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Studies on a novel mammalian ER transmembrane J-protein, DNAJB14

新規小胞体膜 J タンパク質 DNAJB14 の機能に関する研究

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Graduate School of Biological Sciences　Doctoral Thesis Abstract

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Abstract

The J-protein/heat shock protein 40 (Hsp40) family is a conserved prokaryotic and eukaryotic protein group whose members share a J-domain that often promotes the binding of a Hsp70 family member to unfolded proteins and enhances ATPase activity of the Hsp70 protein. The J-protein/Hsp70 teams play important roles in a variety of cellular processes, including protein synthesis, folding, and degradation.

The endoplasmic reticulum (ER) is a cellular compartment where newly synthesized secretory and membrane proteins are folded, assembled, and modified. Proteins that failed to be correctly folded are pulled out from the ER to the cytosol, and poly-ubiquitinated for degradation by the proteasome. This process is known as the ER-associated degradation (ERAD).

DNAJB12 is a mammalian ER-localized transmembrane J-protein having its J-domain facing the cytosol. Recent studies from this and other laboratories have shown that DNAJB12, together with Hsc70 (a cytosolic Hsp70), is involved in the ERAD of misfolded ER membrane proteins. *In silico* search of mammalian genomes has identified DNAJB14, which exhibits a high similarity to DNAJB12. However, the function and basic character of DNAJB14 remained entirely unknown. Therefore, in this study, I aimed to reveal the function of this protein.

To study the expression of *DNAJB14,* I performed RT-PCR using total RNA from mouse tissues. Interestingly, in contrast to *DNAJB12* that is ubiquitously expressed, *DNAJB14* mRNA is relatively abundant in testis but present at low levels in most tissues, implying its specific role in this tissue. Curiously, semi-quantitative RT-PCR analysis with the mouse cell line NIH3T3 showed that the transcription of both *DNAJB14* and *DNAJB12* is not affected by either heat shock or ER stress.

To characterize DNAJB14, I raised antibodies specific for the N- and C- termini of this protein. The immunostaining and proteinase K protection assay, using the antibodies and HeLa cells overexpressing DNAJB14, revealed that DNAJB14 is an ER-localized, single membrane spanning protein having its J-domain in the cytosol. Further, co-immuno-precipitation showed that DNAJB14, like DNAJB12, interacts with Hsc70 via its J-domain. Thus, DNAJB14 resembles DNAJB12 in both its architecture and its sub-cellular localization.

Next, I proceeded to ask whether DNAJB14 also accelerates the degradation of misfolded membrane proteins by performing cycloheximide chase experiments. Strikingly, the overexpression of DNAJB14 greatly enhanced the degradation of CFTRΔF508, a misfolded polytopic membrane protein, in a manner dependent on the proteasome. Thus, DNAJB14 can promote the ERAD of this protein. Similarly, DNAJB14 accelerated the degradation of T-cell receptor α subunit, a single membrane-spanning protein. However, this protein failed to stimulate the degradation of null-Hong Kong (NHK) variant of α1-antitrypsin (A1AT), a misfolded ER luminal protein. Hence, DNAJB14, like DNAJB12, can promote specifically the ERAD of misfolded membrane proteins.

In summary, my work has established that the mammalian ER possesses two single membrane-spanning J-proteins having its J-domain in the cytosol (DNAJB14 and DNAJB12) and that they can promote the ERAD of misfolded membrane proteins. Whether they have evolved to play distinct roles *in vivo* must await further studies.

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List of Abbreviations

A1AT-NHK:	α1-antitrypsin-null Hong Kong variant
CFTR:	Cystic fibrosis transmembrane conductance regulator
CFTRΔF508:	A CFTR mutant having a deletion of phenylalanie 508
CNX:	Calnexin
EndoH:	Endoglycosidase H
ER:	Endoplasmic reticulum
ERAD:	ER-associated degradation
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
Hsc70:	70 kDa heat shock cognate protein
Hsp40:	40 kDa heat shock protein
Hsp70:	70 kDa heat shock protein
Hsp47:	47 kDa heat shock protein
IP:	Immunoprecipitation
RT-PCR:	Reverse transcription polymerase chain reaction
TCRα:	T cell-receptor α subunit

I. Introduction

1.1 J-proteins

The heat shock protein 40 (Hsp40)/J-proteins, a protein group conserved in all kingdoms of life, play vital roles in various cellular processes such as protein synthesis and maturation, protein translocation across membranes, and misfolded protein degradation (Walsh *et al.,* 2004; Qiu *et al.,* 2006). Thus far, the human genome is suggested to encode total of 47 J-proteins (Table 1) (Walsh *et al.,* 2004; Qiu *et al.,* 2006; Kampinga and Craig, 2010), implying the diverse roles played by J-proteins in cells. J-proteins are defined by the presence of a J-domain containing the His-Pro-Asp signature tripeptide. Via this domain, J-proteins bind heat shock protein 70 (Hsp70) and activate the ATPase of Hsp70, allowing Hsp70 to recognize and fold its client proteins (Jiang *et al.,* 2007; Kampinga and Craig, 2010).

J-proteins are classified into three subgroups according to their domain organization (Fig. 1). Type A J-proteins have three domains in the following order: a J-domain, a glycine/phenylalanine-rich region, and a cysteine-rich domain. Type B J-proteins take the same domain organization as type A J-proteins, but lack the C-terminal cysteine-rich domain. Type C J-proteins contain only a J-domain and lack both of the other domains in their structures (Fig. 1) (Walsh *et al.,* 2004; Qiu *et al.,* 2006; Kampinga and Craig, 2010).

In eukaryotic cells, J-proteins are distributed in many subcellular compartments such as cytosol, nucleus, mitochondria and endoplasmic reticulum (ER) (Walsh *et al.,* 2004; Qiu *et al.,* 2006; Vos *et al.,* 2008). Among them, the ER is a cellular compartment where secretory and transmembrane proteins are synthesized and modified (Hebert and Molinari, 2007). Various ER-localized J-proteins contribute

to protein homeostasis in this compartment. For example, ERdj3 (also called DNAJB11) binds a nascent protein or a protein that is unable to fold in the ER. This binding recruits BiP, an ER-resident Hsp70, to the target protein. The recruited BiP, then, assists the folding of the target protein or maintains the soluble state of the protein before degradation. (Shen and Hendershot, 2005). Furthermore, JPDI (also called ERdj5) promotes the degradation of misoxidized proteins that arise in the ER (Hosoda *et al.*, 2003; Ushioda *et al.,* 2008). (See also below for the general mechanism of the degradation of misfolded ER proteins).

1.2 ER-associated degradation (ERAD)

When proteins fail to attain their correctly folded state in the ER, they are recognized, dislocated from the ER to the cytosol, and polyubiquitinated for degradation by the proteasome in a process collectively called ER-associated degradation (ERAD) (Fig. 2) (Wiertz *et al.,* 1996; Tsai *et al.,* 2002; Vembar and Brodsky, 2008; Hegde and Ploegh, 2010). ERAD of proteins requires the cooperation of various enzymes. They include Hsp70s, J-proteins, and proteins involved in ubiquitin proteasome pathway, such as E3 ubiquitin ligases (Table 2).

When misfolded proteins arise in the ER membrane, these proteins are also degraded by ERAD. Interestingly, this process is stimulated by Hsc70, an Hsp70 family member in the cytosol (Yang *et al.,* 1993; Ward, *et al.,* 1995; Younger, *et al.,* 2006; Morito *et al.,* 2008). As described above, Hsp70 generally requires a specific J-protein to recognize their substrate proteins and exert their functions. However, the J-protein involved in this process had remained unknown.

However, recently, the Kohno lab and other lab discovered that this missing protein is DNAJB12 (Fig. 3). DNAJB12 is an ER-localized type II, transmembrane J-

protein, having its J-domain in the cytosol. This protein physically interacts with Hsc70 in the cytosol and promotes the degradation of misfolded ER membrane proteins via the proteasome (Yamamoto *et al.,* 2010; Grove *et al.,* 2011). Thus, DNAJB12 plays a vital role in the quality control of ER membrane proteins (Fig. 3).

1.3 Objectives of the research

Interestingly, recent computational analyses have revealed that human and mouse genomes encode a protein called DNAJB14 that exhibits a high sequence similarity to DNAJB12 (Qiu *et al.,* 2006; Vos *et al.,* 2008; Hageman and Kampinga, 2009; Kampinga and Craig, 2010). Yet, the function of this protein remained entirely unknown.

Here, I investigated the basic character and function of this protein to find that DNAJB14 is, like DNAJB12, an ER-localized, type II transmembrane protein, having its J-domain in the cytosol. Further, I presented evidence that DNAJB14 physically interacts with Hsc70 in the cytosol and promotes the degradation of misfolded transmembrane proteins via the proteasome. Based on these findings, I propose that the ER of mammalian cells has two analogous transmembrane J-proteins that can promote the ERAD of misfolded membrane proteins.

Nevertheless, I found some differences in the properties between DNAJB14 and DNAJB12. I will also discuss the implication of the observed differences.

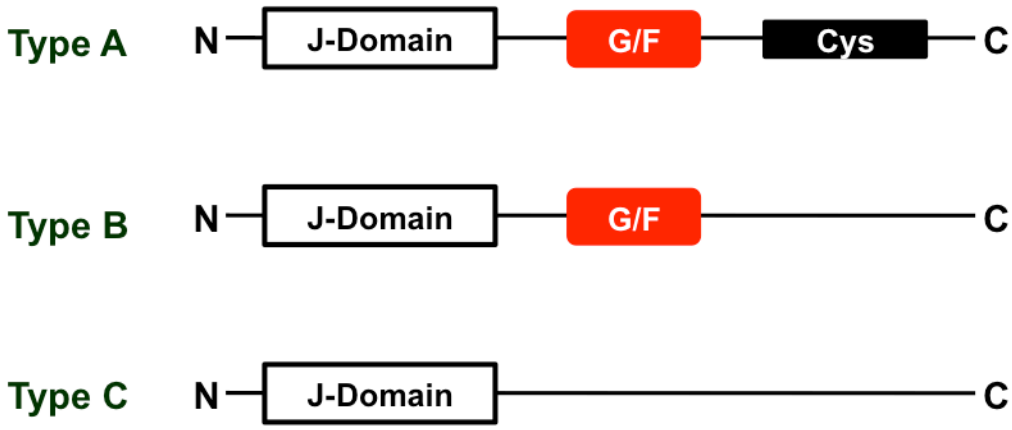

Fig. 1. Structural classification of J-proteins. The type A, B and C J-proteins are defined by their domain organization. Open box, J-domain shared by all three subgroups; closed red box, glycine/phenylalanine-rich domain shared by type A and B J-proteins; closed black box, cysteine-rich region that is present only in type A J-protein.

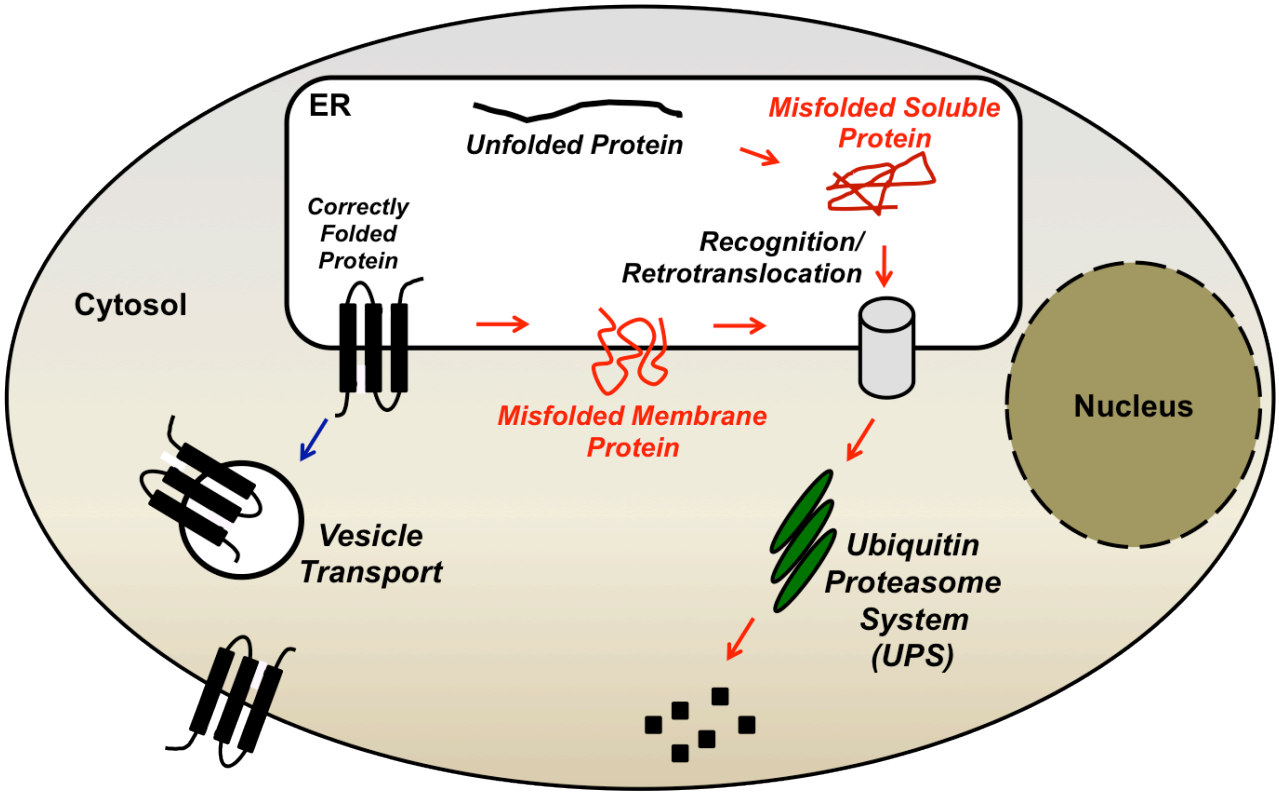

Fig. 2. ER-associated degradation (ERAD). Misfolded proteins that arise in the ER are recognized, dislocated from the ER into the cytosol, poly-ubiquitinated and degraded by the ubiquitin proteasome system in a process collectively called ERAD. Only correctly folded proteins can exit from the ER for transport to their final destinations.

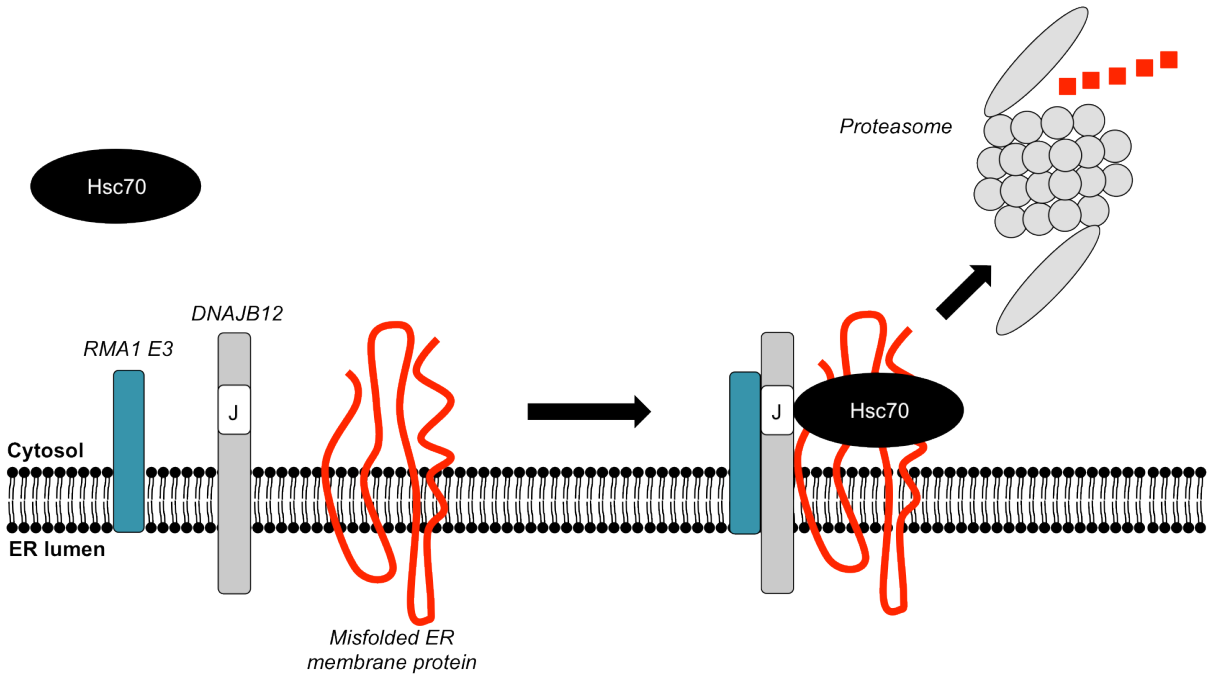

Fig. 3. A model for DNAJB12-mediated degradation of misfolded membrane proteins. DNAJB12 recognizes misfolded membrane proteins in the ER. Upon recognition, DNAJB12 and its substrate may form a heterotetrameric complex with Hsc70 (an Hsp70 family member in the cytosol) and RMA1 (an E3 ubiquitin ligase). The recognized misfolded membrane protein may then be poly-ubiquitinated by RMA1 and targeted to the proteasome in the cytosol for degradation.

Table 1. Overview of human J-proteins.

Protein	Amino acids	Other names	Yeast orthologue	Localization	Function or predicted function
DnaJA1	397	*DJ-2, DjA1, HDJ2, HSPF4, HSJ2, HSDJ, hDj-2*	Ydj1	Cytosol, Nucleus	Hormone receptor maturation
DnaJA2/ DnaJA2b	412/415	*CPR3, DNJ3, HIRIP4, DNAJ3, mDj3*		Cytosol, Nucleus	G-protein signaling
DnaJA3	480	*TID1, hTid-1, Tidl1*	Mdj1	Cytosol, Mitochondria(i)	Signaling
DnaJA4	397	*Dj4, Hsj4*		Cytosol, Nucleus	Protein folding
DnaJB1/ DnaJB1b	340/339	*HSPF1, Hsp40*	Sis1	Cytosol, Nucleus	Protein refolding
DnaJB2	324/277	*HSJ1, HSPF3, DnaJB10*		Cytosol, ER(m)	Protein degradation
DnaJB3	242	*Hsj3, Msj1, MSJ-1*		Cytosol	Protein folding in sperm
DnaJB4	337	*DNAJW, Djb4, HLJ1*		Cytosol, Nucleus	
DnaJB5	348/241	*Hsc40, HSP40-3*		Cytosol, Nucleus	
DnaJB6	326/341	*HHDj1, HSJ-2, MSJ-1, Mrj, mDj4*		Cytosol, Nucleus	Anti-aggregation
DnaJB7	309	*HSC3, Dj5, mDj5*		Cytosol, Nucleus	
DnaJB8	232	*MGC33884, mDj6*		Cytosol, Nucleus	Anti-aggregation
DnaJB9	223	*ERdj4, MDG1, mDj7*		ER(m)	
DnaJB11	358	*ABBP-2, ERdj3, hDj9*		ER(i)	Protein folding, mRNA editing
DnaJB12	375	*DJ10, mDj10*	Hlj1	ER(m)	ERAD
DnaJB13	316	*Tsarg*		Cytosol	
DnaJB14	379	*EGNR9427, FLJ14281*		ER(m)	ERAD (*This study)*
DnaJC1	554	*DNAJL1, HTJ1, ERj1p, ERdj1*		ER(m)	Protein translocation, translation
DnaJC2	621	*MPP1*	Zuo1	Cytosol	Translation
DnaJC3	504	*HP58, P58IPK, PRKR1*	HRC558	ER(i)	Translation under stress
DnaJC4	135	*HSPF2, MCG18*			
DnaJC5, b, g	198/199/ 189	*CSP, CSP-beta, CSP-gamma*		Exosome	Exocytosis
DnaJC6	913	*DJC6, auxilin, mKIAA0473*	Swa2	Cytosol	Clathrin uncoating
DnaJC7	484	*TPR2, TTC2*	STI1	Cytosol	Hormone maturation
DnaJC8	264	*SPF31*		Cytosol	Protein phosphorylation
DnaJC9	260	*JDD1, SB73*		Nucleus	
DnaJC10	793	*ERdj5, JPDI*		ER(i)	ERAD

Table 1. (*continued*)

Protein	Amino acids	Other names	Yeast orthologue	Localization	Function or predicted function
DnaJC11	559	*FLJ10737*		Mitochondria(i)	
DnaJC12	198/107	*JDP1*			Estrogen signaling
DnaJC13	2243	*RME-8*		Endosome	Endosome trafficking
DnaJC14	702	*DRiP78, HDJ3, LIP6*		Cytosol, ER(m), Mitochondria	Cell surface export
DnaJC15	150	*DNAJD1, HSD18, MCJ*		Golgi	Degradation oncoproteins
DnaJC16	782	*RP4-680D5.1*			
DnaJC17	304	*FLJ10634*	Cwc23		
DnaJC18	358	*MGC29463*			
DnaJC19	116	*TIM14, TIMM14*	Pam18	Mitochondria(i)	Protein translocation
DnaJC20	235	*HSC20, HscB*	Jac1	Mitochondria(i)	FeS cluster biogenesis
DnaJC21	202	*DnaJA5*	Jjj1		
DnaJC22	341	*FLJ13236*		Cytosol	Endocytosis
DnaJC23	760	*SEC63L, ERdj2*	Sec63	ER(m)	Protein translocation
DnaJC24	149	*DPH4*	Jjj3	Cytosol	Dipthamide synthesis
DnaJC25	360	*Gng10, ERdj7, ERj7*		ER(m)	
DnaJC26	1311	*GAK*		Cytosol, Nucleus	Clathrin uncoating
DnaJC27	273	*RBJ, Rabj*			
DnaJC28	454	*Orf28*			
DnaJC29	4432	*SACS, Sacsin*		Cytosol, Mitochondria	Protein degradation
DnaJC30	226	*WBSCR18*			

Note: (i), located in the inside of the organelle; (m), a membrane-associated or transmembrane protein. Adapted from Qiu *et al.* (2006) and Kampinga and Craig (2010).

Table 2. Human and yeast proteins implicated in ERAD.

Process in ERAD	Human proteins	Alternative name	Localization	Yeast homologs
Recognition	ER ManI	-	ER membrane	Mns1
	EDEM1-3	-	ER lumen	Htm1
	PDI	-	ER lumen	Pdi
	BiP	GRP78	ER lumen	Kar2
	GRP94	-	ER lumen	-
	ERdj5	JPDI	ER lumen	-
	OS-9	-	ER lumen	Yos9
	SEL1L	-	ER membrane	Hrd3
Translocation	Sec61 complex	-	ER membrane	Sec61 complex
	Derlin1-3		ER membrane	Der1
Poly-ubiquitination (E3 ubiquitin ligases)	HRD1	Synoviolin	ER membrane	Hrd1/Der3
	TEB4	MARCHVI	ER membrane	Doa10
	RMA1	RNF5	ER membrane	-
	CHIP	STUB1	Cytosol	-
Extraction and delivery to proteasome	p97	VCP	Cytosol	Cdc48
	UFD1		Cytosol	Ufd1
	NPL4		Cytosol	Npl4
	Ubxd8	ETEA	ER membrane-associated	Ubx2
Miscellaneous	HERP	Mif1	ER membrane	Usa1
	VIMP	ERASIN	ER membrane	-

II. Materials and Methods

2.1 RNA extraction

Eight-week old male mice were sacrificed and used to prepare tissues. The dissected tissues were lysed in RNAiso (Takara) using Mixer Mills MM300 (Retsch) and total RNA was extracted from the lysate, following the instructions provided by the manufacturer. Total RNA preparation from mouse fibroblast cell line, NIH3T3, followed the same instructions. All first strand cDNAs were synthesized using Superscript™ II Reverse Transcriptase (Invitrogen) and Oligo-dT primer.

2.2 Northern analysis of *DNAJB14*

Total RNAs were purified from mouse tissues, separated by agarose MOPS/formaldehyde gel electrophoresis, and then blotted onto nylon membrane by capillary transfer. The blotted membrane was hybridized with a DIG-labeled probe to detect *DNAJB14* mRNA. The probe to detect *DNAJB14* mRNA was prepared by PCR reaction that was carried out in the presence of DIG-dUTP (Roche) using KAPA Taq DNA polymerase, *DNAJB14* cDNA as a template, and mJB14-3'-F (5'-CGTACTCCTTATACCCCAGATCTGG-3') and mJB14-3'-R (5'-TCCTTGTAGAGACTGGTCAGCCG-3') as primers. For the PCR, a cycle (94^{o}C for 45 sec, 55^{o}C for 30 sec and 72^{o}C for 30 sec) was repeated 32 times. The hybridized membrane was further incubated with anti-Digitonin-AP Fab fragments (Roche) and then treated with CSPD substrate (Roche) to detect *DNAJB14* mRNA. The same membrane was then stripped and re-probed with *β-actin* probe. The probe to detect *β-actin* mRNA was synthesized using the same procedures as described above except that the reaction cycle was repeated 25 times using pHFβA-1 (Gunning *et al.,* 1983)

as a template and hβ-Actin-F (5'-AACTGGAACGGTGAAGGTGACA-3') and hβ-Actin-R (5'-ACTGGTCTCAAGTAGTGTACAGG-3') as primers.

2.3 Cloning of *DNAJB14* cDNA and plasmid construction

To amplify the full coding sequence of *DNAJB14* cDNA, total RNA from brain, small intestine, large intestine, lung, muscle, testis, and thymus was used for the first strand cDNA synthesis. The synthesized cDNAs were subjected to PCR using PrimeStar HS DNA polymerase (Takara), a forward primer, mDnaJB14-F (5'-TTAGAATTC*ATG*GAGGGCAACCGCGACGAG-3') and a reverse primer, mDNAJB14-R primer (5'-CCGGAATTC*TTA*TCCCCCCTTGTAGAGAC-3'); the underline indicates an *Eco*RI site, the italic letters indicate the start or stop codon. For the amplification, a cycle (94^{o}C for 45 sec, 55^{o}C for 30 sec and 72^{o}C for 1 min) was repeated 30 times. These conditions were suitable particularly for the amplification of high GC content DNA. The amplified PCR products were treated with *Eco*RI in primers, and then cloned into the *Eco*RI site of a mammalian expression vector, pCAGGS, creating pCAGGS-mDNAJB14. The full coding sequences of *DNAJB14* was also cloned into the *Eco*RI-*Xho*I sites of pcDNA3.1(+) after amplifying the coding sequence by using the mDnaJB14-F primer and a reverse primer, mDNAJB14-XhoI-R primer (5'-CCGCTCGAG*TTA*TCCCCCCTTGTAGAGAC-3'), the underline indicates an *Xho*I site, the italic letters indicate the stop codon. The resulting plasmid was named pcDNA-mDNAJB14.

To introduce substitution mutations into the *DNAJB14* gene of a plasmid, pCAGGS-mDNAJB14, I followed the instruction described in Quikchange Site-Directed Mutagenesis Kit (Stratagene) except that PrimeStar GXL DNA polymerase was used as an enzyme for PCR. To introduce a D138N mutation, I used following

primers: mDNAJB14-D138N-F (5'-TTTCATCCAAACAAAAACCACGCCCCTGG-3'), and mDNAJB14-D138N-R (5'-GTGGTTTTTGTTTGGATGAAACTTCAAAGC-3') ; the underlines indicate the position of mutation. To introduce N-glycosylation sites into the *DNAJB14* gene, following primers were used: mDNAJB14-S281N-F (5'-CTTATACCCCAGAAACGGATCAGGACAAAC-3') and mDNAJB14-S281N-R (5'-GTCCTGATCCGTTTCTGGGGTATAAGGAG-3') for the S281N mutation, and mDNAJB14-A360N-F (5'-GAAGGCGGATAACCTGAGCATGGAGAACTG-3') and mDNAJB14-A360N-R (5'-CTCCATGCTCAGGTTATCCGCCTTCCTCC-3') for the A360N mutation.

To construct pCAGGS-mDNAJB12 encoding a non-tagged version of DNAJB12, the coding sequence of *DNAJB12* without tag was amplified from pCAGGS-HA-DNAJB12-Flag (Yamamoto *et al.,* 2010) by PCR using PrimeStar GXL DNA polymerase (Takara) and following two primers: mDNAJB12-F (5'-GCAGAATTC*ATG*GAATCCAACAAGG-3') and mDNAJB12-R (5'-ATAGAATTC*CTA*TCCATGCAGGGAGG-3'); the underlines indicate an *Eco*RI site, the italic letters represent the start or stop codon. For the amplification, a cycle (94^{o}C for 45 sec, 58^{o}C for 30 sec, and 68^{o}C for 1 min) was repeated 30 times. The resulting DNA fragment was cloned into the *Eco*RI site of pCAGGS vector, generating pCAGGS-mDNAJB12.

2.4 Semi quantitative RT-PCR

To compare the levels of *DNAJB14* and *DNAJB12* mRNA in mouse tissues, the first strand cDNA was synthesized using 1 μg of total RNA prepared from mouse tissues. The resulting cDNA was then used as a template for PCR to evaluate the level

of each mRNA. Primers used were: mJB14-3'-F (5'-CGTACTCCTTATACCCCAGATCTGG-3'), and mJB14-3'-R (5'-TCCTTGTAGAGACTGGTCAGCCG-3') for *DNAJB14*; mJB12-5'-F (5'-ATCCAACAAGGATGAAGCCGAGCG-3') and mJB12-5'-R (5'-TGCTTGACCCTTTTCACAGCTG-3') for *DNAJB12*; and hβ-Actin-F (5'-AACTGGAACGGTGAAGGTGACA-3') and hβ-Actin-R (5'-ACTGGTCTCAAGTAGTGTACAGG-3') for β-actin. For the PCR, KAPA Taq DNA polymerase was used and the following cycle (94^{o}C for 45 sec, 55^{o}C for 30 sec, and 72^{o}C for 30 sec) was repeated 32 cycles for the detection of *DNAJB14* or *DNAJB12* mRNA and 25 cycles for the detection of *β-actin* mRNA.

To compare the levels of *DNAJB14* and *DNAJB12* mRNA in NIH3T3 cells under heat or ER stress, the following primers were also used: mHSP47-F (5'-ACATCCTCCTGTCACCCTTG-3') and mHSP47-R (5'-AACCTCTCATCCCAGTGTGG-3') for *HSP47*; mXBP1-F (5'-GAGAACCAGGAGTTAAGAACACG -3') and mXBP1-R (5'-GAAGATGTTCTGGGGAGGTGAC-3') for *XBP1*; and mHSPA5-F (5'-ATGATGAAGTTCACTGTGGTGG-3') and mHSPA5-R (5'-GAAGGGTCATTCCAAGTGCG-3') for *BiP*. The numbers of PCR cycles used were: 22 for *Hsp47;* 24 for *BiP* and *β-actin;* 27 for *DNAJB12;* 33 for *DNAJB14* and *XBP1*.

The PCR product was separated by gel electrophoresis, stained with ethidium bromide, and detected with LAS4000 (GE Life Sciences). The band intensities were then quantified using Multigauge (Fujifilm).

2.5 Cell culture and transfection

Mouse fibroblast NIH3T3 and human cervical cancer HeLa cell were cultured at 37^{o}C under 5% CO_2-air in Dulbecco's eagle minimum enriched medium (DMEM) supplemented with 10% fetal bovine serum.

NIH3T3 and HeLa cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) and Effectene (Qiagen), respectively. The exception is that we used Lipofectamine 2000 for transfection of HeLa cells with a plasmid in the experiment determining the topology of DNAJB14. For the transfection, I followed the manufacturers' instructions.

For cycloheximide chase experiment, HeLa cells grown on a 6-well culture plate for 24 h were transfected with 300 ng of a plasmid encoding an appropriate ERAD substrates and 5 ng of a plasmid encoding DNAJB14 or DNAJB12. At 24 h after the transfection, cycloheximide was added at a final concentration of 150 μg/ml to start the cycloheximide chase experiment. The expression plasmids used are: pEGFP-ΔF508-CFTR (Johnston *et al*., 1998), pcDNA3.1-TCRα (Yu *et al.*, 1997), pcDNA3.1-NHK (Yamamoto *et al.*, 2010), pCAGGS-mDNAJB12 (this study), pCAGGS-mDNAJB14 (this study), and pCAGGS-mDNAJB14-D138N (this study).

2.6 Antibodies

The anti-mouse DNAJB14-N antibody which recognizes the N-terminus of mouse DNAJB14 was prepared by immunizing guinea pigs with a polypeptide that has an amino acid sequence corresponding to the N-terminal one hundred amino acid residues of DNAJB14. To prepare the polypeptide, the nucleotide encoding the corresponding region was cloned from pCAGGS-DNAJB14 into an expression vector, pQE30 (Qiagen). The resulting plasmid, pQE-5'-mDNAJB14, was used to

produce the polypeptide in *E. coli* Rosetta™ strain (Novagen). The polypeptide was then purified with Prepforesis (Atto) and used to immunize guinea pigs. The anti-mouse DNAJB14-C antibody which recognizes the C-terminus of mouse DNAJB14 was prepared in the same manner except that a polypeptide that has an amino acid sequence corresponding to the C-terminal one hundred amino acid residues of DNAJB14, expressed from pQE-3'-mDNAJB14, was used to immunize guinea pigs.

Guinea pig anti-mouse DNAJB12 antibody has been described (Yamamoto *et al*., 2010). The other antibodies used in this study are: rabbit anti-GFP antibody (MBL), mouse anti-HA 12CA5 antibody (Boehringer Manheim), rabbit anti-β-COP antibody (Oncogene Research Products), rabbit anti-calnexin antibody (Stressgen), rat monoclonal anti-Hsc70 antibody (Stressgen), rabbit anti-A1AT antibody (DAKO), and rabbit anti-GAPDH antibody (Cell Signaling). Mouse monoclonal anti-ERp57 antibody has been prepared by Dr. Akio Tsuru in the Kohno lab.

For immunoblotting experiments, appropriate secondary antibodies were used. As secondary antibodies for immunofluorescence microscopy, I used Alexa Fluor 647 goat anti-mouse IgG (highly cross-adsorbed) and Alexa Fluor 488 anti-rabbit IgG (highly cross-adsorbed) (Invitrogen).

2.7 Immunofluorescence staining

HeLa cells, grown on a cover slip, were fixed in 1% acetic acid in ethanol, permeabilized with 1% Triton-X100 in phosphate-buffered saline (PBS), and then blocked with PBS containing 3% BSA. The resulting cover slip was incubated with a primary antibody diluted in blocking solution, washed three times with PBS, incubated again with an appropriate secondary antibody diluted in blocking solution, and finally washed three times with PBS. The cover slip was then mounted with

Prolong Gold Antifade Reagent (Invitrogen), and placed on a glass slide. Images were captured using a confocal fluorescence microscope, FV1000 (Olympus).

2.8 Immunoprecipitation

HeLa cells overexpressing DNAJB14 were collected with a scraper, lysed in NP-40 lysis buffer [1% (v/v) NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% (w/v) SDS] supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml benzamidine, and 1 mM PMSF), and 20 μM MG132, and subjected to centrifugation at 14,000 rpm, 4°C for 5 minutes to remove cell debris. The resulting supernatant was incubated with Protein G-Sepharose (GE life sciences) for 30 minutes at 4°C. After removing the beads by centrifugation at 2,000 rpm, 4°C for 5 minutes, the supernatant was incubated with anti-DNAJB14 antibody at 4°C for overnight and immunoprecipitated using Protein G-Sepharose. The immunoprecipitates were washed three times with PBS containing 1% Triton X-100, and then eluted by incubation in 2xSDS-PAGE sample buffer at 55°C for 15 minutes. The proteins were separated by SDS-PAGE and detected by immunoblotting.

2.9 Protein preparation, and immunoblotting

Cells were lysed in NP-40 lysis buffer, and centrifuged to remove cell debris. The supernatants was diluted 3:4 with 4x SDS-PAGE sample buffer containing 6 M Urea, and incubated at room temperature for 30 minutes. The proteins were separated by SDS-PAGE, blotted onto an Immobilon-P PVDF membrane (Millipore), incubated with appropriate primary and secondary antibodies, and detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare) and X-ray film. The signals were then analyzed by image analysis program ImageJ (NIH).

2.10 Microsome preparation and topology assay

To prepare microsomes, HeLa cells overexpressing DNAJB14 were harvested by centrifugation, and homogenized by 27-guage syringe in HES buffer (10 mM HEPES-KOH [pH7.4], 1 mM EDTA, 0.25 mM sucrose and 0.008% digitonin) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml benzamidine, and 1 mM PMSF), and 20 μM MG132. The lysate was centrifuged at 14,000 rpm, 4^{o}C for 5 minutes to remove cell debris. The microsomes were collected from the supernatant by centrifugation at 44,000 rpm for 2 hours at 4^{o}C, and re-suspended in HES buffer without digitonin. The resulting microsomes were then subjected to topology assay using proteinase K. For the assay, proteinase K and Triton X-100 were used at final concentrations of 10 μg/ml and 1%, respectively. The enzyme reaction was carried out at 4^{o}C for 40 minutes.

To observe N-glycosylation of DNAJB14 mutants, cell lysates were prepared from HeLa cells expressing DNAJB14 S281N or DNAJB14 A360N, treated with Endoglycosidase H at 37^{o}C for 1 h, and then subjected to SDS-PAGE and western blot analysis.

III. RESULTS

3.1 *DNAJB14* is a paralogue of *DNAJB12* and conserved widely among vertebrates.

Genomic studies on mammals have predicted the existence, in their genomes, of the *DNAJB14* gene that encodes a protein highly homologous to DNAJB12 (Qiu *et al.,* 2006; Vos *et al.,* 2008; Hageman and Kampinga, 2009; Kampinga and Craig, 2010). The deduced amino acid sequence of mouse DNAJB14 shows 50% identity to that of mouse DNAJB12 (Fig. 4A). The sequence comparison suggests that both DNAJB14 and DNAJB12 share a very similar domain organization: a J-domain is followed by a glycine/phenylalanine-rich region and a transmembrane segment (Fig. 4A and B). Particularly conserved are these three domains. Notably, the amino acid sequence of mouse DNAJB14 is much closer to that of human DNAJB14 (93% identity) than to that of mouse DNAJB12 (50% identity). Thus, *DNAJB14* likely has evolved as a paralogue of *DNAJB12*. The orthologues of *DNAJB14* and *DNAJB12* are widely found among vertebrates in the National Center for Biotechnology Information protein database (www.ncbi.nlm.gov/protein).

To examine whether *DNAJB14* is transcribed *in vivo*, I amplified a 1,140-base pair cDNA corresponding to the coding sequence of *DNAJB14* by performing RT-PCR (Fig. 4C). The nucleotide sequence of the cDNAs, obtained from mouse testis, brain and muscle, completely matched with the predicted sequence of the mouse *DNAJB14* mRNA, confirming that *DNAJB14* is actually transcribed in mouse tissues.

3.2 *DNAJB14* transcript is expressed weakly in mouse tissues, being most abundant in testis.

To study the expression of *DNAJB14*, I examined the tissue distribution of the *DNAJB14* transcript by northern hybridization and RT-PCR. Detection of *DNAJB14* mRNA by northern blot analysis revealed that *DNAJB14* mRNA is most abundant in testis (Fig. 5A, lane 9). I also examine the tissue distribution of *DNAJB14* mRNA by RT-PCR to confirm that *DNAJB14* is most abundant in testis (Fig. 5B, lane 9). It should be noted that much lower, but detectable, levels of *DNAJB14* mRNA were present in other tissues (Fig. 5B).

Using the same total RNA samples, I also detected *DNAJB12* and *β-actin* mRNA using RT-PCR. The semi-quantitative analysis of their band intensities showed that the levels of *DNAJB12* mRNA were greater than those of *DNAJB14* mRNA in all samples examined (Fig. 5C). Thus, in contrast to *DNAJB12* mRNA that was widely expressed in mouse tissues, *DNAJB14* mRNA was expressed more weakly, being most abundant in testis.

3.3 Neither heat stress nor ER stress affects the expression of *DNAJB14* mRNA.

To further study the expression of *DNAJB14*, I examined the effect of heat stress on the expression of *DNAJB14* mRNA in NIH3T3 by incubating the culture of NIH3T3 cells at 42^{o}C for 45 min and then 37^{o}C for another 2 h. The level of *Hsp47* mRNA, encoding a typical ER-resident heat shock protein (Nagata *et al.,* 1986; Takechi *et al.,* 1994), was significantly increased after the treatment, confirming that the cells were indeed stressed with heat (Fig. 6A). However, this treatment failed to affect the levels of *DNAJB14* and *DNAJB12* mRNAs, suggesting that their expression was not induced by heat shock. I next studied the effect of ER stress on the expression

of *DNAJB14* mRNA by treating the culture of NIH3T3 cells with 5 mM dithiothreitol (DTT) for 3 h. This treatment indeed triggered the splicing of *XBP1u* mRNA, a cellular response to the ER stress, confirming that the treated cells indeed experienced this stress (Fig. 6B) (Yoshida *et al.,* 2001). Consistently, upon DTT treatment, the level of *BiP* mRNA, encoding an ER-stress inducible protein, was increased. However, the same treatment failed to affect the levels of *DNAJB14* and *DNAJB12* mRNAs (Fig. 6B). Thus, the expression of *DNAJB14* and *DNAJB12* is insensitive to both heat and ER stresses.

3.4 Preparation of antibodies for DNAJB14

To analyze molecular characteristics and function of DNAJB14, I prepared two kinds of antibodies that are specific to DNAJB14. One of them is anti-DNAJB14-N antibody, which recognizes the N-terminus of DNAJB14 (Fig. 7A). This antibody specifically detected a band that runs at around 42-kDa on an immuno-blot (Fig. 7B, lane 2). The same band was also detected by anti-DNAJB14-C antibody that recognizes the C-terminus of DNAJB14 (Fig. 7A and B, lane 5). Thus, this band at 42-kDa represents the full-length DNAJB14. These anti-sera did not detect any specific band even when I used the cell lysate prepared from HeLa cells overexpressing DNAJB12 (Fig. 7B, lanes 3 and 6), demonstrating the high specificity of both DNAJB14-N and DNAJB14-C antibodies.

3.5 DNAJB14 is an ER-localized, type II transmembrane protein.

To study the subcellular localization of DNAJB14, I overexpressed HA-DNAJB14 in HeLa cells and visualized the protein with anti-HA antibody. As shown in Fig. 8A, HA-DNAJB14 was detected in a pattern that overlapped with calnexin, an

ER marker, indicating that this protein is located in the ER. DNAJB12, which shows a high sequence similarity to DNAJB14, is also an ER-localized protein (Yamamoto *et al.,* 2010; Grover *et al.,* 2011). Thus, I compared the subcellular localization of DNAJB14 with that of DNAJB12. The fluorescence signal derived from HA-DNAJB14 exhibited a pattern that is identical to that of DNAJB12-FLAG (Fig. 8B). Hence, I concluded that both DNAJB14 and DNAJB12 are localized in the ER.

Next, I proceeded to determine the membrane topology of this protein using both DNAJB14-N and DNAJB14-C antibodies. For this purpose, the microsomes were treated with proteinase K, and the digestion products were analyzed by immuno-blotting with these antibodies. Upon treatment with proteinase K, only anti-DNAJB14-C antibody detected a proteolytic fragment of 15-kDa (Fig. 8C, lane 5). The failure of the anti-DNAJB14-N antibody to detect this fragment (Fig. 8C, lane 2) and the size of the protected fragment indicate that proteinase K digested the N-terminus of DNAJB14 including the J-domain. Disruption of the barrier function of membrane by treating the microsomes with Triton X-100, a detergent, caused the disappearance of this fragment, indicating that the C-terminus of DNAJB14 resides in the luminal side of the microsomes (Fig. 8C, lane 6). The right panel of Fig. 8C shows a control experiment demonstrating that a luminal protein, ERp57, was efficiently degraded only in the presence of Triton X-100 (compare lane 9 with lanes 7 and 8), which indicates the integrity of the purified microsomes. These immunofluorescence staining and protease protection assay indicate that DNAJB14, like DNAJB12, is an ER-localized type II transmembrane protein with its J-domain facing the cytosol.

To further confirm the localization and membrane topology of DNAJB14, I inserted an N-glycosylation site at two different positions near the C-terminus of this protein. For this purpose, I mutated either a serine residue at position 281 (S281) or an

alanine residue at position 360 (A360) to asparagine (N) to generate a conserved motif for N-linked glycosylation, N-X-S/T (Moremen *et al.,* 2012), in DNAJB14 (Fig. 9A). The introduction into DNAJB14 of either one of the two mutations (S281N and A360N) caused the appearance of a new band that runs slightly slower than the band corresponding to the wild-type DNAJB14 (Fig. 9B, compare lanes 5 and 8 with lane 2). Upon treatment of the cell lysates with Endoglycosidase H (EndoH) that cleaves asparagine-linked mannose rich oligosaccharides from glycoproteins, the slowly migrating band was converted to the band corresponding to the wild-type DNAJB14 that is non-glycosylated. This finding indicates that the slowly migrating band represents DNAJB14 that has been glycosylated at one of the newly introduced N-glycosylation sites. Importantly, N-glycosylation of polypeptide occurs upon localization of a glycosylation site into the lumen of the ER and only N-glycosylated ER-resident proteins exhibit EndoH sensitivity (Moremen *et al.,* 2012). Thus, the observed N-glycosylation of DNAJB14 mutants at positions 281, and 360 and the sensitivity of the N-glycosylated DNAJB14 mutant proteins to EndoH digestion indicate that these two residues are located within the lumen of the ER. These findings further confirm the subcellular location and membrane topology of DNAJB14.

3.6 DNAJB14 interacts with Hsc70

J-proteins interact with Hsc70 family members to perform their functions. The Kohno Lab and others have shown that DNAJB12 interacts with Hsc70, a cytosolic Hsp70 family member, via its J-domain, to exert its function (Yamamoto *et al.,* 2010; Grove *et al.,* 2011). Since, like DNAJB12, DNAJB14 has its J-domain in the cytosol, I surmised that DNAJB14 may also interact with Hsc70 in the cytosol using its J-

domain. To examine the interaction between DNAJB14 and Hsc70, I purified DNAJB14 from HeLa cells overexpressing DNAJB14 by immunoprecipitation using anti-DNAJB14-C antibody and tested whether Hsc70 was co-purified with DNAJB14. As shown in Fig. 10, lane 2, Hsc70 was co-immunoprecipitated with DNAJB14. This binding requires an intact J-domain of DNAJB14, because the interaction was lost when I mutated the conserved HPD motif of DNAJB14 to HPN (D138N) (Fig. 10, lane 3). This finding is consistent with the general notion that HPD motif of J-domain is important for the recognition of Hsp70 by J-protein (Kampinga and Craig, 2010). I, thus, concluded that, like DNAJB12, DNAJB14 uses its J-domain to interact with Hsc70 in the cytosol.

3.7 Interaction of DNAJB14 with E3 ubiquitin ligases

E3 ubiquitin ligases play important roles in ERAD by promoting the poly-ubiquitination of their target proteins. A previous study has shown that DNAJB12 physically and functionally interacts with RMA1, an E3 transmembrane ubiquitin ligase, to promote the degradation of a misfolded membrane protein (Grove *et al.,* 2011). Since DNAJB14 resembles DNAJB12 in its architecture, subcellular localization, and ability to interact with Hsc70, I surmised that DNAJB14 may also bind RMA1.

To test this hypothesis, I overexpressed both RMA1-FLAG and HA-DNAJB14 in HeLa cells and examined their physical interaction by co-immunoprecipitation experiment. Consistent with the previous report, HA-DNAJB12 was co-immunoprecipitated with RMA1-FLAG (Fig. 11, 4th panel, lane 5), demonstrating the physical interaction between DNAJB12 and RMA1. However, under the same conditions, HA-DNAJB14 failed to be co-immunoprecipitated with

RMA1 (Fig. 11, 4th panel, lane 6), suggesting that DNAJB14 may not physically interact with RMA1.

I further investigated whether DNAJB14 can bind other E3 ligases. A few members of E3 ligases have been reported to be involved in ERAD in mammalian cells. Among them, Hrd1 is the most common E3 ubiquitin ligase which participates in the degradation of some transmembrane ERAD substrates (Smith *et al.,* 2011). To examine whether DNAJB14 can bind Hrd1, HeLa cells overexpressing both HA-DNAJB14 and Hrd1-myc were subjected to co-immunoprecipitation experiment. However, I was unable to detect the physical interaction between Hrd1-myc and either HA-DNAJB14 or HA-DNAJB12 (Fig. 12, lanes 6 and 5). Thus, an E3 enzyme that cooperates with DNAJB14 remains to be determined.

3.8 DNAJB14 promotes the degradation of CFTRΔF508 via the ubiquitin-proteasome system

DNAJB12 is involved in the ERAD of misfolded transmembrane proteins (Yamamoto *et al.,* 2010; Grove *et al.,* 2011). Due to the similarities between DNAJB14 and DNAJB12 in their structure and molecular characteristics, I thought that DNAJB14 may also promote the ERAD of proteins. To test this hypothesis, I first looked at the effect of DNAJB14 overproduction on the degradation of CFTRΔF508, a mutant of CFTR (cystic fibrosis transmembrane conductance regulator). CFTR is a polytopic plasma membrane chloride channel. The presence of this mutation, ΔF508, in CFTR leads to the development of cystic fibrosis. This mutant is widely used as a model substrate to study the degradation of membrane proteins on the ER due to its greatly diminished folding ability. In fact, DNAJB12 promoted the ERAD of this protein (Yamamoto *et al.,* 2010; Grove *et al.,* 2011).

To study the effect of DNAJB14 overexpression on the degradation of CFTRΔF508, I performed cycloheximide chase experiment. To do this, I treated HeLa cells expressing CFTRΔF508 with cycloheximide to block the synthesis of new proteins and followed the fate of the existing protein for different chase periods. At the zero time point, CFTRΔF508 was found as an ER-localized form (B-form), which was gradually degraded during chase (Fig. 13A). Strikingly, the overexpression of DNAJB14 (Fig. 13AB) enhanced the degradation of CFTRΔF508. Thus, like DNAJB12 (Fig. 13AB), DNAJB14 can enhance the degradation of this membrane protein.

To get insight into the degradation enhancing activity of DNAJB14, I examined whether DNAJB14 requires the ubiquitin-proteasome system to promote the degradation of CFTRΔF508. For this end, I studied the effect of MG132, an inhibitor of the proteasome, on the DNAJB14-dependent degradation of CFTRΔF508. Remarkably, treatment of HeLa cells with MG132 greatly retarded the DNAJB14-dependent degradation of CFTRΔF508 (compare the third panel with fourth panel in Fig. 14A, and the red solid line with red dotted line in Fig. 14B), revealing the importance of the ubiquitin-proteasome pathway in the degradation. Consistently, in the presence of MG132, the rate of CFTRΔF508 degradation in DNAJB14-overproducing cells was comparable to that of cells without DNAJB14-overexpression (compare red dotted line with black dotted line in Fig. 14B). Based on these findings, I concluded that DNAJB14 requires the ubiquitin-proteasome pathway to promote the degradation of the misfolded membrane protein.

To further characterize the DNAJB14-dependent degradation of CFTRΔF508, I examined whether this degradation requires the ability of DNAJB14 to interact with Hsc70. For this purpose, I utilized DNAJB14 D138N mutant that was deficient in its

interaction with Hsc70 (*see* Fig. 10). This mutation caused the slight retardation of the DNAJB14-dependent degradation of CFTRΔF508 (Fig. 15AB). Indeed, a significant difference was observed, at the 30 min chase time point, in the level of CFTRΔF508 between the cells overexpressing the wild-type DNAJB14 and those overexpressing the D138N mutant when the values were analyzed by Student t-test (p-value <0.05).

However, intriguingly, when compared with cells without DNAJB14-overexpression, cells overexpressing DNAJB14 D138N mutant exhibited an increased ability to degrade CFTRΔF508 (Fig. 15AB), indicating that DNAJB14 D138N mutant still retained some of its activity. The implications of this finding will be discussed later in the discussion section.

3.9 DNAJB14 can also enhance the degradation of another ERAD membrane substrate.

DNAJB12 enhances the degradation of misfolded membrane proteins. Since DNAJB14 promoted the degradation of CFTRΔF508, I examined whether DNAJB14 can stimulate the degradation of another ERAD substrate, α subunit of T-cell receptor (TCRα). In contrast to CFTR which is a multiple membrane-spanning protein, TCRα is a small single membrane-spanning protein. This protein is misfolded and degraded by ERAD when produced in the absence of its partner subunit (Yu *et al.,* 1997). Under such conditions, TCRα was indeed gradually degraded in HeLa cells (Fig. 16A, *upper panel,* and 16B). Importantly, like in the case of DNAJB12 (Yamamoto *et al.,* 2010; Grover *et al.,* 2011), the overexpression of DNAJB14 also accelerated the degradation of TCRα (Fig. 16A, *lower panel,* and 16B). These findings indicate that DNAJB14 can promote the degradation of a variety of membrane proteins.

I next tested whether DNAJB14 can enhance the degradation of an ER luminal protein. α1-antitrypsin protein is a serine protease inhibitor that is secreted into the blood plasma. The null-Hong Kong variant of α1-antitrypsin (A1AT-NHK) cannot fold properly in the ER and, thus, this protein is eliminated from the ER by ERAD (Sifers *et al.,* 1988). Therefore, this protein has been used as a model substrate of ERAD of an ER luminal soluble protein. The overexpression of DNAJB14 did not affect the stability of this protein (Fig. 17A and B). Thus, like DNAJB12, DNAJB14 can specifically enhance the ERAD of membrane proteins.

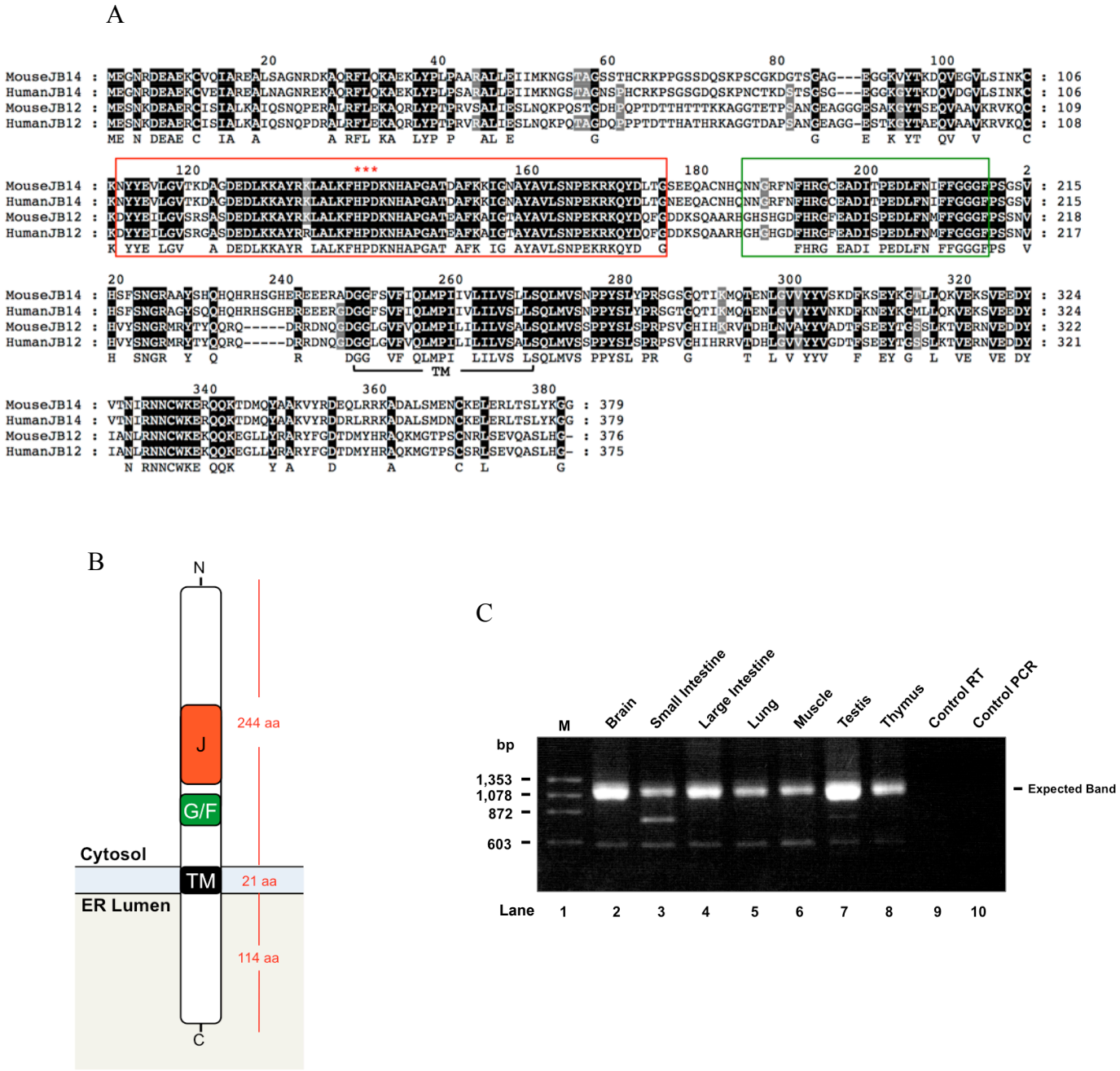

Fig. 4. *DNAJB14*, a paralogue of *DNAJB12*, is expressed in mouse tissues. (A) Sequence alignments of DNAJB14 and DNAJB12 from mouse (*Mus musculus*) and human (*Homo sapiens*). The J-domain (red box), HPD motif (red asterisks), a glycine/phenylalanine-rich region (green box) and transmembrane domain (TM) are indicated. (B) Predicted membrane topology of DNAJB14. J-domain, red; a glycine/phenylalanine-rich region, green; transmembrane domain, black; amino acids, aa. (C) Detection of DNAJB14 transcript by RT-PCR. Total RNA from mouse tissues was subjected to RT-PCR to obtain a product having the expected size of 1140 bp. Control RT indicates RT-PCR reaction using total RNA from testis, performed without using the primer required for the synthesis of the first strand cDNA.

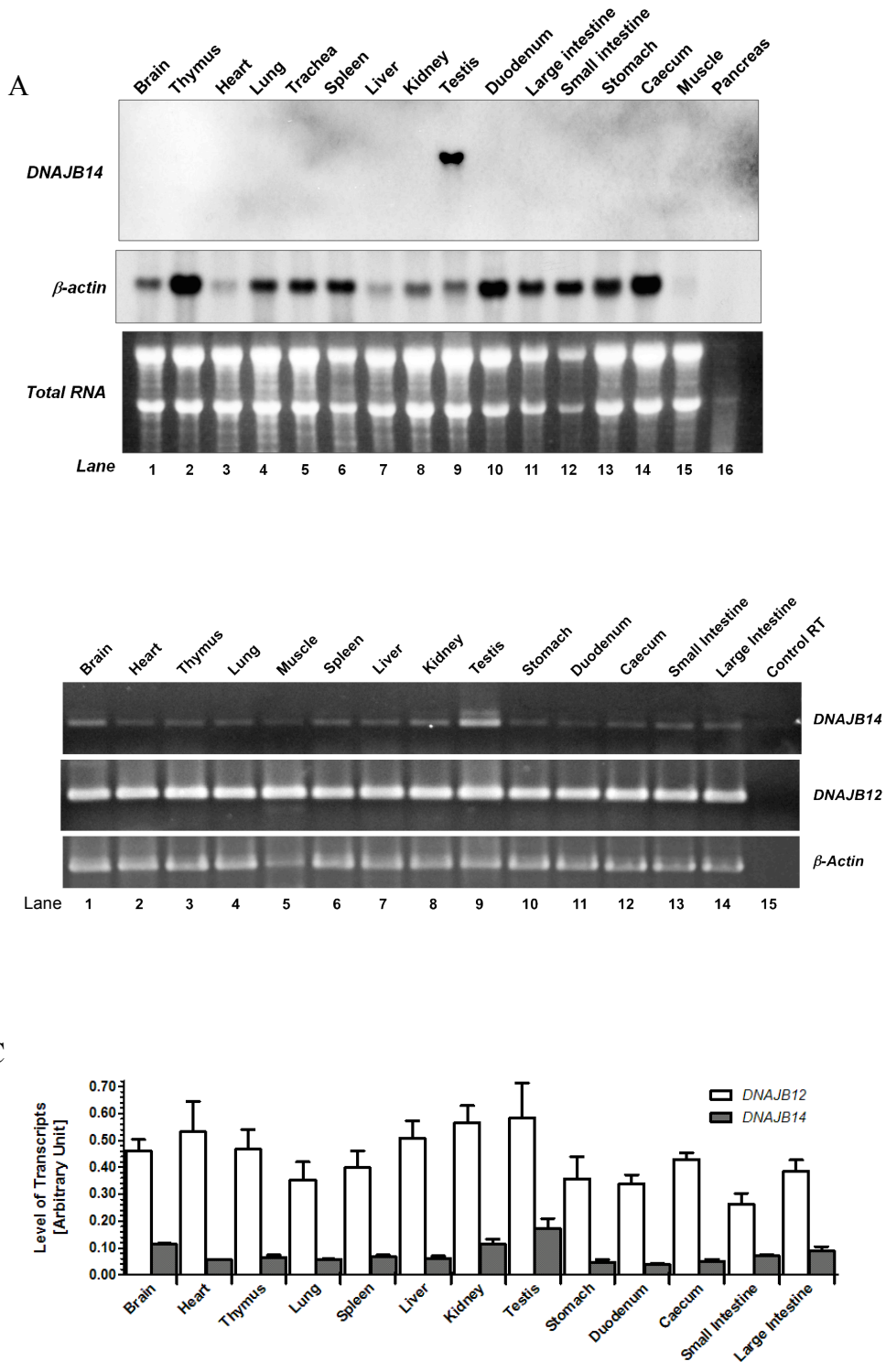

Fig. 5. Tissue distribution of *DNAJB14* transcript examined by northern analysis and RT-PCR. (A) Total RNA from mouse tissues was subjected to northern analysis using DIG-labeled probes to detect *DNAJB14* (*upper*) and *β-actin* (*middle*) mRNAs. Total RNA was visualized with ethidium bromide in the lower panel. (B) Tissue distribution of *DNAJB14* mRNA examined by RT-PCR. Total RNA from the indicated tissues was subjected to RT-PCR with primers specific to *DNAJB14, DNAJB12* and *β-actin*, and separated by agarose gel electrophoresis. The band intensities of *DNAJB14* and *DNAJB12* were recorded and normalized by the intensities of *β-actin*, and plotted onto a graph on panel C. Control RT indicates RT-PCR using total RNA from testis, performed without synthesizing the first strand cDNA. To analyze the expression of *DNAB12* and *DNAJB14* mRNA, the same volume of samples obtained after RT-PCR was loaded in each lane.

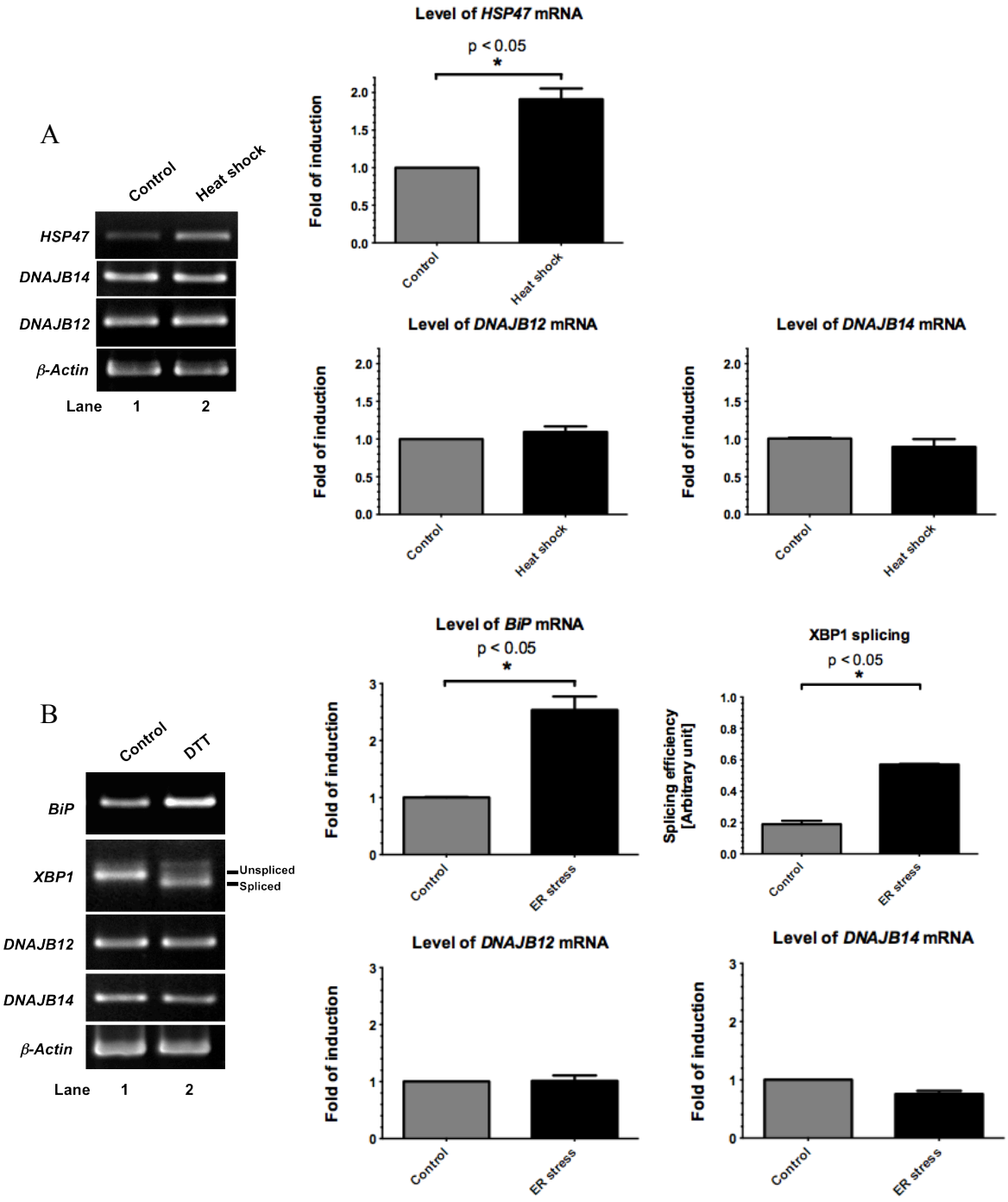

Fig. 6. Expression of *DNAJB14* mRNA is insensitive to heat or ER stress. (A) Effect of heat stress on the level of *DNAJB14* mRNA. NIH3T3 cells were subjected to heat stress by incubating the culture at 42°C for 45 min, and then at 37°C for 2 h before preparing total RNA. RT-PCR was used to compare the levels of *Hsp47*, *DNAJB12*, *DNAJB14* and *β-actin* mRNA before and after the stress. The RT-PCR products were then analyzed by agarose gel electrophoresis (left panel). The band intensities of *Hsp47*, *DNAJB12* and *DNAJB14* were quantified and normalized by the intensities of *β-actin*. The obtained values were then plotted onto bar graphs (right panels). (B) Effect of ER stress on the level of *DNAJB14* mRNA. Total RNAs were prepared from NIH3T3 cells that had been treated with 5 mM DTT for 3 h and processed as described above. RT-PCR was also used to detect the splicing of *XBP1* mRNA, which occurs upon ER stress. To calculate the splicing efficiency of *XBP1* mRNA, the intensity of the spliced band was divided by the sum of the intensities of the spliced and unspliced bands. Error bars indicate standard deviations from the average of triplicate experiments.

A

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1   MEGNRDEAEKCVQIAREALSAGNRDKAQRFLQKAEKLYPLPAARALLEIIMKNGSTAGSS 60
61  THCRKPPGSSDQSKPSCGKDGTSGAGEGGKVYTKDQVEGVLSINKCKNYYEVLGVTKDAG 120
121 DEDLKKAYRKLALKFHPDKNHAPGATDAFKKIGNAYAVLSNPEKRKQYDLTGSEEQACNH 180
181 QNNGRFNFHRGCEADITPEDLFNIFFGGGFPSGSVHSFSNGRAAYSHQHQHRHSGHEREE 240
241 ERADGGFSVFIQLMPIIVLILVSLLSQLMVSNPPYSLYPRSGSGQTIKMQTENLGVVYYV 300
301 SKDFKSEYKGTLLQKVEKSVEEDYVTNIRNNCWKERQQKTDMQYAAKVYRDEQLRRKADA 360
361 LSMENCKELERLTSLYKGG                                         379
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B

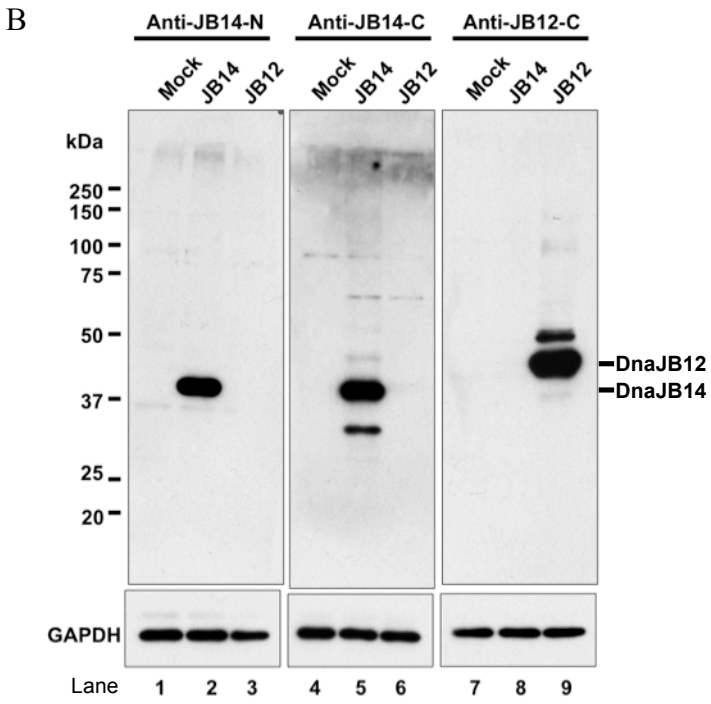

Fig. 7. Preparation of anti-DNAJB14 antibodies. (A) The sequences of the polypeptides used to immunize Guinea Pig. The polypeptide having the amino acid sequence highlighted with a red underline was used to prepare the DNAJB14-N antibody. To prepare the DNAJB14-C antibody, the polypeptide having the sequence highlighted with a green line was used. The grey box with italic letters represents the J-domain of DNAJB14. The conserved HPD motif was highlighted with bold letters. The black box refers to the transmembrane domain. (B) HeLa cells were transfected with pcDNA-mDNAJB14 (to express DNAJB14) or pCAGGS-HA-mDNAJB12-Flag (to express DNAJB12-Flag). The cell lysates were prepared, and subjected to western blot analysis using DNAJB14-N or DNAJB14-C antibodies. Mock, JB14, and JB12 indicate HeLa cells transfected with an empty vector, a DNAJB14-overexpresssion plasmid and a DNAJB12-overexpression plasmid, respectively.

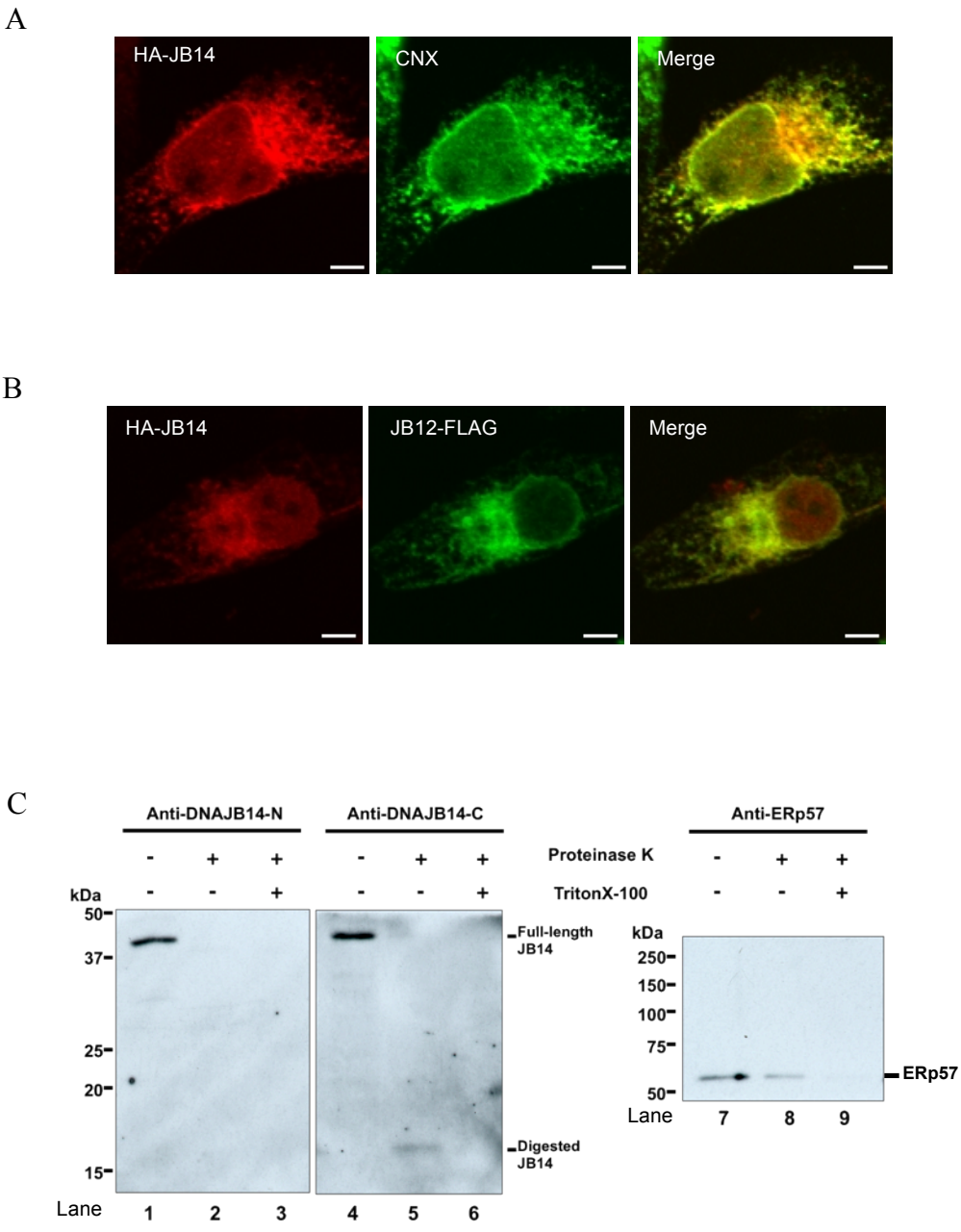

Fig. 8. DNAJB14 is an ER-localized, type II transmembrane protein. (A) Subcellular localization of DNAJB14 was studied by immunostaining HeLa cells expressing HA-DNAJB14 (from pCAGGS-HA-mDNAJB14) with antibodies specific to HA (*left*) and to Calnexin (CNX; an ER marker) (*middle*). The two images were merged on the right. Bars indicate 5 μm. (B) HeLa cells expressing both HA-DNAJB14 (from pcDNA-HA-mDNAJB14) and DNAJB12-FLAG (from pCAGGS-mDNAJB12-Flag) were immunostained with anti-HA (*left*) and anti-FLAG (*middle*) antibodies, respectively, to compare the subcellular localization of these two proteins. (C) Membrane topology of DNAJB14. Microsomes, extracted from HeLa cells expressing DNAJB14 (from pcDNA-mDNAJB14), were treated with proteinase K. The digestion products were then separated by SDS-PAGE and detected by immunoblotting using anti-DNAJB14-N (*left*), anti-DNAJB14-C (*middle*), and anti-ERp57 (*right*) antibodies. Where indicated, Triton X-100 was added to break microsomes before the digestion reaction.

A

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1   MEGNRDEAEKCVQIAREALSAGNRDKAQRFLQKAEKLYPLPAARALLEIIMKNGSTAGSS 60
61  THCRKPPGSSDQSKPSCGKDGTSGAGEGGKVYTKDQVEGVLSINKCKNYYEVLGVTKDAG 120
121 DEDLKKAYRKLALKFHPDKNHAPGATDAFKKIGNAYAVLSNPEKRKQYDLTGSEEQACNH 180
181 QNNGRFNFHRGCEADITPEDLFNIFFGGGFPSGSVHSFSNGRAAYSHQHQHRHSGHEREE 240
241 ERADGGFSVFIQLMPIIVLILVSLISQLMVSNPPYSLYPRSGSGQTIKMQTENLGVVYYV 300
301 SKDFKSEYKGTLLQKVEKSVEEDYVTNIRNNCWKERQQKTDMQYAAKVYRDEQLRRKADA 360
361 LSMENCKELERLTSLYKGG                                         379
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B

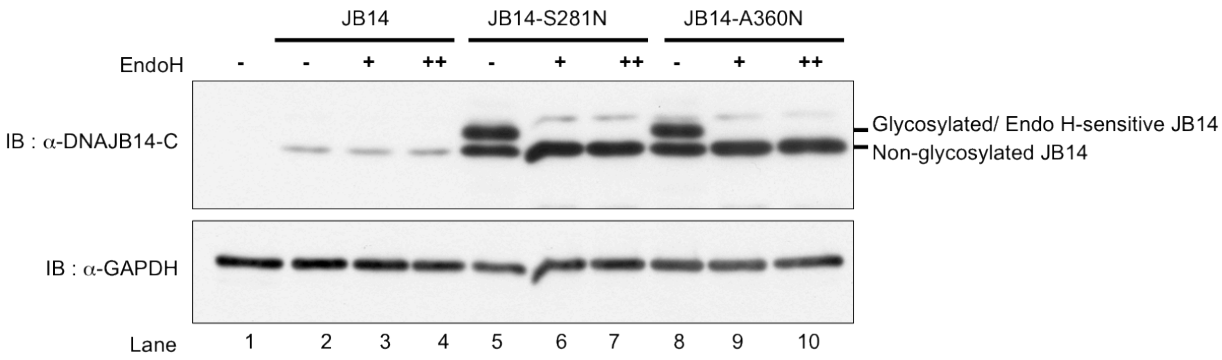


Fig. 9. Introduction of N-glycosylation sites for the analysis of the membrane topology and localization of DNAJB14. (A) The positions, where an N-glycosylation motif was introduced, were marked with red underlines. In each position, the mutated residue (S281 or A360) is highlighted with a bold letter. The grey box with an HPD motif represents the J-domain. The black box refers to the putative transmembrane domain. (B) HeLa cells expressing either the wild-type DNAJB14 (from pCAGGS-mDNAJB14), DNAJB14 S281N (from pCAGGS-mDNAJB14-S281N) or DNAJB14 A360N (from pCAGGS-mDNAJB14-A360N) were lysed, treated with EndoH, and subjected to immuno-blotting with anti-DNAJB14-C antibody (*upper*) and anti-GAPDH antibody (*lower*), respectively.

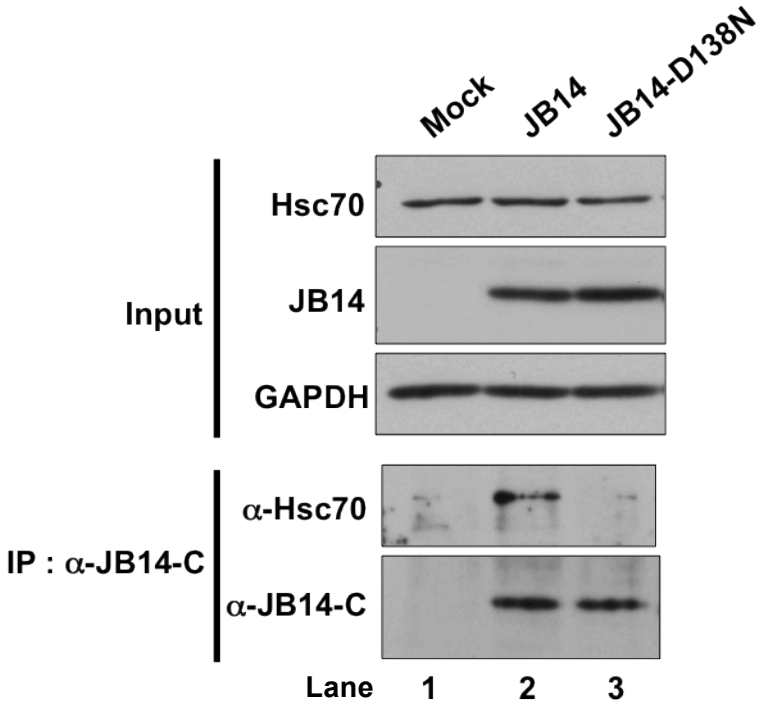


Fig. 10. Interaction of DNAJB14 with Hsc70. Cellular extracts were prepared from HeLa cells expressing the wild-type DNAJB14 (from pCAGGS-mDNAJB14) (JB14) or a J-domain mutant (from pCAGGS-mDNAJB14-D138N) (JB14 D138N). The lysates were subjected to immunoprecipitation using anti-DNAJB14-C antibody and the resulting immunoprecipitates were analyzed by immunoblotting using the indicated antibodies to study the interaction between DNAJB14 and Hsc70.

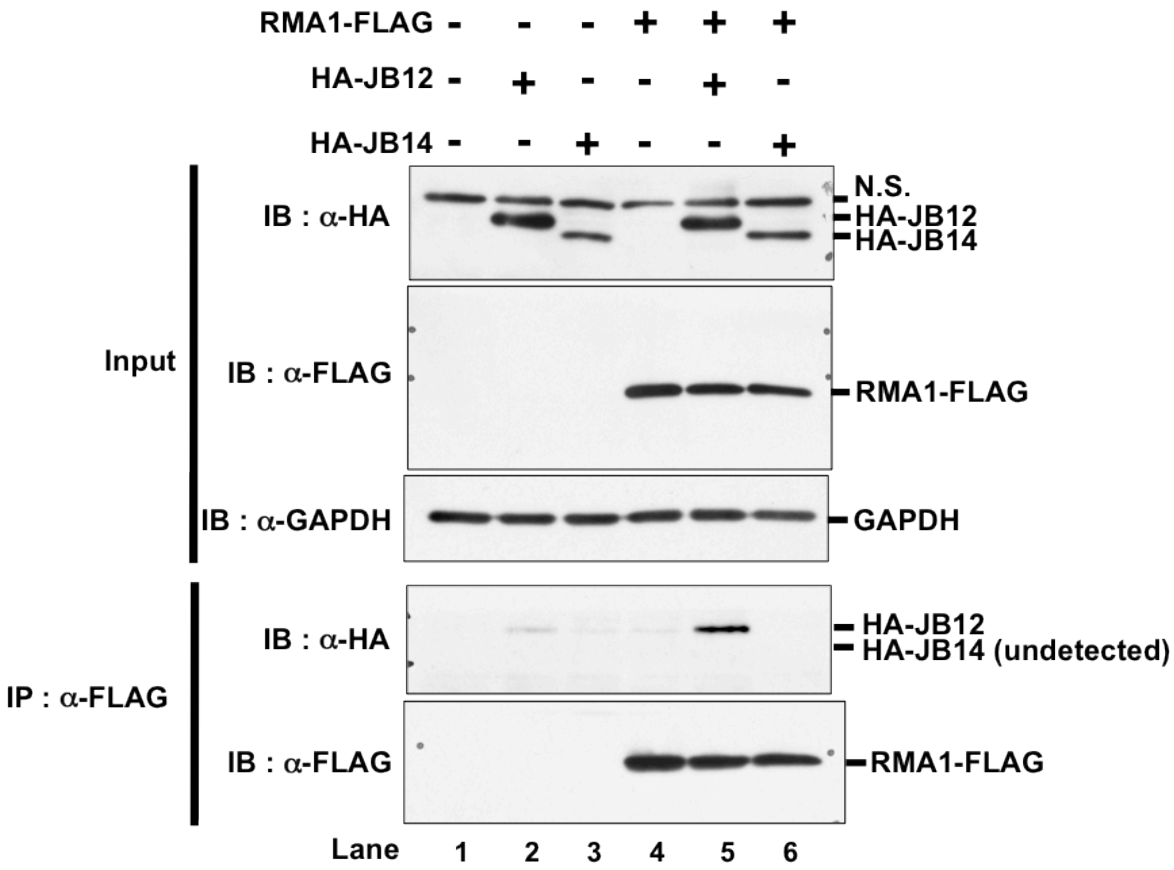


Fig. 11. Detection of a physical interaction between RMA1 and DNAJB12 but not between RMA1 and DNAJB14. Cellular extracts prepared from HeLa cells, expressing both RMA1-FLAG (from pcDNA3.1(+)-RMA1-flag), and either HA-DNAJB14 (from pCAGGS-HA-mDNAJB14) or HA-DNAJB12 (from pCAGGS-HA-mDNAJB12), were subjected to immunoprecipitation using anti-FLAG antibody. The immunoprecipitates were then subjected to immunoblotting analysis using the indicated antibodies to study the physical interaction between the two proteins. N.S.; a non-specific band.

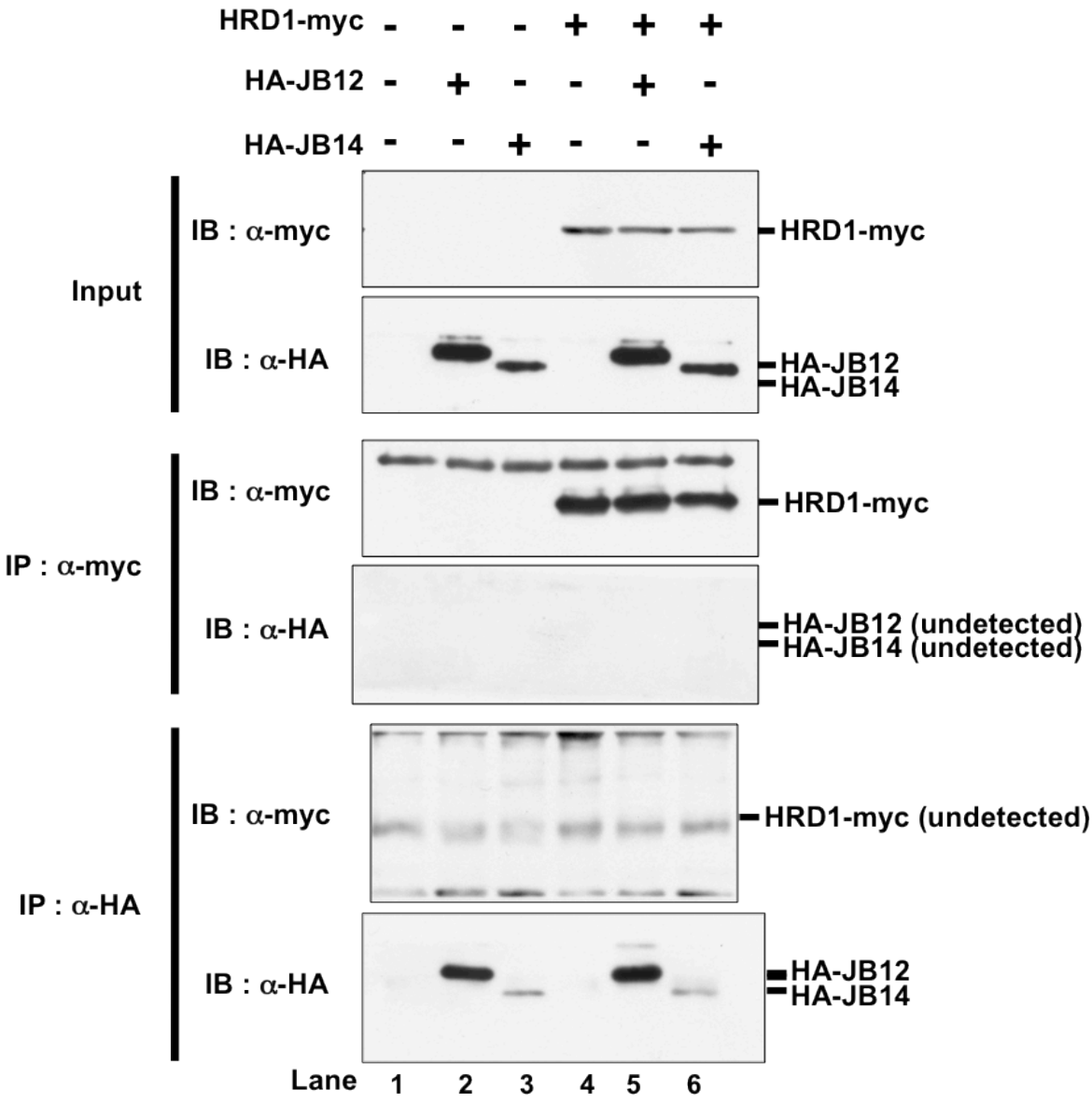


Fig. 12. Co-immunoprecipitation experiment fails to detect a physical interaction between DNAJB14 and Hrd1. Cellular extracts prepared from HeLa cells expressing both Hrd1-myc (from pcDNA3.1(+)-Hrd1-myc), and either HA-DNAJB14 (from pCAGGS-HA-mDNAJB14) or HA-DNAJB12 (from pCAGGS-HA-mDNAJB12) were subjected to immunoprecipitation using anti-myc (*middle*) or anti-HA antibody (*lower*). The immunoprecipitates were then subjected to immunoblotting using the indicated antibodies to study physical interaction between two proteins.

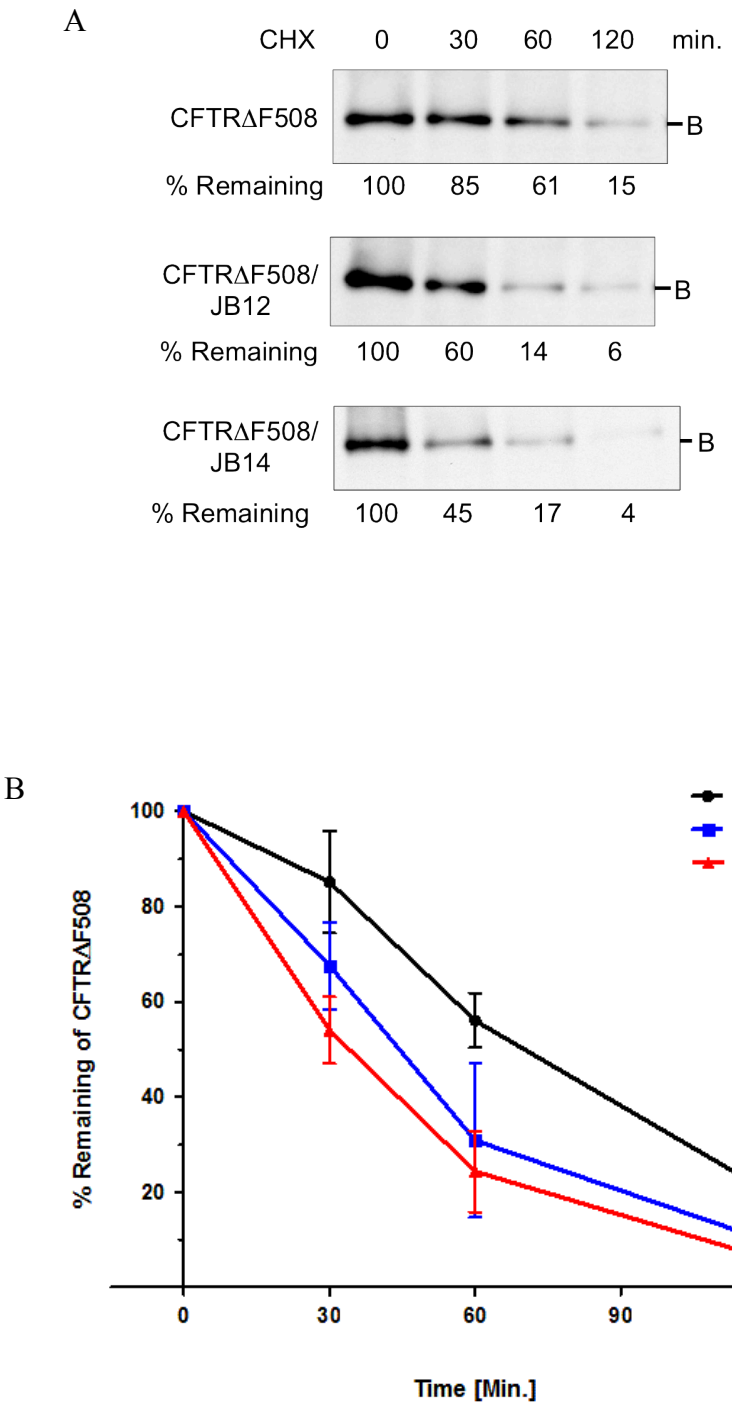


Fig. 13. DNAJB14 can enhance the degradation of CFTRΔF508. (A) To study the effect of DNAJB14 overexpression on the degradation of CFTRΔF508, HeLa cells were transfected with both pEGFP-ΔF508-CFTR (to express CFTRΔF508-GFP) and either pCAGGS-mDNAJB14 (to express DNAJB14), or pCAGGS-mDNAJB12 (to express DNAJB12). After 24 h, cells were treated with 150μg/ml cycloheximide to block the synthesis of proteins and chased for 0, 30, 60 and 120 min. Cells were then lysed and the resulting cellular lysates were separated by SDS-PAGE and subjected to immunoblotting with the anti-GFP antibody to detect CFTRΔF508-GFP. JB12, DNAJB12; JB14, DNAJB14; B, core-glycosylated, ER-localized form of CFTRΔF508-GFP. The band intensities were quantified and normalized with the intensity at zero time point. Results from three individual experiments were plotted into a graph (B). Error bars indicate standard deviations from the average of three independent experiments.

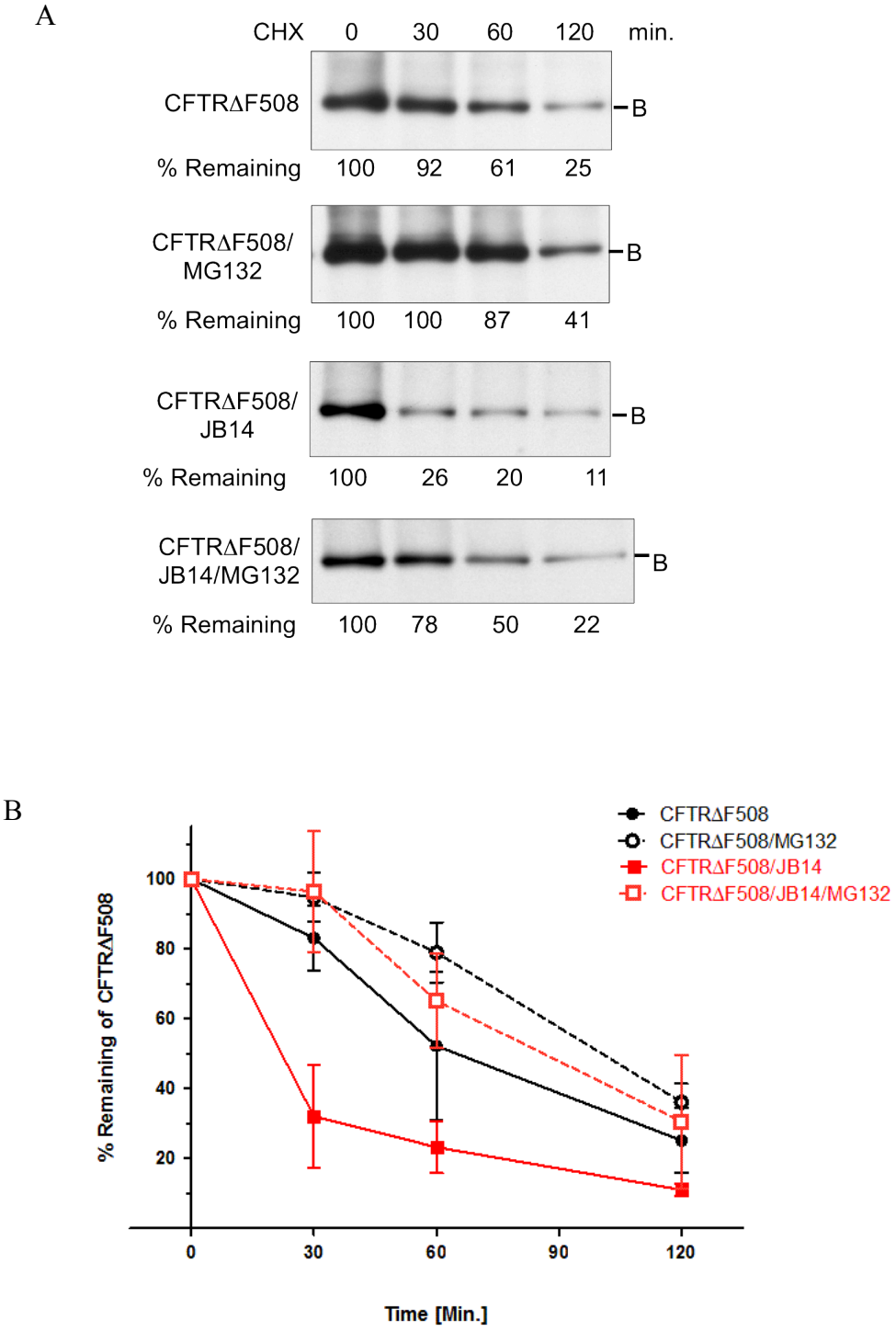
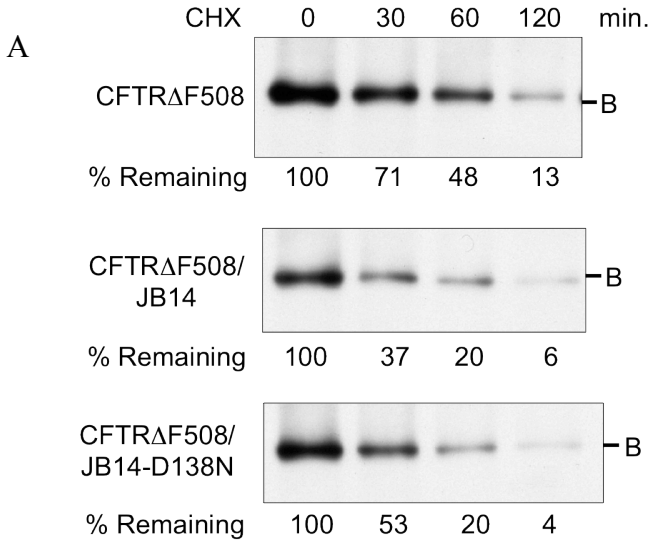


Fig. 14. Effect of a proteasome inhibitor, MG132, on the DNAJB14-dependent degradation of CFTRΔF508. (A) To study the involvement of the ubiquitin-proteasome system in the DNAJB14-dependent degradation of CFTRΔF508, HeLa cells expressing CFTRΔF508-GFP alone (from pEGFP-ΔF508-CFTR) or both CFTRΔF508-GFP (from pEGFP-ΔF508-CFTR) and DNAJB14 (from pCAGGS-mDNAJB14) were processed as described in Fig.13, except that cells were treated with 20 μM MG132, a ubiquitin-proteasome inhibitor, at zero time point. The data from three individual experiments were plotted into a graph on panel B. Error bars indicate standard deviations from the average of three independent experiments.



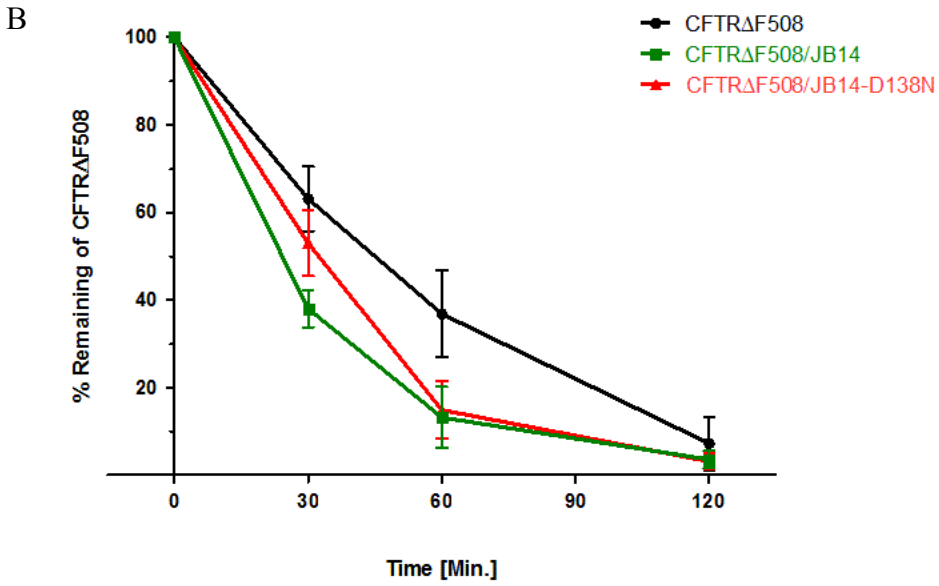


Fig. 15. Effect of a mutation in the J-domain of DNAJB14 on the degradation of CFTR Δ F508. (A) To study the effect of a mutation in the J-domain of DNAJB14 on the degradation of CFTRΔF508, HeLa cells expressing both CFTRΔF508-GFP (from pEGFP-ΔF508-CFTR) and either DNAJB14 D138N (from pCAGGS-mDNAJB14-D138N), or DNAJB14 (from pCAGGS-mDNAJB14) were processed as described in Fig. 13. Data from three individual experiments were plotted into a graph (B). Error bars indicate standard deviations from the average of three independent experiments.

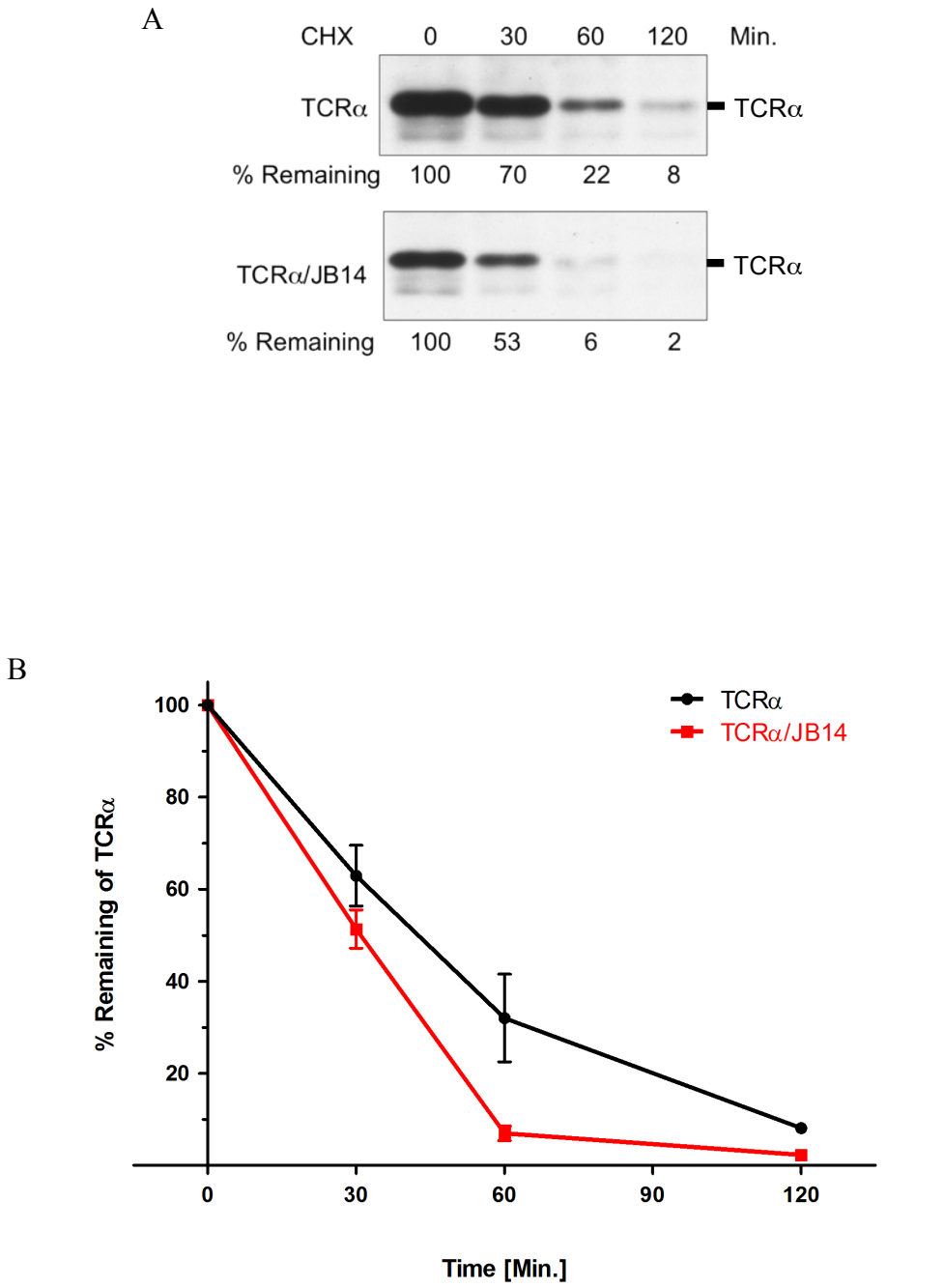


Fig. 16. DNAJB14 can accelerate the degradation of a misfolded membrane protein, TCRα. (A) HeLa cells expressing TCRα (from pcDNA3.1-TCR α) alone or both TCRα (from pcDNA3.1-TCR α) and DNAJB14 (from pCAGGS-mDNAJB14) were chased with 150 μg/ml cycloheximide for 0, 30, 60 and 120 min. The protein extracts were prepared, separated by SDS-PAGE, and analyzed by immunoblotting using anti-HA antibody, to detect TCRα. The band intensities were recorded and fitted into a bar graph (B). Error bars represent standard deviation from triplicate experiments.

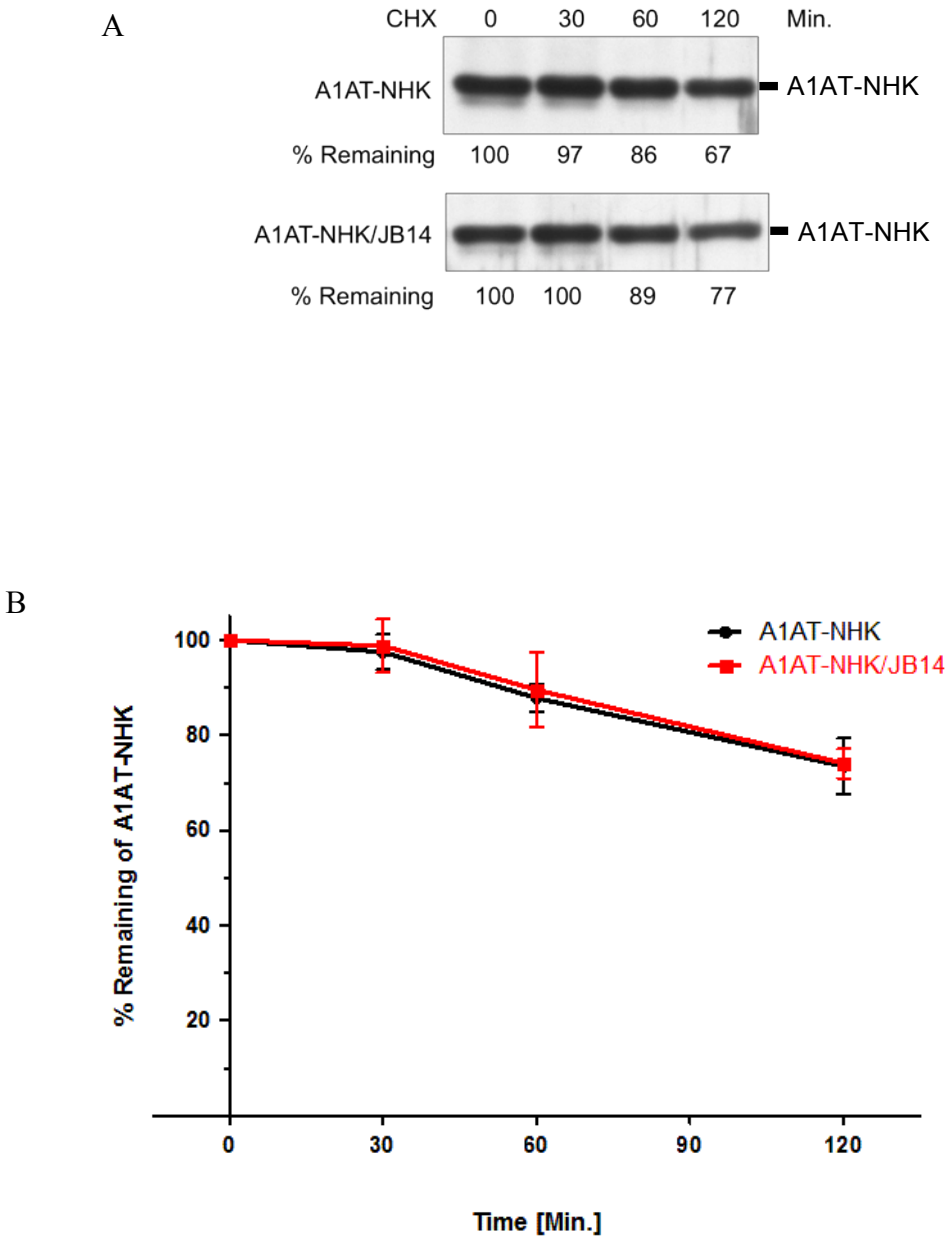


Fig. 17. DNAJB14 does not promote the degradation of a soluble, ER-luminal ERAD substrate, A1AT–NHK. (A) HeLa cells overexpressing the null-Hong Kong variant of α1-antitrypsin (A1AT-NHK) alone (from pcDNA3.1-NHK) or both A1AT-NHK (from pcDNA3.1-NHK) and DNAJB14 (from pCAGGS-mDNAJB14) were chased with 150 μg/ml cycloheximide for the indicated times. The protein extracts were prepared, separated by SDS-PAGE and analyzed by immunoblotting using anti-A1AT antibody to study the effect of DNAJB14 overexpression on the degradation of NHK-A1AT. The band intensities were recorded and fitted into a bar graph (B). Error bars represent standard deviation from triplicate experiments.

IV. Discussion

Protein quality control systems play important roles in the maintenance of cellular homeostasis by degrading misfolded proteins that arise in the cells (Goldberg and St. John, 1976). When misfolded proteins, such as CFTRΔF508, arise in the ER membrane, they are pulled out of the ER, poly-ubiquitinated, and degraded by the proteasome in the cytosol (Vembar and Brodsky, 2008; Houck and Cyr, 2012). DNAJB12 is an ER membrane-localized J-protein having a J-domain in the cytosol. This protein plays an important role in the quality control of membrane proteins by stimulating the degradation of misfolded membrane proteins (Yamamoto et al., 2010; Grove et al., 2011). Here, I presented evidence that the mammalian ER has another membrane-bound J-protein (DNAJB14) that, like DNAJB12, can promote the degradation of misfolded membrane proteins.

My data indicated that DNAJB14 resembles DNAJB12 in many ways. Firstly, the amino acid sequence of mouse DNAJB14 shares 50% identity with that of mouse DNAJB12 (Fig.1). Secondly, like DNAJB12, DNAJB14 is an ER-localized single membrane spanning protein having a J-domain facing the cytosol and uses its J-domain to interact with Hsc70 in the cytosol (Figs. 8-10). Thirdly, the expression of *DNAJB14* and *DNAJB12* is insensitive to both heat and ER stresses (Fig. 6). Fourthly, DNAJB14 can also promote the degradation of misfolded ER membrane proteins but not that of a misfolded ER luminal protein (Fig. 13-17). Finally, like DNAJB12, DNAJB14 requires the function of the proteasome to promote the degradation of a membrane protein (Fig. 14).

Then, why does the mammalian ER possess two analogous proteins (DNAJB14 and DNAJB12) that both can promote the degradation of membrane

proteins? In contrast to *DNAJB12* mRNA that was widely expressed in mouse tissues, *DNAJB14* mRNA was expressed more weakly, being most abundant in testis (Fig. 5). This finding may imply that DNAJB14 has a specific role in this tissue. In addition, the amino acid sequence of mouse DNAJB14 is closer to that of human DNAJB14 than to that of mouse DNAJB12. These observations imply that, during evolution, these two analogous genes have evolved separately to perform distinct functions. Nevertheless, apparently, further studies are needed to clarify this point.

In the ubiquitin-proteasome system, an E3 ubiquitin ligase promotes the poly-ubiqutination of a subset of specific target proteins for degradation. A previous study has shown that, among the E3 enzymes, an ER-membrane-localized E3 enzyme, RMA1, physically interacts and cooperates with DNAJB12 to promote the degradation of CFTRΔF508 (Grove *et al.,* 2011*)*. I have not identified the E3 ubiquitin ligase which physically and functionally interacts with DNAJB14. In fact, in contrast to DNAJB12, DNAJB14 failed to interact with RMA1 in my co-immunoprecipitation experiment (Fig 11). Thus, it may be possible that DNAJB14 binds another ER-resident E3 ligase and thus uses a different mechanism to degrade misfolded membrane proteins.

Then, what could be the E3 ligase that cooperates with DNAJB14 to promote the degradation of membrane proteins?

Since DNAJB12 interacts with RMA1, which is a transmembrane E3 ligase, I speculated that DNAJB14 might require a transmembrane E3 ligase other than RMA1 (Fig. 18). Thus far, the ER-localized transmembrane E3 ligases, including RMA1, Hrd1 and gp78, have been reported to be important for the degradation of CFTRΔF508 (Younger *et al.,* 2006; Ballar *et al.,* 2010; Houck and Cyr, 2012). Thus, I surmised that Hrd1 and gp78 could be candidates.

Among these two transmembrane E3 ligase, Hrd1 is a common E3 ligase for the ERAD of various membrane proteins (Smith *et al.,* 2011). Thus, I expected that Hrd1 could be a primary candidate for the E3 ligase that functions with DNAJB14. However, my co-immunoprecipitation experiment failed to detect a physical interaction between DNAJB14 and Hrd1 (Fig. 12). Thus, both Hrd1 and RMA1 may not be the E3 ligase that works with DNAJB14.

The other tranmembrane E3 ligase, gp78, has been reported as a downstream co-mediator for RMA1-dependent CFTRΔ508 degradation (Morito *at al.,* 2008). However, I have not yet tested whether gp78 physically interacts with DNAJB14.

Besides those transmembrane E3 enzymes, a soluble cytosolic E3 ligase, CHIP, has also been reported to poly-ubiquitinate many misfolded secretory and membrane proteins, including CFTRΔ508 (Connell *et al.,* 2001; Jiang *et al.,* 2001; Murata *et al.,* 2001; Younger *et al.,* 2006). However, I also failed to observe an interaction between DNAJB14 and CHIP by my preliminary co-immunoprecipitation experiment (data not show).

To summarize my discussion on E3 enzyme, I hypothesize that DNAJB14 and DNAJB12 use a different E3 ligase each other (Fig. 18). However, I have not identified the E3 ubiquitin ligase which physically and functionally interacts with DNAJB14.

My result indicated that, like DNAJB12, DNAJB14 stably associates with Hsc70 (Fig. 10). This stable association was lost by a mutation in the J-domain of DNAJB14 (Fig. 10). However, like a J-mutant of DNAJB12 (Yamamoto *et al.,* 2010), this DNAJB14 mutant retained some of its ability to promote the degradation of CFTRΔF508 (Fig. 15). Thus, as I have already discussed for the J-mutant of DNAJB12 (Yamamoto *et al.,* 2010), it may be possible that DNAJB14 can exert some

of its functions without interacting with Hsc70. Alternatively, the DNAJB14 mutant may still be able to weakly interact with Hsc70 *in vivo* to promote the degradation of membrane proteins.

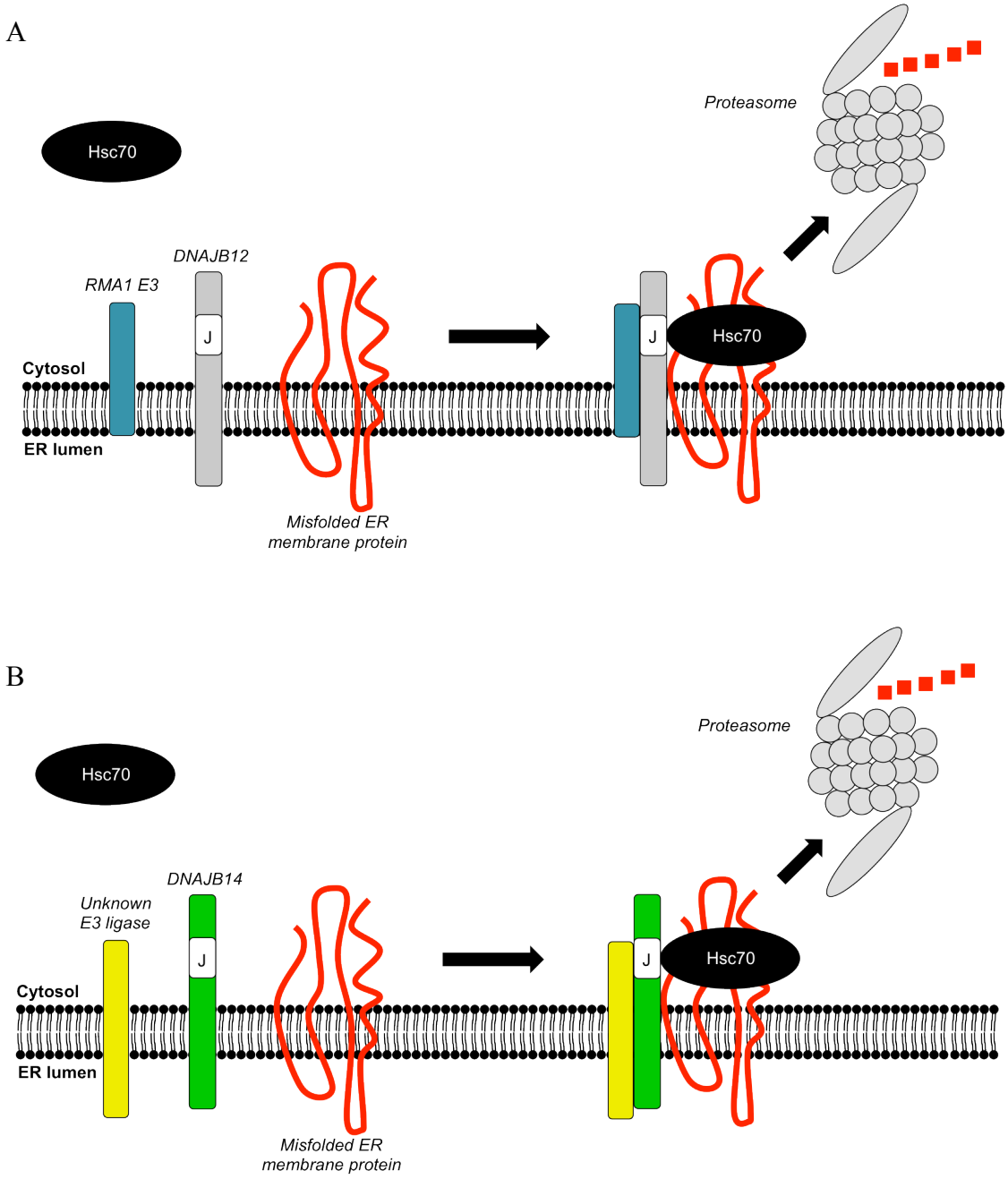


Fig. 18. A model for DNAJB14-mediated degradation of misfolded membrane proteins. (A) DNAJB12 recognizes and targets misfolded membrane proteins for degradation by the proteasome in a manner dependent on Hsc70 and RMA1 E3 ligase. (B) DNAJB14 can also promote the ERAD of misfolded membrane proteins. The E3 ligase that cooperates with DNAJB14 has remained to be determined: DNAJB14 may use an E3 ligase other than RMA1 to perform the function. Although Hsc70 is depicted in the model, whether Hsc70 is required for the DNAJB14-dependent degradation of membrane proteins remains ambiguous.

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Pattarawut Sopha

VI. References

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List of Publications

Lab name (Supervisor)	Molecular and Cell Genetics (Prof. Kenji Kohno)		
Name (surname) (given name)	Sopha Pattarawut	Date	(2012/09/20)

First-author publication(s) from your doctoral research
(Title, authors, year of issue, name of the journal, volume, page)

1. A novel mammalian ER-located J-protein, DNAJB14, can accelerate ERAD of misfolded membrane proteins.
Pattarawut Sopha, Hiroshi Kadokura, Yo-hei Yamamoto, Masato Takeuchi, Michiko Saito, Akio Tsuru and Kenji Kohno. (2012) *Cell Struct. Funct.* (in press)

Other co-authored publication(s) during your doctoral research
(Title, authors, year of issue, name of the journal, volume, page)

1. Domain compatibility in Ire1 kinase is critical for the unfolded protein response. Juthakorn Poothong, Pattarawut Sopha, Randal J Kaufman and Witoon Tirasophon. (2010) *FEBS Lett.* 584: 3203-3208.