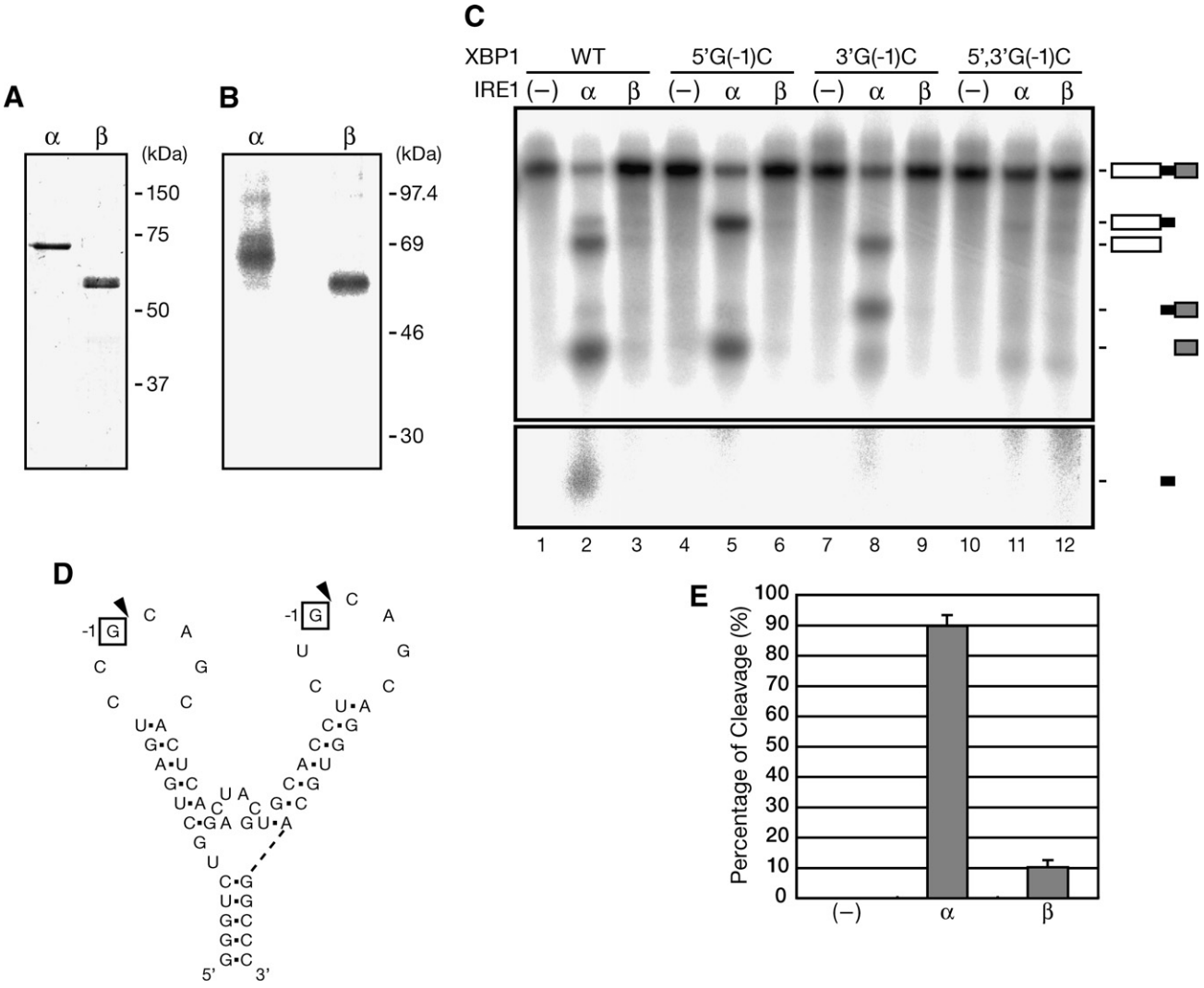

Fig. 2. Purification of rIRE1 and in vitro *XBP1* mRNA cleavage. (A) Purified hIRE1 proteins were subjected to SDS–PAGE. The gel was stained with Coomassie Brilliant Blue. (B) To check their autophosphorylation abilities, the purified proteins were incubated with [γ-^{32}P] ATP, separated by SDS–PAGE and visualized by autoradiography. (C) To assess the cleavage activity of IRE1s against *XBP1* mRNA, the *XBP1* transcripts were incubated with rIRE1 at 30 °C for 30 min. After incubation, the RNA fragments were resolved on a urea-denatured polyacrylamide gel and visualized by autoradiography. Nucleotide substitutions in mutant mRNAs 5′ G(−1)C, 3′ G(−1)C and 5′, 3′ G(−1)C are described in the text. The icons to the right of the figure indicate the fragments of *XBP1* mRNA produced by IRE1 cleavage. The boxes and line indicate the exon and intron of *XBP1* mRNA, respectively. (D) Predicted secondary structure of human *XBP1* mRNA in the region surrounding the IRE1 cleavage site (represented by arrowheads). (E) Quantification of the RNA fragments shown in C, lanes 1–3. Error bars indicate the S.D. of triplicate experiments.

hIRE1α (α/βR) in which the RNase domain was swapped with that of hIRE1β (aa: 777–925) (Fig. 1E). Cleavage was not detected in cells overexpressing any other mutant (Fig. 1E). We note α/βR was less abundant than βWT, yet still showed 28S rRNA cleavage activity. Unexpectedly, weak 28S rRNA cleavage was detected in cells overexpressing αWT (Fig. 1E, lane 2), suggesting that hIRE1α possesses low-level 28S rRNA cleavage activity. This result indicates that the RNase domain of hIRE1β functions better to cleave 28S rRNA in vivo, and suggests that the functional differences between hIRE1α and hIRE1β depend on their respective RNase domains.

3.3. Higher activity of hIRE1α in XBP1 mRNA cleavage

Next, to assess the cleavage activity of the various IRE1s against substrate RNAs, we performed in vitro RNA cleavage assays. The recombinant hIRE1 proteins contained the cytosolic effector region of hIRE1α or hIRE1β fused with a hexahistidine tag at the N terminus. The rIRE1s were purified on Co^{2+}-loaded resin (Fig. 2A), and tested to confirm they retained autophosphorylation function (Fig. 2B), as both IRE1s are known to have *trans*-autophosphorylation activity [10]. To assess their cleavage activities against *XBP1* mRNA, purified rIRE1s were incubated with an in vitro transcript of *XBP1*. Cleavage of *XBP1* was clearly detected when rIRE1α was present, whereas weak activity was observed with rIRE1β (Fig. 2C, lanes 1–3, Fig. 2E). This conforms to a previous report showing that rIRE1β could cleave the *XBP1* mRNA [7]. The activity of rIRE1β seemed very weak in our system, but we note the same rIRE1β preparation retains phosphorylation ability (Fig. 2B) and could cleave 28S rRNA (Fig. 3). This indicates that rIRE1β was functional and that hIRE1α has much higher cleavage activity against *XBP1* mRNA than hIRE1β. Both the 5′ and 3′ cleavage sites of *XBP1* mRNA form characteristic stem-loop structures as shown in Fig. 2D. Nucleotide (−1) in each loop is reported to be crucial for IRE1α-specific cleavage [8]. To confirm that the *XBP1* transcript was cleaved specifically by IRE1, rIRE1s were incubated with *XBP1* mutant transcripts 5′G(−1)C (substituting cytosine [C] for guanine [G] at the −1 nucleotide position of the 5′-cleavage site), 3′G(−1)C (substituting C for G at the 3′-cleavage site), or 5′,3′G(−1)C (substituting C for G at both the 5′ and 3′-cleavage sites). With the 5′G(−1)C and the 3′G(−1)C transcript, rIRE1-dependent cleavage was detected in only the 3′ and 5′ loop, respectively. No cleavage was detected with the 5′,3′G(−1)C transcript (Fig. 2C, lanes 4–12). These results support the idea that IRE1α was specifically responsible for the cleavage of *XBP1* mRNA.

3.4. Higher activity of hIRE1β in 28S rRNA cleavage

To assess their cleavage activities against 28S rRNA, we incubated rIRE1s with polysomes isolated from HeLa cells. Cleavage of 28S rRNA was detected when either rIRE1 was present. However, IRE1β was more active in cleaving 28S rRNA than IRE1α (Fig. 3). We noticed that this cleavage was specific to the 28S rRNA: 18S rRNA remained intact during the reaction (Fig. 3A, lower panel). These results indicate that hIRE1β directly catalyses site-specific cleavage of 28S rRNA. Unexpectedly, we observed that rIRE1α could also cleave 28S rRNA. This result is consistent with the activity observed in vivo (Fig. 1E, lane 2) but the difference in activity between rIRE1α and rIRE1β is smaller than seen in the in vivo

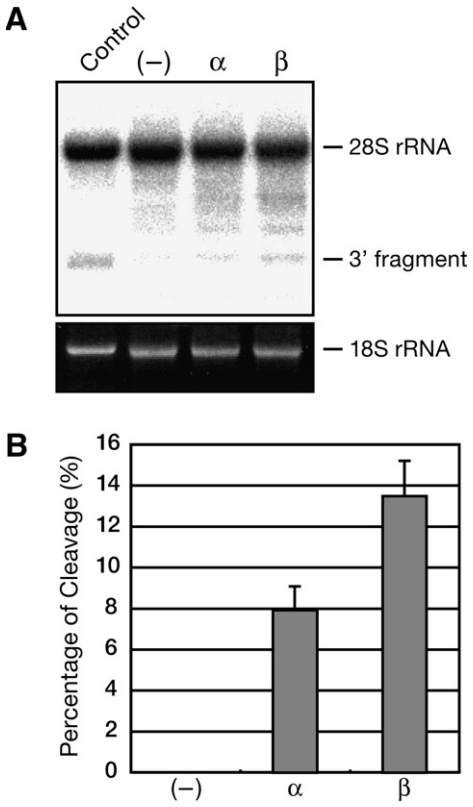

Fig. 3. In vitro 28S rRNA cleavage. (A) Polysomes were incubated with rIRE1 at 30 °C for 30 min. 28S rRNA cleavage was detected by Northern blot analysis using the 3′ end specific probe. Lane Control contains total RNA isolated from hIRE1β-overexpressing HeLa cells (Fig. 1E, lane 6). (B) Quantification of Northern blots shown in A. Error bars indicate the S.D. of triplicate experiments.

assay, suggesting the presence of other factors determining IRE1 substrate specificity, or different conditions in vivo that might affect IRE1 activity, such as interaction with ribosomes on the ER membrane. Our 28S rRNA cleavage assay also suggests that the specific cleavage reaction requires the proper ribosomal subunit tertiary structure, since this reaction occurred only when the intact ribosome was used as a substrate. It seems reasonable to suggest that the hIRE1β cleavage site of 28S rRNA exists within the L1 protuberance of the 60S subunit and is associated with the formation of the ribosomal E site [11,12].

We conclude from the experiment described above that IRE1α and IRE1β have different functions and suggest that this functional difference is determined by their substrate specificities of their respective RNase domains. What is the physiological significance of these different substrate specificities in the ER stress response? Recently, *Drosophila* IRE1 was shown to mediate the rapid degradation of specific ER-localized mRNAs, which may help to decrease the burden of incoming proteins in the ER [13]. The identification of a novel mammalian IRE1α cleavage target was also reported [14]. Considering that IRE1α is expressed in various tissues, whereas IRE1β expression is limited to the digestive tissues [4,15], it is reasonable to imagine the existence of a tissue-specific ER stress response pathway mediated by IRE1β-specific RNA cleavage. Future research is required to identify IRE1β-dependent

cleavage targets and to elucidate the IRE1β-dependent ER stress response pathway.

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