Adaptor Aly and co-adaptor Thoc5 function in the Tap-p15-mediated nuclear export of *HSP70* mRNA

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In metazoans, nuclear export of bulk mRNA is mediated by Tap-p15, a conserved heterodimeric export receptor that cooperates with adaptor RNA-binding proteins. In this article, we show that Thoc5, a subunit of the mammalian TREX complex, binds to a distinct surface on the middle (Ntf2-like) domain of Tap. Notably, adaptor protein Aly and Thoc5 can simultaneously bind to non-overlapping binding sites on Tap-p15. *In vivo*, Thoc5 was not required for bulk mRNA export. However, nuclear export of *HSP70* mRNA depends on both Thoc5 and Aly. Consistent with a function as a specific export adaptor, Thoc5 exhibits *in vitro* RNA-binding activity and is associated with *HSP70* mRNPs *in vivo* as a component of the stable THO complex. Thus, through the combinatorial use of an adaptor (e.g., Aly) and co-adapter (e.g., Thoc5), Tap-p15 could function as an export receptor for different classes of mRNAs.

Introduction

Eukaryotic cells are organized in distinct subcellular compartments, each of which is surrounded by a specific organellar membrane. The nuclear membrane separates the nucleus from the cytoplasm, but nuclear pore complexes (NPCs) perforate the nuclear envelope to allow for nucleo-cytoplasmic transport between these two compartments. A huge variety of cargoes (proteins, RNAs, RNPs) is transported through the NPCs by binding to specific nucleo-cytoplasmic transport receptors, which decode the targeting signals (NLS or NES) in the various transport cargoes. Many of these receptors belong to the importin-β/karyopherin family of transport factors, which require the small GTPase Ran for the directionality of nucleo-cytoplasmic transport (for recent reviews, see Kohler and Hurt, 2007; Terry and Wente, 2007).

Nuclear export of mRNA is unique in the sense that the shuttling export receptor is not a member of the importin-β family and does not directly depend on RanGTP. However, the mRNA export receptor is evolutionarily conserved and is called Mex67-Mtr2 in the budding yeast *Saccharomyces cerevisiae* and Tap-p15 (or NXF1-NXT1) in metazoans (Segref *et al*, 1997; Gruter *et al*, 1998; Santos-Rosa *et al*, 1998; Kang and Cullen, 1999; Katahira *et al*, 1999; Herold *et al*, 2000). Although the transporters exhibit RNA-binding activity by themselves *in vitro* (Santos-Rosa *et al*, 1998; Katahira *et al*, 1999; Liker *et al*, 2000), they predominantly select their mRNA cargoes through interactions with adaptor RNA-binding proteins. Tap exhibits a modular domain organization, in which the region including the amino terminal (N-) and leucine-rich repeat (LRR-) domains, and the middle (M-) and carboxyl terminal (C-) domains, are assigned as binding sites of adaptor RNA-binding proteins and FG-nucleoporins, respectively (see Figure 1A) (Katahira *et al*, 1999; Bachi *et al*, 2000; Strasser *et al*, 2000; Stutz *et al*, 2000; Fribourg *et al*, 2001; Huang *et al*, 2003).

The evolutionarily conserved TREX complex is required for coupled transcription elongation and nuclear export of mRNAs, and provides an example of an mRNA-specific adaptor (Reed and Hurt, 2002; Aguilera, 2005; Reed and Cheng, 2005; Kohler and Hurt, 2007). The yeast complex is composed of the THO transcription elongation complex (Hpr1, Tho2, Mft1 and Thp2), Tex1, Sub2 and Yra1 (Strasser *et al*, 2002). The complex is loaded onto mRNAs co-transcriptionally, facilitating assembly of nascent transcripts into export competent mRNPs. Yra1, which is recruited onto mRNAs through interaction with the DEAD-box type RNA helicase Sub2, physically interacts with Mex67-Mtr2 and functions as an adaptor (Strasser and Hurt, 2000, 2001; Abruzzi *et al*, 2004). Accordingly, yeast TREX mutants show a nuclear export defect for bulk poly(A)$^+$ RNAs and are synthetically lethal with many mutants of the mRNA export machinery (Strasser *et al*, 2002).

An equivalent complex exists in metazoans (Reed and Hurt, 2002; Reed and Cheng, 2005; Kohler and Hurt, 2007). The human TREX complex is composed of the hTHO subcomplex (hHpr1, Thoc2, Thoc5, Thoc6 and Thoc7), TEX1, UAP56 and Aly. A related complex has also been identified in the fruit fly *Drosophila melanogaster* (Strasser *et al*, 2002; Rehwinkel *et al*, 2004; Masuda *et al*, 2005; Reed and Cheng, 2005). Aly, similar to its yeast orthologue Yra1 (Strasser and Hurt, 2000), is recruited to mRNA through the interaction with UAP56 (the Sub2 orthologue) and directly interacts with Tap-p15. In *Xenopus* oocytes, Aly was shown to be a limiting factor for nuclear export of mRNAs (Stutz *et al*, 2000; Zhou *et al*, 2000; Luo *et al*, 2001; Rodrigues *et al*, 2001). Despite these structural and functional conservations, gene

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knockdown experiments performed with cultured fruit fly cells have shown that only the UAP56 orthologue, but not the other THO/TREX components (including *Drosophila* Aly), is essential for bulk poly(A)$^+$ RNA export (Gatfield *et al*, 2001; Herold *et al*, 2001; Gatfield and Izaurralde, 2002; Farny *et al*, 2008). It has also been shown that nuclear export of only a subset of mRNAs is affected by depletion of the TREX components (Rehwinkel *et al*, 2004; Farny *et al*, 2008). These data suggest that various nuclear mRNA export pathways, which may be dictated by different adaptor RNA-binding proteins, exist in higher eukaryotes.

In this study, we show that the human TREX component Thoc5 binds directly to the middle domain of Tap, which exhibits an Ntf2-like fold. Our data further indicate that Thoc5, along with the other THO components, although not required for bulk mRNA export, is crucial for nuclear export of a specific mRNA (*HSP70*) in conjunction with the adaptor protein Aly. *In vitro*, Thoc5 exhibits RNA-binding activity and can shuttle between the nucleus and cytoplasm. Biochemical studies showed that Thoc5 binds to the Ntf2-like domain of Tap, whereas Aly is recruited to the N- and LRR-domain of Tap. Together, these findings suggest that Thoc5 functions in the nuclear export of *HSP70* mRNA as a co-adaptor in close overlap with the general adaptor protein Aly. Thus, by the recruitment of an adaptor (Aly) and co-adaptor (Thoc5) to non-overlapping binding sites, Tap-p15 could be involved in the nuclear export of different classes of mRNAs.

Results

The TREX component Thoc5 binds to the Ntf2-like (middle) domain of the Tap-p15 heterodimer

Several interacting proteins of the Tap mRNA export receptor have been identified in yeast two-hybrid screens, including FG-nucleoporins and hCG1 (Katahira *et al*, 1999). Notably, one of the functionally unknown Tap-interacting factors found in these screens turned out to be Thoc5, a component of the human TREX complex (this protein was previously designated as 'anonymous gene product' (Xle *et al*, 1993). The insert of the two-hybrid prey plasmid interacting with the Tap bait protein contained a sequence of human Thoc5 that encompassed amino acids 164–683. Further two-hybrid analyses suggested that the M-domain of Tap, which has an Ntf2-like fold, interacts with Thoc5 (Supplementary Figure S1).

To show a direct interaction between Tap and Thoc5, both proteins were expressed in *Escherichia coli* and GST pull-down assays were performed. The full-length Tap-p15 heterodimer, as well as fragments containing the middle (M) domain, effectively enriched Thoc5 from *E. coli* whole cell lysates (Figure 1B, lanes 6, 8, 10), whereas a fragment consisting of the LRR- and part of the N-domain (residues 96-371) known to be involved in adaptor binding (e.g., Aly, 9G8 or SRP20; Rodrigues *et al*, 2001; Huang *et al*, 2004) did not interact with Thoc5 (Figure 1B, lane 12; for domain organization, see Figure 1A). Further truncation analyses showed that the Ntf2-like domain of Tap (residues 372–551) bound to p15 is necessary and sufficient for binding to Thoc5 (Figure 1C, lanes 2–5).

The M-domain of Tap contains hydrophobic patches on the surface, which bind the phenylalanine residues that are part of the FG repeats (Fribourg *et al*, 2001). To examine whether the binding site of Thoc5 on the Ntf2-like domain of Tap

overlaps with the FG-binding sites, a series of alanine-scan mutations were generated in the M-domain of Tap (Figure 1D). The alanine substitutions (m2–m7), which mapped to the surface of Tap-p15 that is opposite the FG-repeat binding site (Fribourg *et al*, 2001; Yao *et al*, 2007) (see Figure 1D for 3D structure), effectively inhibited binding to Thoc5, but FG repeat binding was unaffected (Figure 1E, lanes 4–7, 11 and 12; and data not shown). These data suggest that Thoc5 is recruited to the Ntf2-like domain of Tap-p15 and not to a region on Tap, which binds to FG repeats of nucleoporins. Notably, the way Thoc5 binds to a specific region on the Ntf2-like domain of Tap is reminiscent of the binding of pre-ribosomal particles and the Nup84p complex to a related surface on the homologous Mex67-Mtr2 heterodimer (Yao *et al*, 2007, 2008).

To determine the region in Thoc5 that binds to Tap-p15, bacterially expressed N- (1–251) and C-terminal (251–683) parts of Thoc5 were subjected to GST pull-down assays. Thoc5 (1–251), but not Thoc5 (251–683), showed a robust interaction with Tap-p15, which was comparable with the binding of full-length Thoc5 (Figure 1F, lanes 4 and 5). The N-terminal domain of Thoc5 is composed of a series of predicted α-helices, which by homology modelling are thought to form an α-solenoid structure (Supplementary Figures S2A and S2B). Such a structure may be crucial for interaction with Tap-p15 (see Discussion).

Thoc5 and Aly are required for HSP70 mRNA export in mammalian cells

To further characterize Thoc5 in mammalian cells, a polyclonal antibody was raised against human Thoc5, which recognized Thoc5 on western blots (Supplementary Figure S3A, lane 2) and in HeLa cells by indirect immunofluorescence. As anticipated, Thoc5 was found concentrated in the splicing factor-rich nuclear compartment that contains the SC35 marker protein as well as Aly and hHpr1 (Supplementary Figure S3B, upper panels) (Zhou *et al*, 2000; Masuda *et al*, 2005).

To find out whether Thoc5 can shuttle between the nucleus and the cytoplasm, we performed an inter-species heterokaryon assay using a mouse L929 cell line stably expressing Thoc5–GFP (Figure 2A, lane 2). hnRNP C, a non-shuttling protein, and human Tap were used as controls to show shuttling between the mouse and *Xenopus* nucleus (Figure 2B). Apparently, Thoc5–GFP, similar to Tap or Aly (Katahira *et al*, 1999; Zhou *et al*, 2000; Rodrigues *et al*, 2001), can shuttle in the heterokaryon assay. As shown in Figure 2C, hHpr1–GFP also shuttles, suggesting that Thoc5 may rapidly shuttle in and out of the nucleus as part of the THO–TREX complex.

To examine whether Thoc5 has an important function in mRNA export, siRNA knockdown experiments were performed. Expression of Thoc5, Aly and Tap was efficiently blocked by siRNA transfection (Figures 3A–C). However, depletion of Thoc5 did not affect nuclear export of poly(A)$^+$ RNAs, which was examined by *in situ* hybridization using Cy-3 labelled oligo-dT probes. In contrast, depletion of Tap-p15 resulted in a strong nuclear accumulation of poly(A)$^+$ RNA. Upon depletion of Aly, $\sim 70\%$ (in the case of siAly-1) and $\sim 20\%$ (in the case of siAly-2), respectively, of the siRNA transfected cells exhibited a strong nuclear accumulation of poly(A)$^+$ RNA (Figure 3D). These data indicate that in cultured mammalian cells, Tap-p15 and Aly,

but not Thoc5, has a crucial function in nuclear export of poly(A)$^+$ RNA.

Previously, it was reported that the *Drosophila* THO–TREX complex is required for nuclear export of heat-shock mRNAs but is dispensable for nuclear export of bulk poly(A)$^+$ RNA (Rehwinkel *et al*, 2004). Therefore, we tested whether Thoc5 is required for the export of *HSP70* mRNA. *HSP70* mRNA was induced in cells upon heat shock and detected in a few nuclear foci by *in situ* hybridization, which were shown to be transcription sites adjacent to the heat-shock genes (Jolly *et al*, 1997, 1999). Our FITC-labelled oligonucleotide probes allowed specific detection of *HSP70* mRNAs in nuclear foci after heat shock (Supplementary Figures S4A and B).

Notably, upon depletion of Thoc5, a robust increase in signal intensity of the nuclear *HSP70* mRNA-containing foci was observed (Figure 4A, see also Supplementary Figure S5; 94 and 87% of cells treated with siThoc5-1 and siThoc5-2, respectively, showed larger nuclear foci). A similar increase in signal intensity of the nuclear foci was observed when the expression of Tap was inhibited by RNAi. Depletion of Aly with the two different siRNAs also resulted in enlargement of nuclear *HSP70* mRNA containing foci in almost all the cells (Figure 4A; 89 and 94% of cells treated with siAly-1 and siAly-2, respectively, showed enlarged nuclear foci). This was in contrast to the effect of Aly depletion on bulk poly(A)$^+$ RNA export. The nuclear foci were not observed in siRNA-
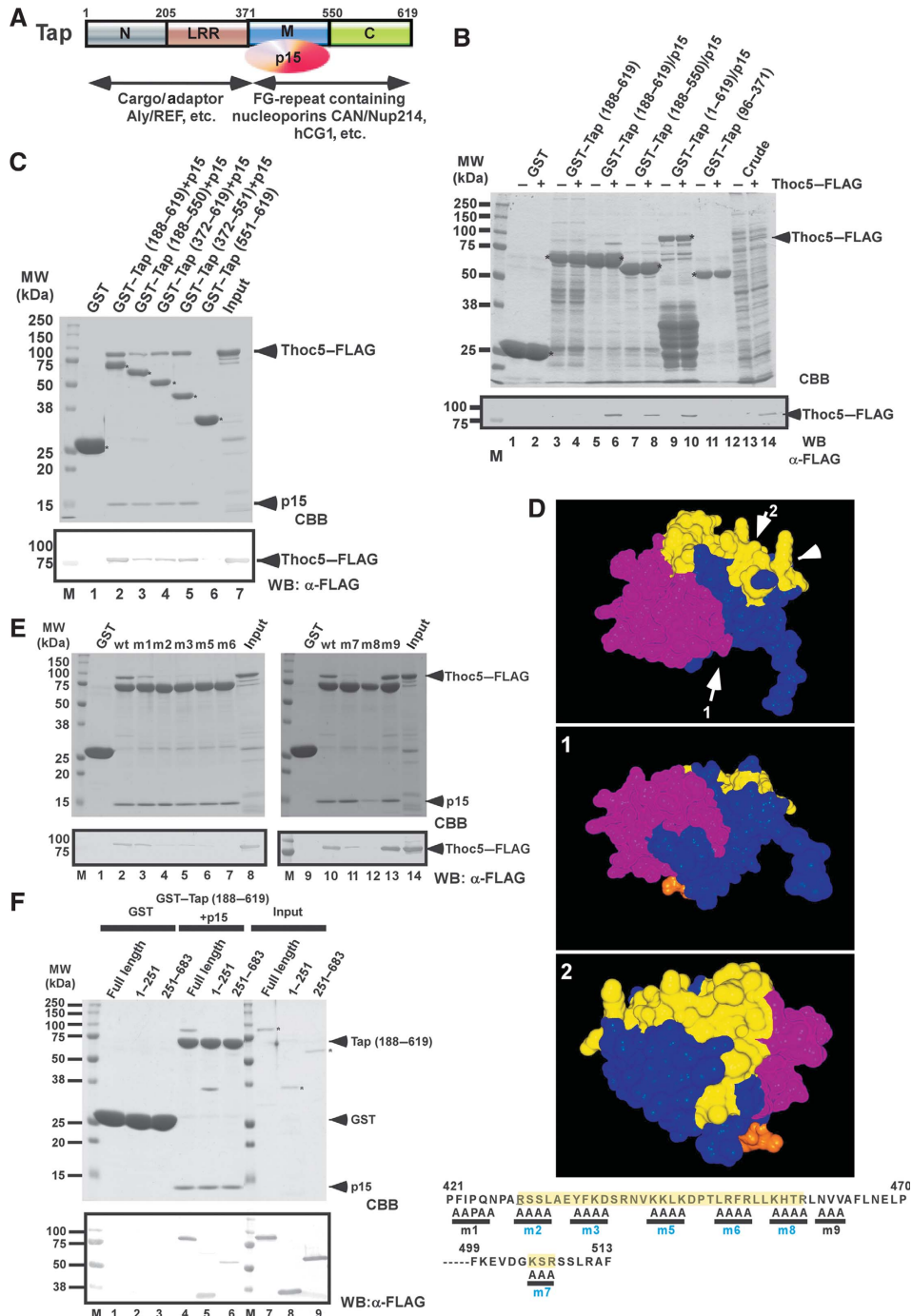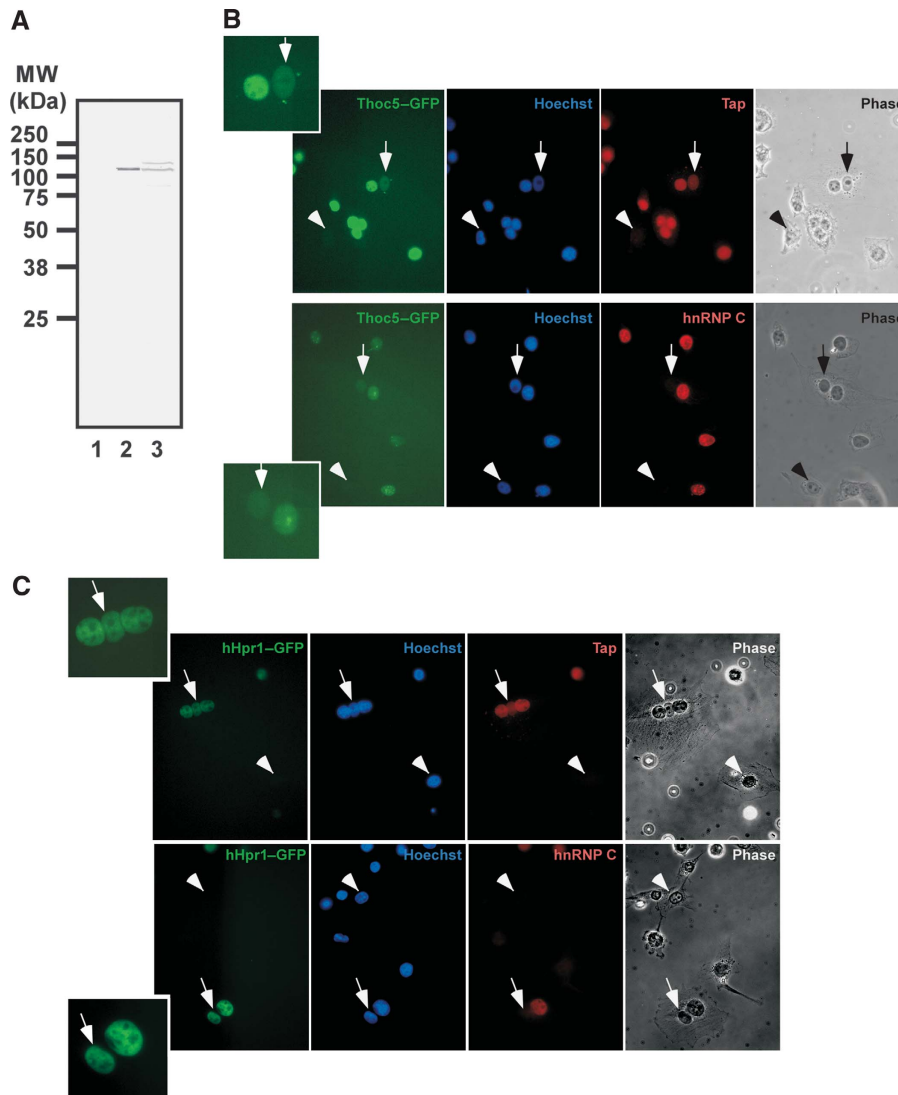
Figure 2 Thoc5 shuttles between the nucleus and the cytoplasm. (**A**) Total cell extracts prepared from parental L929 cells (lane 1) or L929 cell lines stably expressing Thoc5–GFP (lane 2) and hHpr1–GFP (lane 3) were subjected to western blot using anti-GFP antibody. Positions of molecular weight markers are indicated on the left in kDa. (**B, C**) Heterokaryon formation was performed using *Xenopus* A6 cells and the L929 cell lines stably expressing Thoc5–GFP (B) or hHpr1–GFP (C) in the presence of CHX. After incubation for 3 h at 30°C in the presence of CHX, the cells were fixed and immunostained with anti-Tap (shuttling; upper panels) and anti-hnRNP C (non-shuttling; lower panels) antibodies. Nuclei were stained with Hoechst 33342 dye. Arrows indicate *Xenopus* nuclei in fused cells, whereas arrowheads indicate those in unfused cells. Insets show magnified views of the GFP signals in the fused cells.

Figure 1 Thoc5 is a novel interaction counterpart of Tap. (**A**) Domain organization and the known interaction counterparts of the Tap-p15 heterodimer. Numbers on each rectangle indicate amino-acid positions of human Tap (for details, see Segref *et al*, 1997; Liker *et al*, 2000; Rodrigues *et al*, 2001; Reed and Hurt, 2002). (**B**) Crude lysates of *E. coli* with (indicated by +) or without (indicated by −) Thoc5–FLAG expression were added to GSH beads pre-adsorbed to GST or GST fused to various fragments of Tap. Bound proteins were analysed by SDS–PAGE followed by CBB staining (upper panel) and western blot using anti-FLAG antibody (lower panel). In lanes 13 and 14, aliquots of 10% of each input were loaded. Arrowheads indicate the positions of Thoc5–FLAG. Positions of molecular weight markers are indicated on the left in kDa. Note that in this particular gel, p15 migrated to the dye front. (**C**) Purified Thoc5–FLAG was added to GSH beads pre-adsorbed to GST (lane 1) or GST fused to various fragments of Tap (lanes 2–6). Aliquots of 25% of each bound fraction were separated by SDS—PAGE, and protein bands were detected by CBB staining (upper panel) and western blot using anti-FLAG antibody (lower panel). In lane 7, a total of 10% of input was loaded. Arrowheads indicate the positions of p15 and Thoc5–FLAG, whereas asterisks indicate the positions of each GST-fusion protein. Positions of molecular weight markers are indicated on the left in kDa. (**D**) Surface representation showing the Ntf2-like domain of Tap (blue) complexed with p15 (magenta) and FG-containing peptide (orange) (Fribourg *et al*, 2001). Different surfaces are also viewed from orientations indicated by the arrows in the upper-most figure. The regions of Tap that are critical for Thoc5 binding are coloured in yellow. An arrowhead indicates the short loop (aa 505–507) of Tap. The positions of alanine-scan mutations are indicated at the bottom of the figures as a single-letter code. The numbers on top of the sequence indicate the amino-acid positions of Tap. The residues shaded in yellow correspond to the region coloured in yellow in the 3D model. (**E**) Same as in (C), but Tap (188–619) containing the alanine-scan mutations complexed with p15 were used. Arrowheads show the positions of p15 and Thoc5–FLAG on the gel. Positions of molecular weight markers are indicated on the left in kDa. (**F**) Full-length and different domains (aa 1–251 and 251–683) of Thoc5 were expressed in *E. coli* as His₆- and FLAG double-tagged proteins. Purified proteins (5% of input, lanes 7–9) were pulled down by GST (lanes 1–3) or GST-Tap (188-619)-p15 (lanes 4–6). Proteins in bound fractions (25%) were analysed by SDS–PAGE followed by CBB staining (upper) and western blot using anti-FLAG antibody (lower). The positions of Thoc5–FLAG and its derivative are indicated by asterisks, whereas those of Tap (188–619), GST and p15 are indicated by arrowheads. Positions of molecular weight markers are shown on the left in kDa.
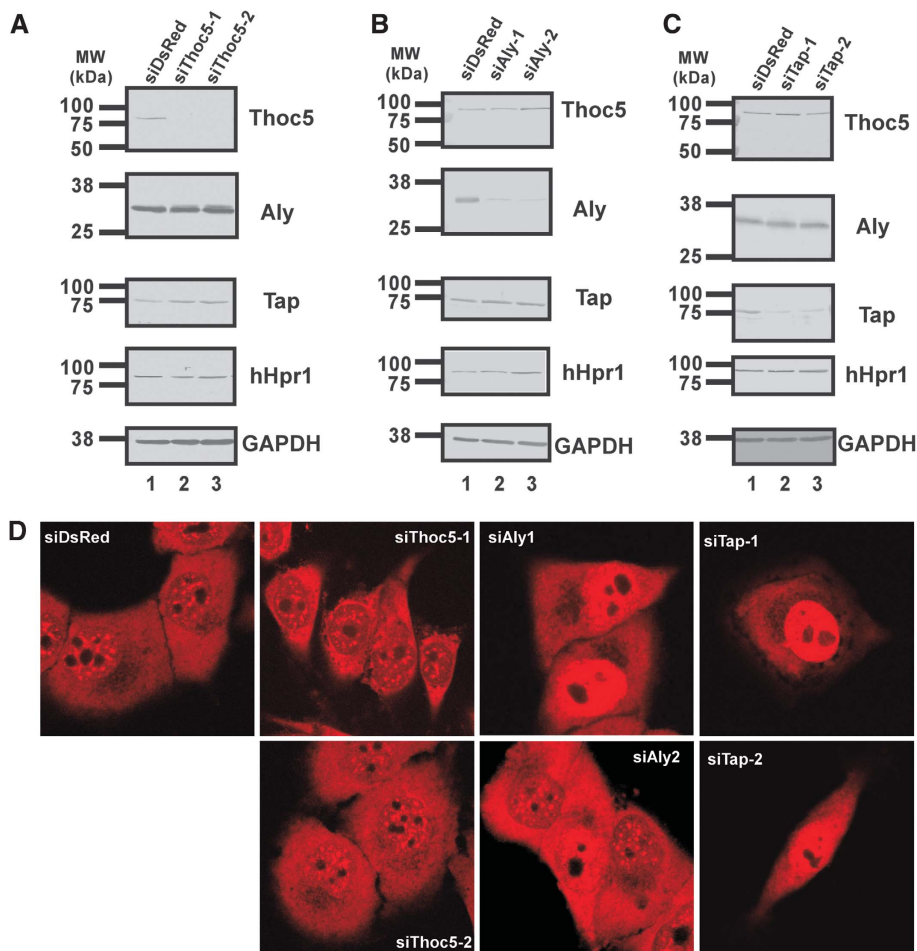
Figure 3 Depletion of Thoc5 does not affect bulk poly(A)$^+$ RNA export in mammalian cells. (**A**) HeLa cells were treated with the indicated siRNAs for 72 h. Total cell extracts were subjected to western blot using the indicated antibodies. For a negative control, siRNA against DsRed protein was used. Positions of molecular weight markers are indicated on the left in kDa. (**B**) HeLa cells were treated with the indicated siRNAs for 72 h. Total cell extracts were subjected to western blot using the indicated antibodies. For a negative control, siRNA against DsRed protein was used. Positions of molecular weight markers are indicated on the left in kDa. (**C**) HeLa cells were treated with the indicated siRNAs for 45 h. Total cell extracts prepared from the cells were subjected to western blot using the indicated antibodies. For a negative control, siRNA against DsRed protein was used. Positions of molecular weight markers are indicated on the left in kDa. (**D**) HeLa cells grown on glass-bottomed dishes were treated with the indicated siRNAs for either 45 h (siRNAs against Tap) or 72 h (others). The cells were fixed and subjected to *in situ* hybridization using Cy3-labelled oligo-dT$_{50}$ probe.

treated cells under unstressed condition (Supplementary Figure S4C). This excludes a possibility that the nuclear foci are caused by the RNAi treatment. Moreover, we did not observe nuclear dot-like accumulation of β-actin transcripts upon siRNA treatment (Supplementary Figures S4D and E). As shown by northern blot analysis, the degree of induction of *HSP70* mRNA expression under heat stress was similar in each of the siRNA-transfected cells, whereas β-actin mRNA expression was severely impaired in Aly-depleted cells (Figure 4B, middle panel). These findings indicate that Thoc5 and Aly have a minor function in the transcription of *HSP70* mRNA and that *HSP70* mRNA retained in close proximity to the gene loci does not undergo significant degradation. A strong increase in nuclear *HSP70* mRNA levels was also observed by cell fractionation (see also Figure 4C for efficiency of fractionation procedure) followed by RT–PCR, when expression of either Thoc5 or Aly was repressed (Figure 4D upper), whereas distribution of β-actin mRNA was affected to a much lesser extent (Figure 4D lower) as expected from the results of *in situ* hybridization.

Taken together, these data indicate that Thoc5 and Aly are required for efficient nuclear export of *HSP70* mRNAs in mammalian cells.

Thoc5 and Aly bind concomitantly to the Tap-p15 heterodimer

The data indicate that Tap-p15, Thoc5 and Aly are required for nuclear export of *HSP70* mRNA in mammalian cells. Although Thoc5 and Aly bind to different domains within Tap-p15, it is possible that they bind concomitantly to the mRNA export receptor. To find out whether these different factors can assemble into a complex, Thoc5 was pre-incubated with increasing amounts of Tap-p15 and added to GST-Aly immobilized on GSH beads. When equimolar amounts of Tap-p15 and Thoc5 were added to GST–Aly, a stoichiometric complex was formed between Thoc5, Aly and Tap-p15 (Figure 5A, lanes 3 and 4). In the absence of Tap-p15, much weaker binding of Thoc5 to GST–Aly, but not GST–hHpr1 or GST, was observed (Figure 5A, lane 2; Figure 5B, lane 3, 5, 7). When a stoichiometric amount of
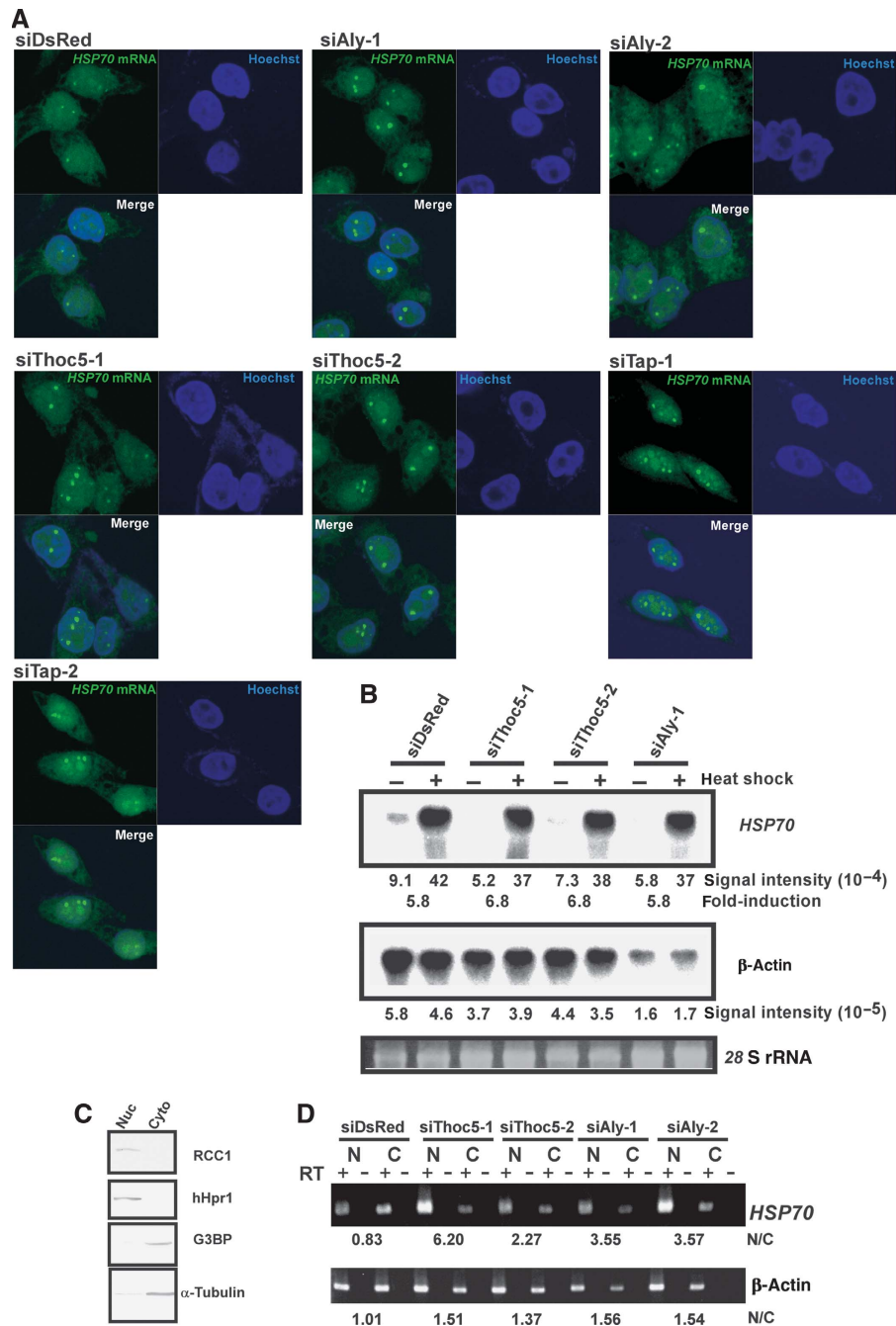
Figure 4 *HSP70* mRNA export requires both Thoc5 and Aly. (**A**) siRNAs against DsRed (siDsRed: negative control), Aly (siAly-1 and -2), Thoc5 (siThoc5-1 and -2) or Tap (siTap-1 and -2) were transfected to HeLa cells. At 48 h (for siTap-1 and -2) or 72 h (for other siRNAs) post-transfection, the cells were subjected to heat shock at 43°C for 1 h, and *in situ* hybridization using FITC-labelled human *HSP70* oligonucleotide probes was performed. (**B**) HeLa cells treated with indicated siRNAs for 72 h were left untreated (indicated by −) or temperature shifted at 43°C for 1 h (indicated by +). Total RNAs (5 μg per lane) prepared from the cells were subjected to northern blot analysis using [^{32}P]-labelled *HSP70* and β-actin cDNA probes. 28 S rRNA detected by ethidium bromide staining served as loading control. (**C**) HeLa cells were fractionated into nuclear (left) and cytoplasmic (right) fractions. Each fraction was subjected to SDS–PAGE followed by western blot using anti-RCC1 and anti-hHpr1 (nuclear markers) or anti-G3BP and anti-α-tubulin (cytoplasmic markers) antibodies. (**D**) HeLa cells treated with the indicated siRNAs for 72 h were temperature-shifted at 43°C for 1 h. Total RNA was isolated from nuclear (N) and cytoplasmic (C) fractions separated as in (C). *HSP70* and β-actin mRNAs in each fraction was detected by RT–PCR using specific primers. Relative nuclear signals (N/C) are shown at the bottom of the panels.

Thoc5 was pre-incubated with a mutant Tap-p15 that lacked a part of the N-domain of Tap (Tap188–619) and was then added to GST–Aly, neither Thoc5 nor Tap-p15 was bound to immobilized GST–Aly (Figure 5A, lane 5). The decrease in binding of Thoc5 to GST–Aly in the presence of the Tap mutant was probably due to competition between 'free' versus Tap-p15-bound Thoc5 for binding to GST–Aly. Thus, Aly and Thoc5 most probably do not have direct contact in the hetero-tetrameric complex. These data suggest that a hetero-tetrameric complex was formed, in which Thoc5 was
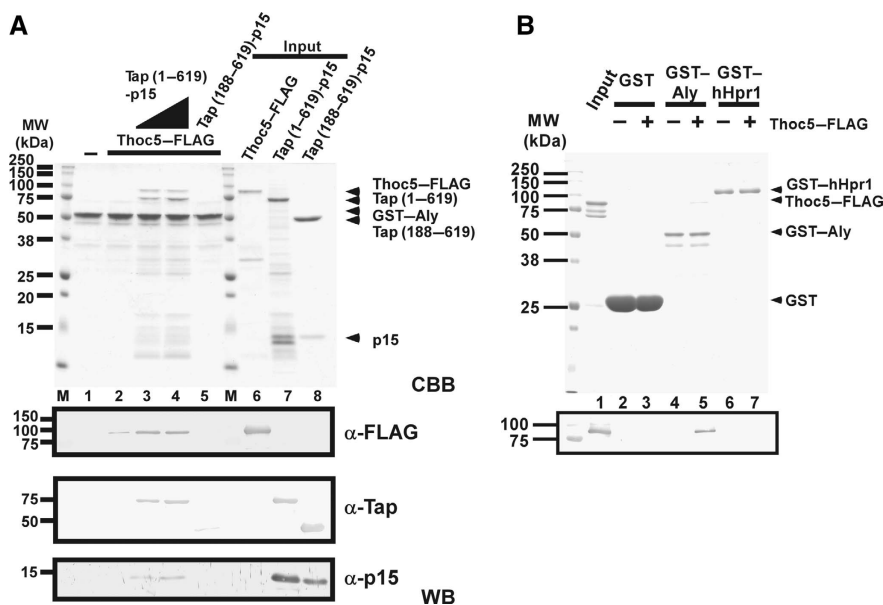
Figure 5 Tap-p15, Thoc5 and Aly constitute a hetero-tetramer. (**A**) Purified Thoc5–FLAG (25 μg) along with increasing amounts of full-length Tap-p15 (lanes 2–4; 0, 8, 16 μg) was incubated with GSH-beads pre-adsorbed to purified GST-Aly (10 μg). In lane 5, Tap (188–619)-p15 (20 μg) was used instead of full-length Tap-p15. The beads were washed and aliquots of the bound fractions (25%) were analysed by SDS–PAGE followed by CBB staining. In lane 1, buffer alone was added in binding reaction for comparison. In lanes 6–8, purified Thoc5–FLAG (1.5 μg), Tap-p15 (2 μg) and Tap (188–619)-p15 (2.5 μg) were run as markers. The bound fractions (10%) were also subjected to western blot using the indicated antibodies. Positions of molecular weight markers are indicated on the left in kDa. (**B**) Purified Thoc5–FLAG (lanes 3, 5, 7) or buffer alone (lanes 2, 4, 6) was incubated with GSH-beads pre-adsorbed to GST (lanes 2 and 3), GST–Aly (lanes 4 and 5) and GST–hHpr1 (lanes 6 and 7). The beads were washed and aliquots of the bound fractions (25%) were analysed by SDS–PAGE followed by CBB staining and western blot using anti-FLAG antibody. In lane 1, 10% of input was loaded.

bound to the M-domain and Aly to the N- and LRR-domain of Tap. Taken together, these biochemical data imply that Thoc5 and Aly can simultaneously bind to the Tap-p15 export receptor.

Thoc5, a novel adaptor RNA-binding protein, is associated with HSP70 mRNP in vivo as a component of THO complex

To find out whether Thoc5 can function as an adaptor RNA-binding protein after recruitment to the Tap-p15 mRNA export receptor, we performed RNA band shift assays. This analysis showed that Thoc5, similar to Aly, caused a robust RNA shift in the assay, showing that Thoc5 exhibits *in vitro* RNA-binding activity (Figure 6A, lanes 5–8). Competition experiments using ribo-homopolymers showed the relative affinities of Thoc5 to different RNAs. The results indicate that Thoc5 exhibits certain preference for different RNA sequences (i.e., poly(rG), poly(U), poly(rA) ≫ poly(rC)). Moreover, binding of Thoc5 to the RNA probe was inhibited by a double-stranded DNA, although less efficiently (Figure 6B).

To test the possibility that Thoc5 is associated with mRNA *in vivo*, we analysed whether *HSP70* mRNA could be co-immunoprecipitated by anti-Thoc5 antibody. To generate *HSP70* mRNA, a HeLa cell culture was heat-shocked followed by cross-linking with formaldehyde to stabilize protein–protein and protein–RNA interactions. Thoc5 was then immunoprecipitated by specific antibodies from whole cell lysates. Antibodies against Aly and an unrelated antigen (mouse IgG) were used for positive and negative controls, respectively. The co-immunoprecipitated RNA was then extracted and

subjected to RT–PCR using human *HSP70*-specific primer sets. *HSP70* mRNA was specifically immunoprecipitated with either anti-Thoc5 or anti-Aly antibodies, but not with control antibodies (Figure 6C, lanes 3, 5, 7, 12, 14, 16). These data indicate that Thoc5, similar to Aly, is associated with *HSP70* mRNA.

To further explore the nature of the *HSP70* mRNP, antibodies against different factors were used in immunoprecipitation. Under heat-shock condition, the THO complex remained stable and the components were efficiently co-immunoprecipitated by the different anti-THO antibodies (Supplementary Figure S3C; see also Figures S3A and B for specificity of the antibodies). *HSP70* mRNA was also co-immunoprecipitated by these antibodies (Figure 6D, lanes 5, 7, 9) as well as by the antibody against Tap (Figure 6D, lane 15). Strikingly, Thoc5-containing *HSP70* mRNP could be re-immunoprecipitated by anti-Tap, anti-Thoc2 and anti-Thoc7 antibodies (Figure 6E, lanes 5, 7, 9). These data strongly suggest that *HSP70* mRNA forms a complex with a novel adaptor Thoc5 and the exporter Tap-p15 *in vivo* and that Thoc5 in *HSP70* mRNP is a part of stable THO complex. In good agreement with the latter conclusion, depletion of Thoc6, another metazoan-specific component of the THO complex, by siRNA, also induced modest, but still significant, nuclear accumulation of *HSP70* mRNA (Figure 7C; see also Supplementary Figure S6) without affecting the expression of the other THO components, including Thoc5 (Figure 7A) and cellular localization of bulk poly(A)$^+$ RNAs (Figure 7B). Taken together, these data support a model that Thoc5 has an adaptor function as a component of stable THO complex.
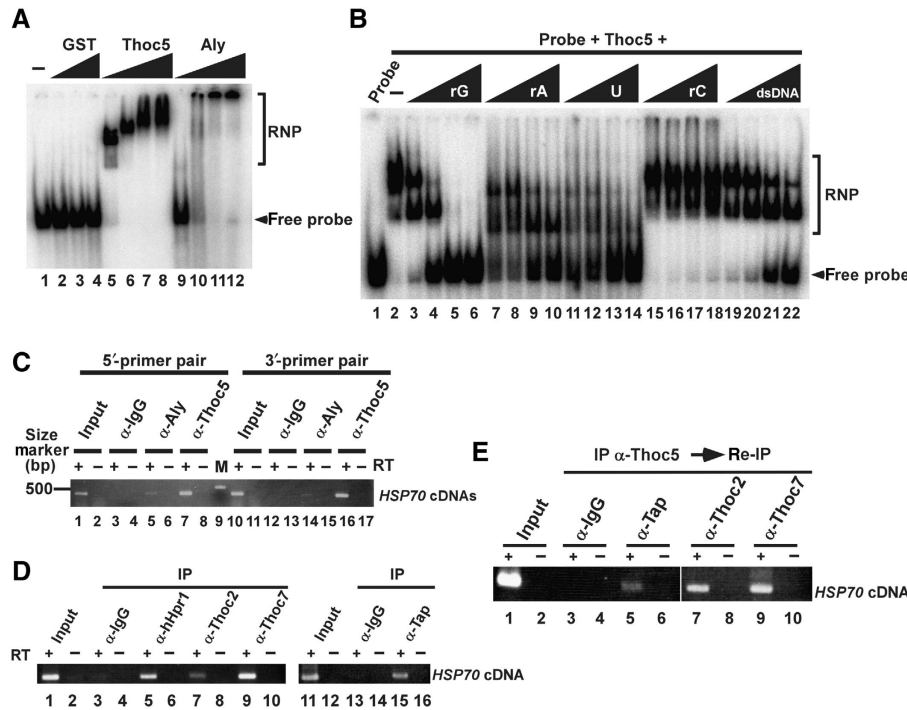
Figure 6 Thoc5 and Aly are components of the *HSP70* mRNP. (**A**) RNA-binding assay was performed using a 91 bp [^{32}P]-labelled RNA encoding pBluescript SK polylinker sequence. Increasing amounts of purified recombinant GST (lanes 2–4: 160, 320, 480 pmol), Thoc5–FLAG (lanes 5–8: 2, 4, 12, 20 pmol) and GST–Aly (lanes 9–12: 0.3, 0.6, 2, 3 pmol) proteins were added to total 10 µl of each binding reaction. Probe alone was run in lane 1. The binding reactions were separated by 6% polyacrylamide gel electrophoresis and visualized by autoradiography. A bracket indicates the positions of the protein–RNA complexes. (**B**) Competition assay of the RNA-binding activity of Thoc5 was performed using increasing amounts (10, 25, 100, 300 ng each) of ribo-homopolymers (lanes 3–18) and a dsDNA (lanes 19–22) as competitors. (**C**) Heat-shocked HeLa cells were subjected to RNA co-immunoprecipitation assay. Co-precipitated RNAs were then subjected to PCR using the indicated primer pairs. RNA isolated from the crude cell extract was also subjected to RT–PCR (lanes 1, 2, 10 and 11; input). In lanes indicated by RT, reverse transcriptase was omitted from the first-strand synthesis reactions. Amplified cDNA fragments were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. DNA size markers were run on lane 9 (indicated by M) and the position of the 500 bp marker is indicated on the left. (**D**) Heat-shocked HeLa cells were subjected to RNA co-immunoprecipitation assay using the indicated antibodies as in (C). (**E**) Heat-shocked HeLa cells were subjected to RNA co-immunoprecipitation assay using anti-Thoc5 antibody. mRNPs released from the anti-Thoc5 beads were re-immunoprecipitated using the indicated antibodies. Co-precipitated RNAs were analysed as in (C).

Discussion

Genetic and biochemical studies have linked the subunits of the TREX complex to the mRNA export machinery in yeast and metazoans (Stutz *et al*, 2000; Zhou *et al*, 2000; Luo *et al*, 2001; Rodrigues *et al*, 2001; Jimeno *et al*, 2002; Strasser *et al*, 2002; Rehwinkel *et al*, 2004; Masuda *et al*, 2005). However, the specific function(s) of the individual TREX components have remained largely unknown. Our results indicate that Thoc5, a subunit of the metazoan TREX complex with no apparent orthologue in yeast, can directly bind to the nuclear export receptor Tap-p15. Notably, Thoc5 can interact with Tap-p15 even in the presence of another bound adaptor protein Aly due to the presence of non-overlapping binding sites on the export receptor. *In vivo*, Thoc5 is not required for bulk mRNA export, but nuclear export of a specific mRNP (*HSP70)* depends on Thoc5, which also involves the general adaptor protein Aly and the exporter Tap. Despite the fact that Thoc5 does not exhibit known RNA-binding motifs, it can bind to RNA *in vitro*. Our data also suggest that Thoc5 is involved in *HSP70* mRNP during nuclear export as a component of stable THO complex. Thus, Thoc5 is a novel adaptor RNA-binding protein in mammalian cells that, in concert with the other adaptor Aly, functions in Tap-p15-mediated export of a specific mRNA.

To date, only the N- and LRR-domains of human Tap (residues 1–371) have been described as binding sites for adaptor proteins involved in mRNA export (Stutz *et al*, 2000; Fribourg *et al*, 2001; Huang *et al*, 2003). However, the yeast mRNA export receptor Mex67-Mtr2 can interact with pre-ribosomal particles or with a nucleoporin (Nup85) through a loop-confined surface on the Ntf2-like domain of Mex67 (Yao *et al*, 2007, 2008). Our studies identified a related surface on the Tap-p15 heterodimer, although with shorter loops, which is opposite the FG-repeat binding domain (Figure 1D) and is involved in binding to Thoc5 and possibly other targets. As suggested for ribosomal subunit export by the Mex67-Mtr2 heterodimer (Yao *et al*, 2007), Tap-p15 could simultaneously bind to FG-nucleoporins and Thoc5 through different surfaces on the Ntf2-like fold, which could be crucial for nuclear export of *HSP70* mRNA. It has been shown that p15 is required for maintenance of the structure of the M-domain (Fribourg *et al*, 2001). Our *in vitro* binding data using Tap monomer and Tap point mutants complexed with p15 also suggest that the structural integrity of the M-domain, but not the presence of p15 *per se*, is important for Thoc5 binding, although we cannot exclude a possibility that p15 also physically interacts with Thoc5.

Through homology modeling, we noted that the N-terminal domain of Thoc5, which is predicted to be rich in
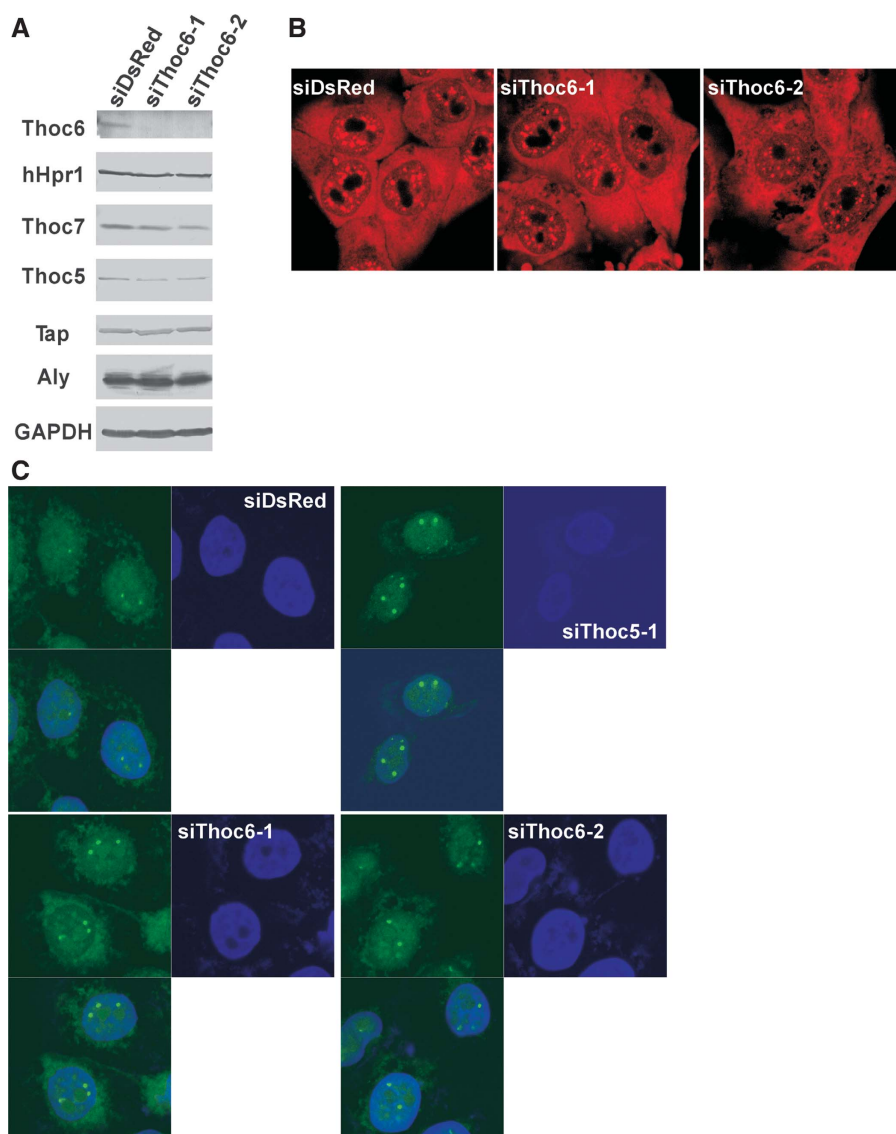
Figure 7 Depletion of Thoc6 also blocks nuclear export of *HSP70* mRNA. (**A**) HeLa cells were treated with the indicated siRNAs for 72 h. Total cell extracts prepared from the cells were subjected to western blot using the indicated antibodies. For a negative control, siRNA against DsRed protein was used. Positions of molecular weight markers are indicated on the left in kDa. (**B**) HeLa cells grown on glass-bottomed dishes were treated with the indicated siRNAs for 72 h. The cells were fixed and subjected to *in situ* hybridization using Cy3-labelled oligo-dT$_{50}$ probe. (**C**) HeLa cells grown on glass-bottomed dishes were treated with the indicated siRNAs against Thoc6 for 72 h. The cells were fixed and subjected to *in situ* hybridization using the FITC-labelled *HSP70* oligonucleotide probes. Cells treated with siThoc5-1 (upper right panel) are shown for comparison of the size of the nuclear foci.

α-helices, could adopt an α-solenoid fold. It was suggested previously that an elongated Nup85 molecule, which also has a series of predicted antiparallel α-helices that could form an α-solenoid (Devos *et al*, 2006), binds through its longitudinal axis to an extended surface on the Mex67-Mtr2 heterodimer (Yao *et al*, 2008). Thus, it is possible that Thoc5 binds in a similar way to a surface on the Tap-p15 heterodimer that could correspond to the loop-defined surface on the Mex67-Mtr2 export receptor.

In some strains of yeast, heat shock rapidly induces inhibition of nuclear export of bulk mRNA (Saavedra *et al*, 1996). In metazoans, heat shock attenuates the expression of non-heat-shock genes, mainly by blocking their splicing and changing the mode of translation (Storti *et al*, 1980; Yost and Lindquist, 1986; Bond, 1988; Shukla *et al*, 1990; Joshi-Barve *et al*, 1992).

Consistent with this observation, most of the heat-shock mRNAs are devoid of introns, which could enable them to by-pass the splicing-dependent inhibition of mRNA export under heat shock (Yost and Lindquist, 1986). On the other hand, the mammalian TREX complex has been reported to be recruited to pre-mRNAs during splicing (Cheng *et al*, 2006). Moreover, TREX subunits are located in splicing factor-rich nuclear speckles and co-enriched with spliceosomes during purification (Zhou *et al*, 2000; Masuda *et al*, 2005; Chen *et al*, 2007; Merz *et al*, 2007). However, our data have showed that both the human THO complex and Aly are associated with naturally intron-less *HSP70* mRNA and hence could have an important function in transcription-dependent loading of the mRNA export machinery to these nascent transcripts (Nojima *et al*, 2007; Yoh *et al*, 2007; Taniguchi and Ohno, 2008).

Another unexpected finding of this study is that nuclear export of *HSP70* mRNA in mammalian cells strictly depends on Aly, but its requirement for bulk poly(A)$^+$ RNA is less pronounced. This finding is similar to the earlier conclusions made in different organisms (Gatfield and Izaurralde, 2002; Longman *et al*, 2003) and may indicate that relative requirement of certain adaptor proteins in nuclear export of different mRNA may differ in higher eukaryotes. In addition to Aly and Yra1, the nucleo-cytoplasmic shuttling Ser/Arg-rich (SR) proteins, such as mammalian 9G8 and SRP20 and yeast Npl3, have been shown to interact with Tap-p15 or Mex67-Mtr2, and thus could function as alternative adaptors (Huang *et al*, 2003, 2004; Gilbert and Guthrie, 2004). Although the relationship between the SR proteins and the TREX complex in metazoans is unknown, in yeast it is thought that the SR-like proteins Gbp2 and Hrb1 are recruited to nascent mRNAs by their physical interaction with the TREX complex (Hurt *et al*, 2004). Thus, it would be interesting to analyse the relationship between Thoc5 and SR proteins, which interact with the N-terminal adaptor binding site on Tap (Huang *et al*, 2004), for nuclear export of different classes of mRNAs.

Another surprising observation in this study was that *HSP70* mRNA requires the co-adaptor Thoc5 for efficient nuclear export. There are several explanations for this observation. One possibility is that due to the absence of introns, *HSP70* mRNA requires a specific adaptor RNA-binding protein for efficient export, which can be recruited by a splicing-independent (e.g., transcription-dependent) mechanism. Another scenario could be that efficient recruitment and stable binding of Aly to *HSP70* mRNA require a second adaptor protein, such as Thoc5, that could stabilize the export receptor-cargo complex under heat shock, which may tend to destabilize protein complexes (e.g., Shukla *et al*, 1990). Finally, in light of the recent findings that transcription and translation can be coupled in eukaryotic cells (Hampsey and Kinzy, 2007; Marr II *et al.*, 2007; Rother and Strasser, 2007), it is tempting to speculate that Thoc5 and/or components of the THO complex could have an additional function in *HSP70* gene expression. The *HSP70* mRNA is translated by a cap-independent mechanism due to an IRES (-like) activity within the 5′-untranslated region in higher eukaryotes (Klemenz *et al*, 1985; McGarry and Lindquist, 1985; Hultmark *et al*, 1986; Joshi and Nguyen, 1995; Hernandez *et al*, 2004). Thus, the recruitment of the TREX complex to the 5′-part of the mRNA could be used not only for nuclear export (Cheng *et al*, 2006), but also for subsequent steps in gene expression, including translation. Genome-wide identification of mRNA species exported through TREX-dependent pathway in mammalian cells will be one of the most important subject of future investigations to further understand the biological functions of the export pathway.

Materials and methods

Reagents
Antibodies against Aly (Abcam), GAPDH (Ambion), hHpr1/Thoc1 (GeneTex), Thoc6 (Abnova), splicing factors (SC35, Sigma), G3BP (BD Biosciences Pharmingen), GFP (Molecular Probes), α-tubulin (Sigma), FLAG peptide tag (Sigma) and mouse IgG (Zymed Laboratories) were commercially acquired. A mouse monoclonal antibody against hnRNP C was a gift from Dr G Dreyfuss. A rabbit polyclonal anti-Thoc5 antibody was raised against the full-length

N-terminally His-tagged Thoc5 protein. Rabbit polyclonal antibodies against Thoc2 and Thoc7 were raised against GST-fusion proteins. Rabbit polyclonal antibodies against Tap and p15 have been described (Katahira *et al*, 1999).

Plasmids
The construction of *E. coli* expression plasmids for GST–Tap (188–619), GST–Tap (96–371), GST–Tap (188–550) and untagged p15 (pET9d-p15) has been described elsewhere (Katahira *et al*, 1999, 2002, 2007). A two-hybrid prey plasmid (pACT2-hCG1) has been reported (Katahira *et al*, 1999). Construction of the human Tap prey plasmids is described in the supplementary data. An *E. coli* expression vector for GST–Aly was a gift from Dr Mutsuhito Ohno (Kyoto University) (Taniguchi and Ohno, 2008). Details of construction of the expression vectors for Thoc5, Aly, hHpr1, Thoc2, Thoc7 and various fragments of Tap are given in the Supplementary data. Point mutations were introduced by the Quick Change kit (Stratagene).

Yeast two-hybrid screening
The methods of yeast two-hybrid screening and *in vivo* β-galactosidase plate assay have been described previously (Katahira *et al*, 1999, 2007).

Protein expression and purification
Recombinant proteins were expressed in *E. coli* BL21(DE3) CodonPlus strain (Stratagene). Expression and purification of GST- or $6\times$ His-fusion proteins were performed as described previously (Katahira *et al*, 1999, 2002, 2007; Taniguchi and Ohno, 2008). Untagged full-length Tap-p15 and Tap (188–619)-p15 were prepared by on-column cleavage of GST-tag by PreScission Protease (GE Healthcare).

Cell culture, transfection and establishment of stable cell lines
HeLa and L929 cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 atmosphere. Transfection of plasmid DNAs was performed by the Effectene Transfection Reagent Kit according to the manufacturer's protocol (Qiagen). L929 cell lines stably expressing Thoc5–GFP and hHpr1–GFP fusion proteins were established as reported previously (Zhou *et al*, 2000).

siRNA treatment
Transfection was performed using the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. Double-stranded siRNAs were synthesized by Eurogentec through NIPPON EGT. The sequences of siRNAs are given in the Supplementary data. At 72 h after the initial transfection, the cells were harvested for western and northern blot analysis or subjected to *in situ* hybridization. We found that siRNA-mediated depletion of Tap impaired cell viability more severely than that of Thoc5 or Aly, and Tap-depleted cells detached from culture dishes almost completely at 72 h post-transfection. Subsequent experiments with Tap-depleted cells were therefore done at an earlier time point (45–48 h post-transfection), when 70–80% of the cells were still viable.

In situ *hybridization*
In situ hybridization was performed by the method of Singer *et al*. (http://www.singerlab.org/protocols). Anti-sense oligodeoxynucleotides internally labelled with FITC were synthesized and purified by JBIOS Co. Ltd. The sequences of each oligodeoxynucleotide are indicated in the Supplementary data.

RNA co-immunoprecipitation assay
A protocol for chromatin immunoprecipitation for mammalian cells (Horie-Inoue *et al*, 2004) was adapted for RNA co-immunoprecipitation assay. The detailed procedure is described in the Supplementary data.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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