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Simultaneous induction of the four subunits of the TRAP complex by ER stress accelerates ER degradation

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The mammalian translocon-associated protein (TRAP) complex comprises four transmembrane protein subunits in the endoplasmic reticulum. The complex associates with the Sec61 translocon, although its function *in vivo* remains unknown. Here, we show the involvement of the TRAP complex in endoplasmic reticulum-associated degradation (ERAD). All four subunits are induced simultaneously by endoplasmic reticulum stresses from the X-box-binding protein 1/inositol-requiring 1α pathway. RNA interference knockdown of each subunit causes disruption of the native complex and significant delay in the degradation of various ERAD substrates, including the α1-antitrypsin null Hong Kong variant (NHK). In a pulse-chase experiment, the TRAP complex associated with NHK at a late stage, indicating its involvement in the ERAD pathway rather than in biosynthesis of nascent polypeptides in the endoplasmic reticulum. In addition, the TRAP complex bound preferentially to misfolded proteins rather than correctly folded wild-type substrates. Thus, the TRAP complex induced by the unfolded protein response pathway might discriminate ERAD substrates from correctly folded substrates, accelerating degradation.

Keywords: ERAD; Sec61 translocon; TRAP complex; UPR; XBP1/IRE1α pathway

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INTRODUCTION

Secretory and membrane proteins are synthesized in the endoplasmic reticulum (ER), in which they are folded and processed or modified into mature proteins. In the ER, a quality control system ensures that only proteins that are folded and processed correctly enter the secretory pathway (Ellgaard & Helenius, 2003). When misfolded proteins accumulate in the ER of mammalian cells, the following four quality control processes operate successively through the activation of unfolded protein response pathways (Rutkowski & Kaufman, 2004): (i) activated PKR-related ER kinase (PERK) phosphorylates the α-subunit of eukaryotic translation initiation factor 2, thus inhibiting translation and preventing accumulation of additional misfolded proteins; (ii) activation of the activating transcription factor (ATF)6 pathway upregulates ER chaperones such as immunoglobulin heavy chain binding protein (BiP) and calreticulin, thus promoting refolding of misfolded proteins; (iii) induction of the X-box-binding protein 1/inositol-requiring 1α (XBP1/IRE1α) pathway produces ER-associated degradation (ERAD) components such as ER degradation-enhancing α-mannosidase-like protein (EDEM; Hosokawa *et al*, 2001), thus promoting degradation of terminally misfolded proteins; and (iv) selective translation of ATF4 results in apoptosis of cells failing to overcome severe ER stress.

The ERAD system for misfolded glycoproteins (Meusser *et al*, 2005; Romisch, 2005) is the best understood of these four responses and comprises three essential processes: (i) recognition of misfolded proteins to be degraded by lectin-like molecules; (ii) retrograde transport of misfolded proteins from the ER to the cytosol through translocating channels; and (iii) degradation of misfolded proteins by the ubiquitin–proteasome system. Despite recent improvements in our knowledge of the ERAD system, many of its underlying mechanisms remain poorly understood.

As many factors in ER quality control are induced by ER stress, we attempted to identify novel genes induced specifically by tunicamycin treatment, which inhibits *N*-linked glycosylation and results in protein misfolding, leading to ER stress. By using suppression subtractive hybridization (Diatchenko *et al*, 1996) before and after tunicamycin treatment, we isolated about 20 genes—the functions of which under the ER stress response are still unknown—with >2-fold induction after ER stress from 480 clones (the screening strategy is shown in supplementary Fig 1 online). We focused our analysis on the translocon-associated protein

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(TRAP) complex γ-subunit. The four-subunit TRAP complex localizes in the ER membrane, associating with the Sec61 translocon as a heterotetramer (Hartmann *et al*, 1993; Menetret *et al*, 2005). The α-, β- and δ-subunits contain a single ER transmembrane region, whereas the γ-subunit spans the membrane four times. A large part of each subunit localizes to the ER lumen.

Here, we show the simultaneous induction of all four subunits of the TRAP complex by ER stresses from the XBP1/IRE1α pathway, and its involvement in the acceleration of misfolded protein ERAD by discriminating them from correctly folded proteins.

RESULTS AND DISCUSSION

In mouse BALB/c 3T3 cells, ER stress induced TRAPγ messenger RNA, whereas cytosolic stress did not (supplementary Fig 2 online). ER stress also induced mRNA synthesis (supplementary Fig 2A online) and protein synthesis (data not shown) of other TRAP complex subunits. After treatment of BALB/c 3T3 cells with tunicamycin, the transcription of genes encoding all four subunits was induced simultaneously (Fig 1A), suggesting a common signalling pathway for induction.

As either the ATF6 or the XBP1/IRE1α pathways might regulate transcription of TRAP subunits, we examined their induction in IRE1α$^{+/+}$ and IRE1α$^{-/-}$ mouse embryonic fibroblast (MEF) cells (Lee *et al*, 2002) by using northern hybridization (Fig 1B). BiP, an ATF6-pathway-induced ER-resident molecular chaperone, is induced in both IRE1α$^{+/+}$ and IRE1α$^{-/-}$ MEF cells after tunicamycin treatment (Lee *et al*, 2002; Yoshida *et al*, 2003). By contrast, IRE1α knockout resulted in almost complete abolition of TRAP complex mRNA induction after ER stress (Fig 1B), indicating that transcription of all four TRAP subunits is induced by the XBP1/IRE1α pathway. This is the first demonstration that ER stress results in simultaneous induction of all subunits in the functional complex, and also in induction by the XBP1/IRE1α pathway. Moreover, the β-subunit of Sec61 translocon was induced at a low level under ER stress conditions (data not shown), and α- and γ-subunits of Sec61 translocon were also reported to be induced during plasma cell differentiation by the XBP1 pathway (Shaffer *et al*, 2004). These observations indicate the possibility that Sec61 translocon and the TRAP complex are simultaneously upregulated by the unfolded protein response pathway.

Many ERAD components are reported to be induced by the XBP1/IRE1α pathway (Yoshida *et al*, 2003; Oda *et al*, 2006), and thus it is possible that the TRAP complex might also be involved in the ERAD pathway. Therefore, we carried out RNA interference (RNAi) knockdown by using short hairpin RNAs (shRNAs) to investigate the function of the TRAP complex *in vivo*. We proposed that knockdown of one subunit would disrupt formation of the TRAP complex, inhibiting its function. Treatment with shRNAs targeted to each of the subunits, with the exception of the TRAPδ subunit, specifically and effectively depleted target mRNAs in human embryonic kidney (HEK) 293 cells (Fig 2A; supplementary Fig 3 online). Two independent shRNA constructs for the TRAPδ subunit failed to effect efficient knockdown. None of the shRNAs affected transcription of non-target TRAP subunit genes (Fig 2A). Western blot analysis showed that TRAPα levels were reduced according to the reduction of TRAPα mRNA (Fig 2B). Surprisingly, TRAPα levels were also reduced in cells transfected with shRNAs for TRAPβ or TRAPγ subunits, whereas mRNA levels remained unaffected. Similar results were also observed for TRAPδ

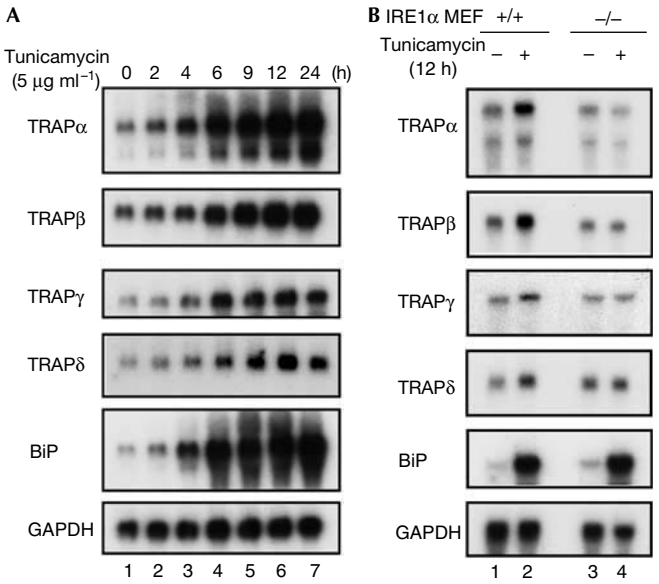


Fig 1 | Endoplasmic reticulum stress causes simultaneous induction of each TRAP complex subunit by the X-box-binding protein 1/inositol-requiring 1α pathway. (**A**) Total RNA was isolated from BALB/c 3T3 cells treated with 5 μg ml^{-1} tunicamycin for the periods indicated. RNA (5 μg) was analysed by northern hybridization using complementary DNA probes specific to each subunit of mouse TRAP (α, β, γ and δ), mouse BiP (induced by endoplasmic reticulum stress) and human GAPDH (loading control). (**B**) Wild-type MEF cells (+/+) or IRE1α-knockout MEF cells (−/−) were treated with 5 μg ml^{-1} tunicamycin for 12 h, followed by northern blot analysis. BiP, immunoglobulin heavy chain binding protein; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IRE, inositol-requiring; MEF, mouse embryonic fibroblast; TRAP, translocon-associated protein.

(Fig 2B). Knockdown of TRAP subunits did not affect the abundance of Sec61β, calnexin or β-actin (Fig 2B). As these results indicated that formation of a native TRAP complex might be affected by missing subunits, we carried out blue-native–polyacrylamide gel electrophoresis (BN–PAGE; Wang & Dobberstein, 1999) on microsomal fractions prepared by treatment with 3% digitonin from cells expressing a specific shRNA. The abundance of native TRAP complex (150 kDa) was reduced on knockdown of the α-, β- or γ-subunits, whereas it remained unchanged by treatment with the negative controls, that is, nonspecific RNAi or TRAPδ subunit shRNA (Fig 2C). Conversely, RNAi of TRAP subunits effected no change in the abundance of the Sec61 complex (140 kDa and 280 kDa; Fig 2C). Thus, depletion of the α-, β- or γ-subunits resulted in disruption of the native TRAP complex, and TRAP components that were not included in the complex might be degraded (Fig 2B).

As ER stress induced all TRAP components by the XBP1/IRE1α pathway, we examined its possible involvement in ERAD. The null Hong Kong variant (NHK) of α1-antitrypsin (α1AT), a serine protease inhibitor secretory protein (Sifers *et al*, 1988; Hosokawa *et al*, 2001), was used as the substrate for ERAD. In pulse–chase experiments, we observed substantial delays in NHK degradation, if any of the TRAP components (α, β or γ) were depleted by RNAi

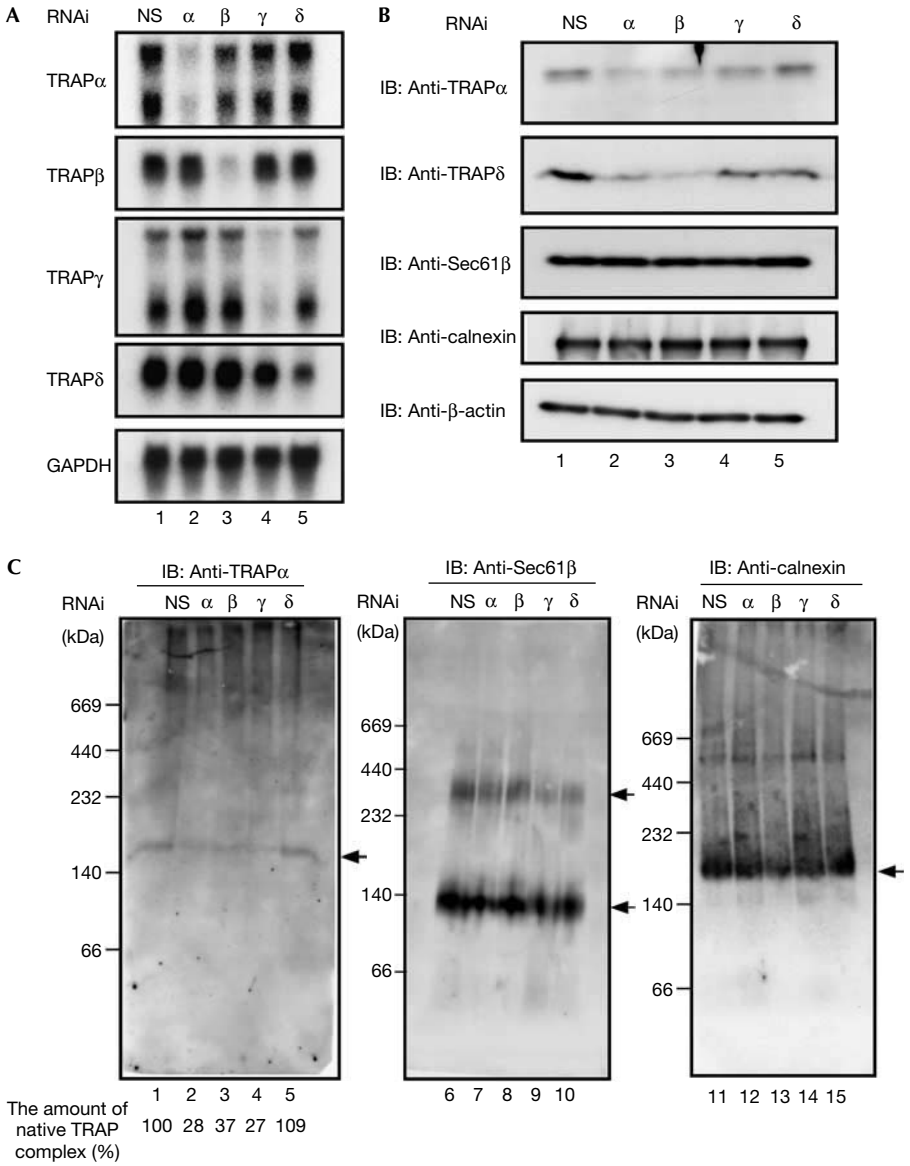


Fig 2 | RNA intereference knockdown of each TRAP subunit disrupts the native TRAP complex in the endoplasmic reticulum. Human embryonic kidney 293 cells were transfected with short hairpin RNA expression vectors against each of the TRAP subunits (pSUPER-α1(α), pSUPER-β1(β), pSUPER-γ1(γ) and pSUPER-δ1(δ)) or a nonspecific sequence (pSUPER-NS (NS)). (**A**) Northern blot analysis of each TRAP subunit after RNAi treatment. Total RNA (10 μg) was used for detection, as described in Fig 1, using probes specific to human TRAP subunits. (**B**) Western blot analysis of each TRAP subunit after RNAi treatment. Cellular proteins were extracted in NP-40 lysis buffer, and each protein was detected by western blotting. (**C**) BN–PAGE analysis of the TRAP complex after RNAi treatment. Microsomal fractions (10 or 2 μg protein) were separated by 5–18% BN–PAGE and detected with TRAPα antibody or Sec61β antibody, respectively. Calnexin was used as the loading control (1 μg of sample). The amounts of native TRAP complex remaining after RNAi treatment of each subunit were quantified from three independent analyses and are shown at the bottom of the left panel. The amount of native TRAP complex in the NS lane was set to 100%. BN–PAGE, blue-native–polyacrylamide gel electrophoresis; IB, immunoblotting; RNAi, RNA interferene; TRAP, translocon-associated protein.

(Fig 3A,B), indicating involvement of the complex in NHK ERAD. As knockdown of the δ-subunit was unsuccessful, no delay in NHK degradation was observed and it represented an additional negative control. RNAi of TRAP components did not affect the rates of cellular clearance and secretion into the medium or glycosylation of wild-type α1AT (Fig 3A,B). To exclude the possibility that the delay of NHK degradation by TRAP RNAi was caused by an indirect effect such as ER disturbance owing to the decrease in the abundant ER membrane proteins, we knocked down another ER membrane protein, ribophorin I. After knockdown of ribophorin I, a partly glycosylated band of NHK was observed because ribophorin I is one of the subunits of oligosaccharyltransferase (supplementary Fig 4A online). However, the degradation of fully glycosylated NHK was not affected

Fig 3 | Knockdown of the TRAP complex delays endoplasmic reticulum-associated degradation of misfolded glycoproteins. Human embryonic kidney 293 cells were transiently co-transfected with plasmids encoding endoplasmic reticulum-associated degradation substrates and short hairpin RNA (pSUPER-NS, pSUPER-α1, pSUPER-β1, pSUPER-γ1 or pSUPER-δ1). Degradation of the substrates was examined by metabolic labelling with [^{35}S]Met/Cys for 15 min and by chasing with normal growth medium for the time periods indicated. Intracellular substrates were detected by immunoprecipitation. (**A**) Degradation of the null Hong Kong (NHK) variant and secretion of wild-type α1-antitrypsin, (**C**) degradation of the C-terminal-truncated soluble form of ribophorin-1 (RI_{332}) and (**D**) degradation of the T-cell receptor α-subunit (TCRα) are shown. Quantification analyses of these are shown in (**B–D**). Radioactivity at chase = 0 h was set to 100%, and relative radioactivity compared with this value is shown in the graph. The averages of five (for NHK), four (for wt α1AT) and three (for RI_{332} and TCRα) independent experiments are plotted and error bars represent s.e.m. CHO refers to *N*-linked glycan. HA, Haemagglutinin; Med, medium; wt α1AT, wild-type α1-antitrypsin.

even after ribophorin I knockdown when compared with nonspecific RNAi (supplementary Fig 4B,C online). These data clearly indicated that the effect on NHK was not caused by an artefact such as disturbance of the ER membrane.

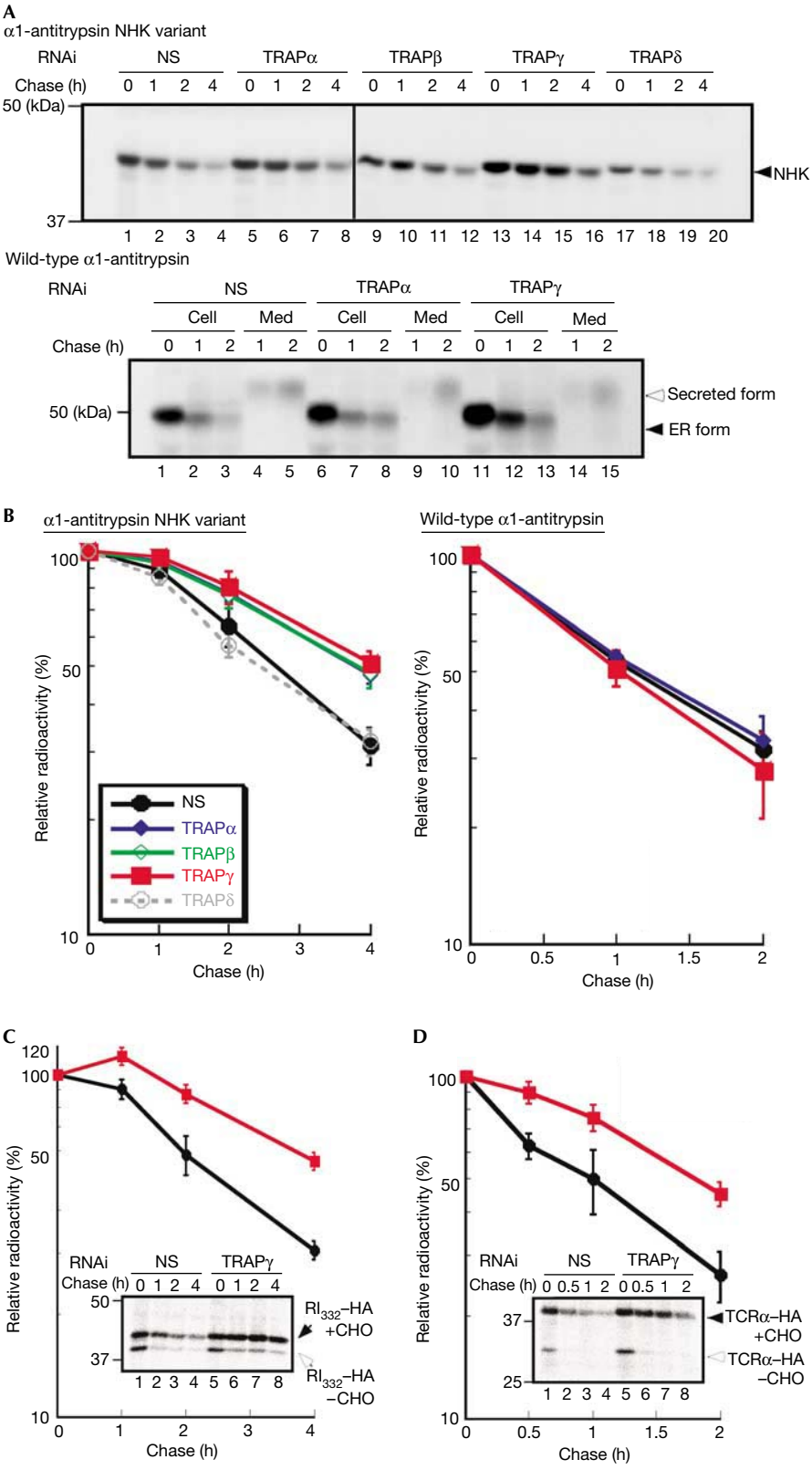
To find other soluble and transmembrane ERAD substrates, we investigated the degradation of a carboxy-terminal-truncated soluble form of ribophorin I (RI_{332}; Fig 3C; de Virgilio *et al*, 1998) and the T-cell receptor α-subunit (TCRα; Fig 3D; Yu *et al*, 1997), after RNAi depletion of TRAP subunits. Degradation of RI_{332} and TCRα was slowed substantially (i.e., half-lives were doubled) after knockdown of the TRAPγ subunit (Fig 3C,D). This supports the suggestion that the TRAP complex is involved in the degradation of various ERAD substrates.

We analysed the interaction between the TRAP complex and misfolded ERAD substrates by using immunoprecipitation. Cells were lysed in 3% digitonin, and substoichiometric ratios of all four subunits of the endogenous TRAP complex were immunoprecipitated with TRAPα antibody (supplementary Fig 5 online; Hartmann *et al*, 1993). Using TRAPα antibody as the first immunoprecipitation, we carried out a sequential immunoprecipitation with α1AT antibody and observed co-immunoprecipitation of endogenous TRAP complex with NHK (Fig 4A, lane 15). This indicates that the ERAD substrate (NHK) is bound to the TRAP complex. Moreover, overexpressed TRAPα–HA (haemagglutinin) and NHK interacted with each other, as shown by co-immunoprecipitation in the presence of 1% NP-40 (supplementary Fig 6A online). Under this condition, subunits of the TRAP complex dissociated and Sec61 translocon also dissociated from the TRAP complex (supplementary Fig 6B online). This indicates that the observed co-immunoprecipitation of NHK and TRAP might reflect direct binding.

Next, we examined the interaction of the TRAP complex with NHK and wild-type α1AT, which is assumed to be folded correctly in the ER, and observed co-immunoprecipitation of endogenous TRAP complex with the former only (Fig 4B, lanes 7 and 9, respectively). In addition, there was only a slight increase in co-immunoprecipitation of wild-type α1AT and the TRAP complex even after secretion of the former was inhibited by osmotic stress (0.6 M sorbitol; supplementary Fig 7 online). NHK was co-immunoprecipitated with the TRAP complex in both the presence and absence of sorbitol (supplementary Fig 7 online), indicating that preferential binding of TRAP to NHK was not merely because NHK was retained in the ER for a longer time.

As the TRAP complex has been reported to accelerate substrate-dependent ER membrane translocation of secretory proteins from the cytosol (Fons *et al*, 2003), we examined whether it interacts with substrates during import into the ER, or with retrograde transport from the ER to the cytosol. We carried out a pulse–chase experiment combined with immunoprecipitation analysis in the presence or absence of lactacystin—a proteasome inhibitor. Little interaction between TRAP and NHK could be detected immediately after pulse labelling, whereas it increased substantially during the chase period (Fig 5A). At 1 h and 2 h of chase, the ratio [TRAP-bound NHK]/[total NHK] increased two- and fourfold, respectively (Fig 5B). In the presence of lactacystin, in which NHK degradation was inhibited, the abundance of bound NHK was much higher than in the absence of lactacystin (Fig 5A, compare lanes 5 and 3). In the presence of lactacystin, the ratio of TRAP-bound NHK to total NHK was also higher after a 2 h chase period compared with that immediately after pulse-labelling (compare lanes 5 and 4). We also quantified calnexin binding of NHK during the chase period. As clearly shown in Fig 5, the binding of NHK to calnexin was rapidly decreased during the chase period, whereas binding to the TRAP complex increased (Fig 5B), indicating that NHK binds to TRAP after it is released from calnexin. These results strongly indicate that the TRAP complex interacts with misfolded ERAD substrates at a late stage—that is, during protein dislocation from the ER and not during import across the ER membrane. Thus, TRAP is necessary for efficient ERAD, discriminating misfolded proteins from correctly folded proteins. Co-immunoprecipitation analysis using the whole-cell lysate of HEK 293 cells showed weak (possibly transient) interaction of the TRAP complex and calnexin (supplementary Fig 8 online), which is consistent with previous reports (Wada *et al*, 1991; Fons *et al*, 2003). Co-immunoprecipitation analysis of the TRAP complex also showed interaction with p97 and EDEM1, which are involved in the ERAD machinery, to an extent similar to that with calnexin, whereas interaction of the TRAP complex with BiP was weaker (supplementary Fig 8 online). As TRAP was first reported to be a transmembrane protein complex that associates with the Sec61 translocon (Hartmann *et al*, 1993; Menetret *et al*, 2005), its function in ERAD might be to recruit misfolded proteins to the Sec61 translocon in the ER membrane.

Both the Sec61 translocon (Pilon *et al*, 1997; Plemper *et al*, 1997) and the Derlin complex (Ye *et al*, 2004) have been proposed as dislocation channels for the transfer of misfolded proteins from the ER to the cytosol. In mammalian cells, Sec61 comprises three subunits (Sec61α, Sec61β and Sec61γ), all of which are ER transmembrane proteins, the first subunit spanning the ER membrane ten times to create the translocation channel (Menetret *et al*, 2005). Electron microscopic observation of the mammalian Sec61 complex, and also X-ray analysis of the SecY complex from archaebacterium *Methanococcus jannaschii*, indicates that the channel pore is too narrow for translocation of folded and/or glycosylated proteins (Van den Berg *et al*, 2004; Menetret *et al*, 2005). Conversely, analysis of the crystal structure of the archaeal SecY complex indicated that some transmembrane components of translocon are flexible, allowing the channel to open to the hydrophobic lipid bilayers of the membrane (Van den Berg *et al*, 2004). This might allow the retrotranslocation of


A
α1-antitrypsin NHK variant
RNAi
NS
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NHK
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Wild-type α1-antitrypsin
Cell
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0 1 2
Secreted form
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
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Relative radioactivity (%)
100
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NS
TRAPα
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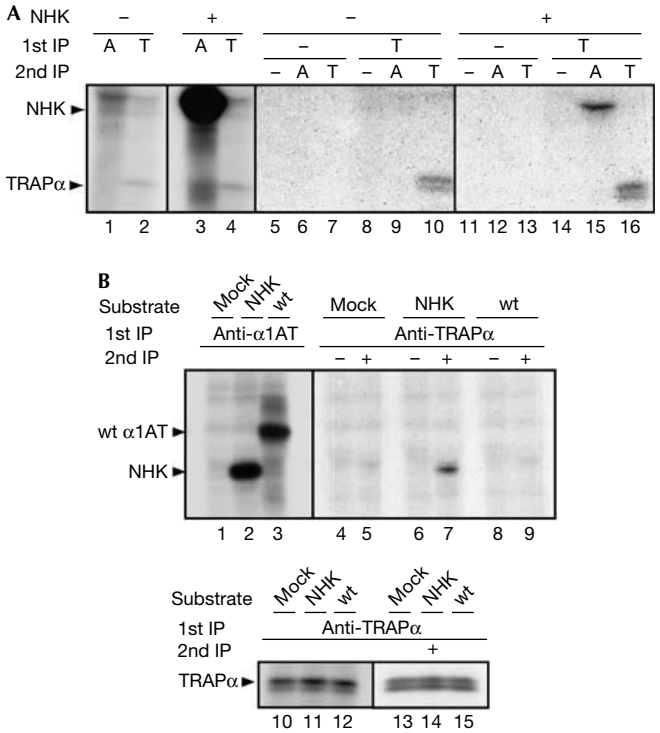

Fig 4 | The endogenous TRAP complex preferentially binds to misfolded endoplasmic reticulum-associated degradation substrates. (**A**) Co-immunoprecipitation (co-IP) of the endogenous TRAP complex with NHK. HEK 293 cells were transfected with NHK (+) or mock plasmid (−), then radiolabelled with [^{35}S]Met/Cys for 2 h, followed by disruption in 3% digitonin lysis buffer. The first IP was carried out with (T) or without (−) TRAPα antibody, and eluates were then subjected to a second IP using α1AT (A), TRAPα antibodies (T) or only protein A beads as a control (−). To detect NHK and the endogenous TRAPα by IP, one-fifth of the volume of the cell lysate used for co-IP was applied to each lane (lanes 1–4). (**B**) Comparison of the affinity of the TRAP complex for NHK and wild-type α1AT. The second IPs were carried out with (+) or without (−) α1AT antibody (upper panel, lanes 4–9), or with TRAPα antibody (lower panel, lanes 13–15). Control IPs were carried out using α1AT (lanes 1–3) and TRAPα (lanes 10–12) antibodies. α1AT, α1-antitrypsin; HEK, human embryonic kidney; IP, immunopreipitation; Met/Cys, methionine/cysteine; NHK, null Hong Kong variant; TRAP, translocon-associated protein; wt, wild type.

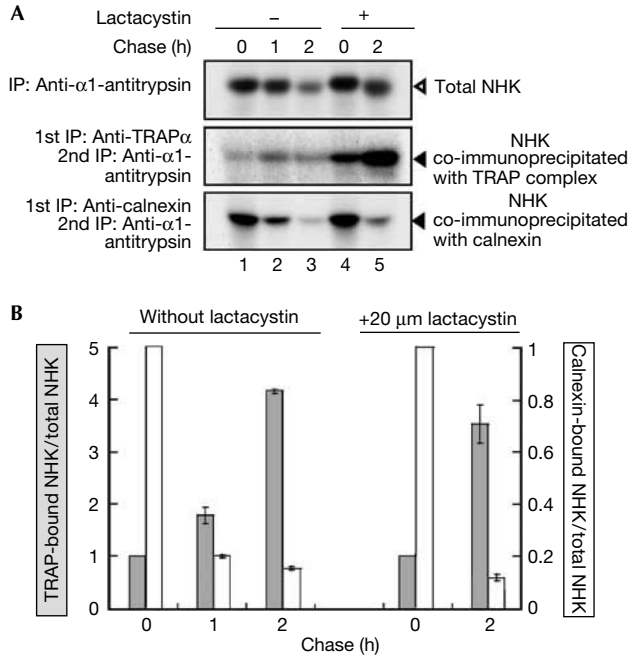

Fig 5 | The null Hong Kong variant is co-immunoprecipitated with the TRAP complex during the chase period. (**A**) Intracellular NHK levels (upper panel) and NHK co-immunoprecipitated with the endogenous TRAP complexes (middle panel) or calnexin (lower panel) in HEK 293 cells transfected with NHK in the presence (+) or absence (−) of lactacystin. The pulse–chase experiment was carried out as in Fig 3, and the time course of NHK degradation and interaction with the TRAP complex was analysed by IP or sequential IP as described in Fig 4A. (**B**) Quantification of NHK co-IP with TRAP (grey bars) or calnexin (white bars) relative to total NHK. The values at chase = 0 h were set as 1 for TRAP-associated (left vertical axis) and calnexin-associated NHK (right vertical axis). The averages of three independent experiments are plotted and error bars indicate s.e.m. ($n = 3$). HEK, human embryonic kidney; IP, immunoprecipitation; NHK, null Hong Kong variant; TRAP, translocon-associated protein.

misfolded ERAD substrates. Recently, a Sec61 dimer was reported to assemble into a tetramer, which associated with two TRAP complexes (Menetret *et al*, 2005). Further investigation is required to determine whether the TRAP complex is involved in the regulation of supramolecular translocon complex formation, the dynamic structure of the channel and/or its pore size.

Mammalian Derlin-1 has recently been implicated in the US11-mediated retrotranslocation of the class I major histocompatibility complex heavy chain (Lilley & Ploegh, 2004; Ye *et al*, 2004), providing the missing link between ER luminal events and those in the cytosol. Although it was proposed that Derlin-1 formed a retrotranslocation channel (Ye *et al*, 2004), we have no direct evidence to confirm this hypothesis. It has also been reported that proteasomes bind directly to the Sec61 complex (Kalies *et al*, 2005), supporting the idea that misfolded proteins are retrotranslocated from the ER to the cytosol through this translocon. Thus, at present, it seems that we are far from any conclusive determination of the retrotranslocation channels working with the ERAD pathway.

In mammalian cells, the XBP1/IRE1α pathway induces certain molecules—for example, EDEMs (1–3) HMG-CoA reductase degradation (HRD)1 and Derlins (1–3), and all of these factors are related to the ERAD pathway (Yoshida *et al*, 2003; Oda *et al*, 2006). The results of this study show that the four TRAP complex subunits are possible ERAD factors induced by the XBP1/IRE1α pathway, and provide strong supportive evidence that ERAD substrates are committed to and retrotranslocated through the Sec61 translocon.

METHODS

Plasmid construction for RNA interference. pSUPER (Oligo-Engine, Seattle, WA, USA) was used to deliver shRNAs. Three independent 19 bp target sequences were designed against each TRAP subunit, and the shRNA sequence for nonspecific RNAi was obtained from Dharmacon (GE Healthcare, Bio-Sciences Corp.,

Piscataway, NJ, USA). To analyse RNAi efficiency using northern hybridization, HEK 293 cells were transfected with pSUPER-expressing constructs or nonspecific shRNA (pSUPER-NS); two constructs were chosen for each subunit. Target sequences are indicated in supplementary Fig 3A online.

Metabolic labelling, immunoprecipitation and blue-native–polyacrylamide gel electrophoresis. HEK 293 cells were radiolabelled with [^{35}S]-EXPRESS protein labelling mixture (PerkinElmer, Life and Analytical Sciences Inc., Wellesley, MA, USA) and chased in normal DMEM/10% fetal bovine serum. Cellular proteins were extracted with 1% NP-40 lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40 and protease inhibitors (1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 2 mM *N*-ethylmaleimide and 0.2 mM AEBSF)) or 3% digitonin (Calbiochem, EMD Biosciences Inc., Darmstadt, Germany) lysis buffer. For sequential immunoprecipitation, immune complexes were released from beads by boiling in elution buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 2% SDS and protease inhibitors) for 10 min. Supernatants were collected and diluted in NP-40 lysis buffer until the final concentration of SDS reached 0.1%. The second immunoprecipitation was carried out using 1% NP-40 as the detergent. Sample preparation and separation by BN–PAGE was carried out as described previously (Wang & Dobberstein, 1999).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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