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Necessary and sufficient factors for the import of transfer RNA into the kinetoplast mitochondrion

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The mechanism of active transport of transfer RNA (tRNA) across membranes is largely unknown. Factors mediating the import of tRNA into the kinetoplast mitochondrion of the protozoon *Leishmania tropica* are organized into a multiprotein RNA import complex (RIC) at the inner membrane. Here, we present the complete characterization of the identities and functions of the subunits of this complex. The complex contains three mitochondrion- and eight nuclear-encoded subunits; six of the latter are necessary and sufficient for import. Antisense-mediated knockdown of essential subunits resulted in the depletion of mitochondrial tRNAs and inhibition of organellar translation. Functional complexes were reconstituted with recombinant subunits expressed in *Escherichia coli*. Several essential RIC subunits are identical to specific subunits of respiratory complexes. These findings provide new information on the evolution of tRNA import and the foundation for detailed structural and mechanistic studies.

INTRODUCTION

The import of transfer RNAs into mitochondria has been documented in a large number of species across the phylogenetic scale (for reviews, see Entelis *et al*, 2001; Bhattacharyya & Adhya, 2004). This process supplies cytosolic tRNAs to replace mutated or deleted organelle-encoded tRNAs for translation of mitochondrial messenger RNAs. However, the mechanism of import seems to have evolved independently in different organisms, resulting in differences in the number and identity of tRNA species that are imported.

Some import factors have been identified in different species. In budding yeast, a single tRNA^{Lys} isoform is imported into the mitochondria; two soluble proteins—the precursor of the mitochondrially imported isoform of lysyl tRNA synthetase (pre-MSK; Tarassov *et al*, 1995a) and enolase (Entelis *et al*, 2006)—are required for this process. Mutational studies also suggested the

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involvement of components TOM20 (translocase outer membrane 20) and TIM44 (translocase inner membrane 44) of the mitochondrial protein import pore (Tarassov *et al*, 1995b). In higher plants, different combinations of tRNAs are imported in different species. Recent studies identified the voltage-dependent anion channel (VDAC), TOM20 and TOM40 as tRNA-binding proteins required for the import of tRNA into potato mitochondria *in vitro* (Salinas *et al*, 2006). In the kinetoplastid protozoa, which includes the disease-causing *Leishmania* and *Trypanosoma* species, more than 24 tRNA species are imported owing to the complete lack of mitochondrial tRNA genes. Early work indicated the *in vitro* requirement of a small RNA-binding protein on the outer mitochondrial membrane of *Leishmania* (Adhya *et al*, 1997), but this protein remains unidentified.

We have previously purified a multiprotein complex (RNA import complex (RIC)) with a molecular mass of approximately 600,000 from the inner mitochondrial membrane of *Leishmania tropica*, which is functional for the import of tRNA into phospholipid vesicles and human mitochondria *in vitro*, as well as in the mitochondria of intact human cells (Bhattacharyya *et al*, 2003; Mahata *et al*, 2005, 2006). This complex is composed of several import components; two such components have been identified so far: RIC1 and RIC8A are import receptors that bind to distinct subsets of tRNAs (Chatterjee *et al*, 2006; Goswami *et al*, 2006). Here, we have attempted to characterize the identities of the remaining subunits of the RIC by using a combination of biochemical and genetic approaches.

RESULTS AND DISCUSSION

In kinetoplastid protozoa four macromolecular complexes of the oxidative phosphorylation pathway have been observed by native gel electrophoresis (Horvath *et al*, 2000a,b; Goswami *et al*, 2006). On the basis of protein sequencing and enzymatic analyses, and our radiolabelling and western blot data (Figs 1B,2C,D), three of these have been shown to correspond to complexes V (F$_1$F$_o$-ATP synthase), IV (cytochrome *c* oxidase) and III (ubiquinol cytochrome *c* reductase; UCR). Complex I (NADH dehydrogenase) is absent from these species, although some of the subunits, such as ND1 and ND7, are detectable in mitochondrial extracts (data not shown). We have presented evidence previously that the fourth, and largest complex in *L. tropica* is identical to the RIC (Chatterjee *et al*, 2006; Goswami *et al*, 2006).
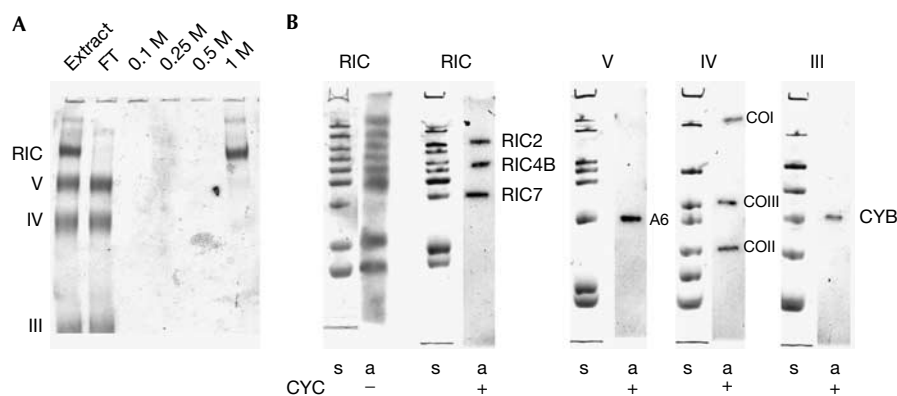
Fig 1 | Radiolabelling of RNA import complex subunits. (A) Binding of RIC to a transfer RNA import signal. A *Leishmania tropica* mitochondrial extract was chromatographed on a tRNA^{Tyr} D arm affinity column. The column was washed with buffer DB containing the indicated concentrations of KCl. Each fraction was concentrated and run on native BN-PAGE. Mitochondrial complexes are indicated on the left. **(B)** Inner membrane complexes, labelled with [^{35}S]methionine in the absence (first two lanes from the left; −) or presence (+) of cycloheximide (CYC) and resolved by BN-PAGE were excised, subjected to SDS–polyacrylamide gel electrophoresis and fluorographed. Mitochondrion-encoded subunits are indicated. a, autoradiograph; BN-PAGE, blue native-polyacrylamide gel electrophoresis; CO, cytochrome oxidase; CYB, cytochrome *b*; FT, flow-through; RIC, RNA import complex; s, Coomassie stain.

The solubilized mixture of complexes was chromatographed on a column containing, as immobilized ligand, an oligoribonucleotide corresponding to the D arm of *Leishmania* tRNA^{Tyr} containing an import signal (Bhattacharyya *et al*, 2000). Complexes III, IV and V flowed through the column, whereas the retained RIC was eluted at a high salt concentration (Fig 1A). The specific affinity of this complex for tRNA provides confirmation of its identity.

The starting point for determining the identities of the subunits of the RIC was the sequencing of the bands of the affinity-purified complex by mass spectrometry. Each band yielded several tryptic peptides that matched open reading frames (ORFs) in the *L. major* database. A total of 122 nuclear-encoded ORFs were recovered from 9 bands (supplementary Table S1 online). Candidate proteins were selected for further study on the basis of their molecular masses, which were close to the experimental value, and high or maximum peptide coverage (supplementary information online).

To detect any mitochondrion-encoded components of the RIC, the resistance of organellar, but not cytosolic, protein synthesis to cycloheximide was used. Mitochondria were isolated from cycloheximide-treated cells and inner membrane complexes were resolved by blue native-polyacrylamide gel electrophoresis (BN-PAGE). The individual bands were excised and resolved into their constituent subunits by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and labelled species were detected by autoradiography. This experiment showed the presence of known mitochondrion-encoded subunits in complexes III, IV and V. Three bands of the RIC (bands 2, 4 and 7) were similarly labelled (Fig 1B), indicating the presence of multiple mitochondrion-encoded subunits.

An antibody against each candidate protein was used to probe western blots of mitochondrial complexes resolved by BN-PAGE (first-dimension blots). Antibodies against proteins encoded by the nuclear genes *LmjF05.0510* (RIC1; Goswami *et al*, 2006), *LmjF35.0100* (RIC8A; Chatterjee *et al*, 2006) and *LmjF21.0340*, *LmjF07.1110*, *LmjF12.0670*, *LmjF35.1540*, *LmjF04.0630* and *LmjF21.1710* (Fig 2A) all reacted with the RIC. Antibodies against mitochondrion-encoded maxicircle unidentified reading frame 1 (MURF1) and MURF2 similarly reacted with the RIC, whereas NADH dehydrogenase subunit 1 (ND1) and ND7 antibodies did not (Fig 2A).

All antibodies except one that reacted with the RIC on first-dimension blots, also reacted with a single species on the second-dimension blots (Fig 2B), thus identifying the principal or sole component of each band (Table 1). The exception was anti-MURF2, which gave two bands at positions corresponding to RIC4 and RIC7, respectively. The same two species were detected in total or mitochondrial cellular extracts (data not shown). The larger protein corresponds to the full-length MURF2 of 355 aa residues (Simpson *et al*, 1998). No other gene homologous to *MURF2* is present in the mitochondrial genome; the smaller species is probably derived from MURF2 by internal initiation or proteolytic processing. Thus, band 4 contains two proteins: one nuclear-encoded and the other mitochondrion-encoded (RIC4A and RIC4B, respectively). Similarly, band 8 contains two nuclear-encoded proteins (RIC8A and RIC8B) of nearly identical size that are distinguishable by immunochemistry. By contrast, antibodies against other candidate nuclear-encoded proteins such as the F$_1$-ATP synthase γ-subunit (*LmjF21.1770*), ribonucleoprotein p18 (*LmjF15.0280*), succinate dehydrogenase FeS protein (*LmjF15.0990*) and cytochrome oxidase subunit VII (*LmjF25.1130*) were not detected to localize to any RIC band (Fig 2B).

Of the ten antibodies that reacted with the RIC on the western blots, four—including the two against mitochondrion-encoded proteins—were specific to this complex, whereas each of the remaining six—all nuclear-encoded—decorated a second mitochondrial complex. These included RIC1/F1α (Goswami *et al*, 2006), RIC8A/UCR6b (Chatterjee *et al*, 2006), RIC3, RIC5, RIC6 and RIC9 (Fig 2A). The last four proteins—RIC1, RIC6, RIC8A and RIC9—are structurally homologous to known respiratory components (Chatterjee *et al*, 2006; Goswami *et al*, 2006; supplementary Figs S1–S4 online). RIC5 (*LmjF12.0670*) is a
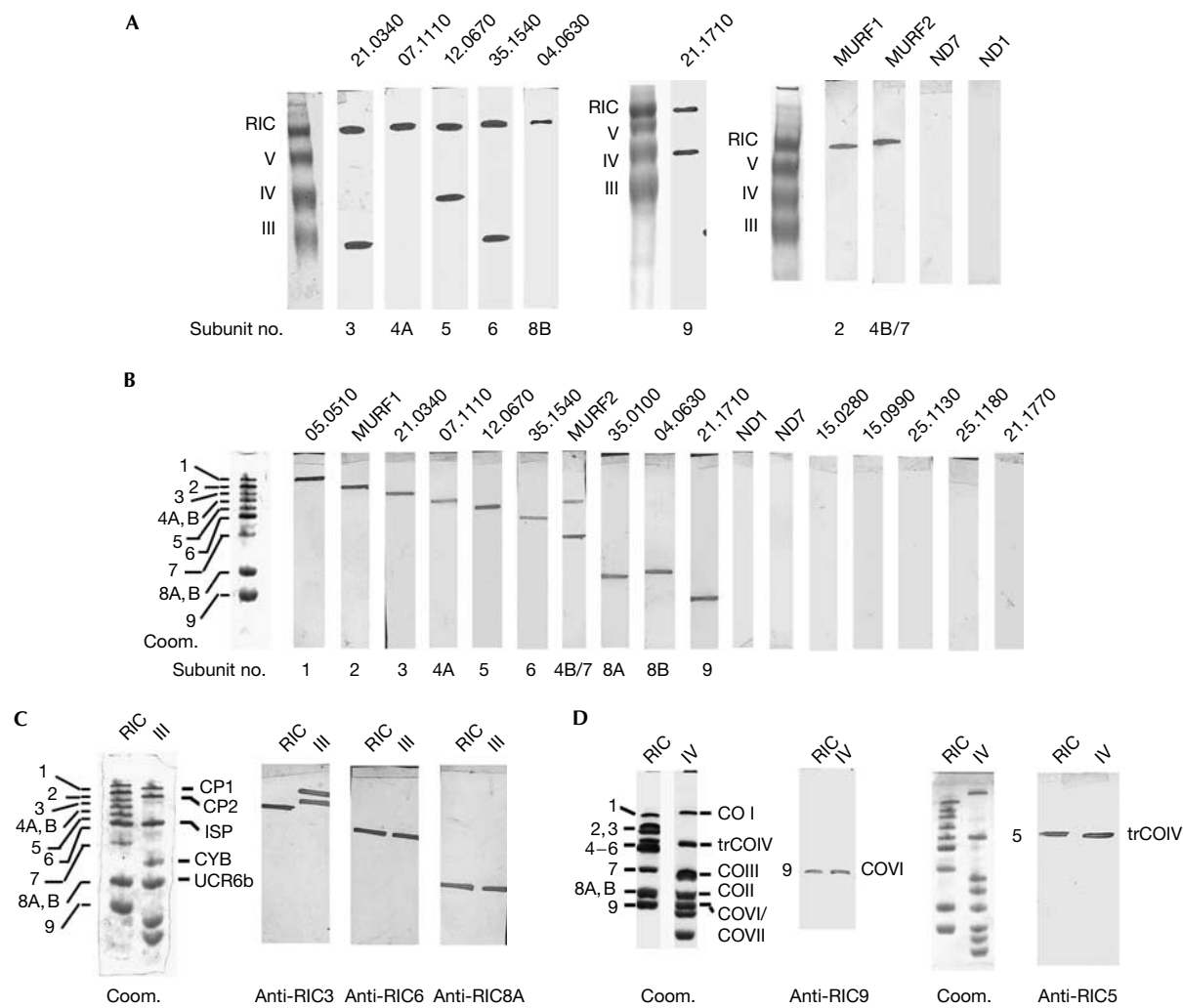
Fig 2 | Identification of RNA import complex. (**A**) First-dimension blots of complexes were probed with antibodies against recombinant proteins encoded by the indicated genes. Each six-digit number is the nuclear-encoded gene ID in the *Leishmania major* sequence database (see Table 1 and supplementary Table S1 online); the others (MURF1, MURF2, ND1 and ND7) are mitochondrion-encoded. (**B**) Two-dimensional blots of RIC probed with the indicated antibodies. Subunit numbers are indicated alongside the Coomassie-stained profile on the left. (**C,D**) Comparative two-dimensional blots of the RIC and complex III (**C**) and of the RIC and complex IV (**D**) probed with antibodies against the indicated RIC subunits. Individual subunits of complexes III, IV and V were identified by western blotting and radiolabelling. CO, cytochrome oxidase; Coom., Coomassie stain; CP, core protein; CYB, cytochrome *b*; ISP, iron sulphur protein; MURF, maxicircle unidentified reading frame; ND, NADH dehydrogenase subunit; RIC, RNA import complex; trCOIV, trypanosome cytochrome oxidase subunit IV; UCR6b, ubiquinol cytochrome *c* reductase subunit 6b.

trypanosomatid-specific protein, termed trCOIV (Maslov *et al*, 2002), which is shared between the RIC and complex IV (Fig 2A,D). These proteins are encoded by single genes (subunits 5, 6, 8A and 9) or by two identical copies of the same gene (subunit 1; Table 1), and are therefore likely to be bifunctional. The sixth protein, encoded by *LmjF21.0340* (RIC3, 467 aa residues), is a member of the M16 family of metalloproteases that include mitochondrial processing peptidase (MPP), and subunits CP1 and CP2 of complex III. An antibody against RIC3 crossreacted with four proteins in total promastigote extracts (Fig 3B); of these, CP1 and CP2 are present in complex III, whereas RIC3 is specific to the import complex (Fig 2C).

The subunit stoichiometry was determined by quantification of Coomassie-stained bands and of ^{35}S-labelled subunits in the absence and presence of cycloheximide (Table 1; supplementary Table S2 online). The RIC is composed of 16–17 polypeptides with a total estimated mass of 580 kDa, which is close to previous estimates of approximately 600 kDa on the basis of sedimentation and electrophoretic mobility (Bhattacharyya *et al*, 2003).

To determine the role, if any, of the detected RIC components in import *in vivo*, it was necessary to knock down each one by using inducible antisense RNA (Goswami *et al*, 2006). This resulted in efficient and specific depletion of the targeted mRNA and protein (Fig 3A,B). On induction of antisense RNA corresponding to RIC3 (*LmjF21.0340*), out of the four M16 metalloprotease family members with sequence similarities and immunological cross-reactivity, only the target protein was depleted (Fig 3B), attesting to the specificity of the targeting procedure.

Table 1 | Identities of RNA import complex subunits

Subunit no.	Size (kDa)	Mole ratio*	Gene identity‡	Gene copy no.	Sequence/structural similarity with known proteins§	Complex with which shared	Role in import
1	62	1	*LmjF05.0510*	2	F_1-ATP synthase subunit α	V	Essential; type I tRNA receptor; tRNA-dependent ATPase; type II tRNA activator
2	56	2	**MURF1**	1	None	None	Not essential[#]
3	47	1	*LmjF21.0340*	1[‖]	Core proteins (complex III)	None[¶]	Not essential
4A	43	1	*LmjF07.1110*	1	None	None	Essential
4B	43	0.5	**MURF2**	1	None	None	Not essential[#]
5	40	1	*LmjF12.0670*	1	None	IV	Not essential
6	35	3	*LmjF35.1540*	1	Complex III iron-sulphur protein	III	Essential
7	28	1	**MURF2**	1	None	None	Not essential[#]
8A	21	2	*LmjF35.0100*	1	Complex III subunit 6b	III	Essential; type II tRNA receptor; negative regulator of type I tRNA
8B	21	1	*LmjF04.0630*	1	None	None	Essential
9	19	3	***LmjF21.1710***	1	Complex IV subunit VI	IV	Essential

*Stoichiometry determination described in the supplementary information online. ‡Nuclear-encoded genes (italics) designated as in the *Leisgmania major* database; mitochondrion-encoded genes (bold italics) as in the kinteoplastid U-insertion/deletion editing database. §BLAST and SWISS MODEL. ‖Multigene family of M16 metalloproteases. ¶Other homologues in complex III. #By *in vitro* import assay. MURF, maxicircle unidentified reading frame.

Knockdown of RIC1 (Goswami *et al*, 2006), RIC8A (Chatterjee *et al*, 2006) and RIC4A, RIC6, RIC8B and RIC9 (Fig 3C) caused a cessation of growth within 24–48 h, with rounding off of the spindle-shaped cells and loss of viability. By contrast, depletion of RIC3 and RIC5 led to only a marginal reduction in growth rate (Fig 3C). Knockdown of all essential subunits produced severe respiratory defects, for example, more than 90% of RIC9-knockdown cells became negative for cytochrome *c* oxidase, and oxygen consumption was reduced from 0.96 to 0.23 fmol/min/cell at 22 °C.

The effect of knockdown on the contents of several mitochondrial tRNA species was monitored. Depletion of subunits 1 (Goswami *et al*, 2006) and 8A (Chatterjee *et al*, 2006) leads to the loss of mitochondrial tRNAs, whereas knockdown of the F1β subunit, which is not a part of the complex (Fig 2B), does not (Goswami *et al*, 2006). Loss of subunits 4A, 6, 8B and 9 resulted in the depletion of mitochondrial tRNAs; cytosolic tRNA levels were unaltered (Fig 4A). By contrast, knockdown of RIC3 or RIC5 had no marked effect on the mitochondrial tRNA pool (Fig 4A).

There was a general inhibition of translation of mitochondrial mRNAs on depletion of the essential subunits; cytosolic translation was unaffected (Fig 4B). Depletion of the non-essential subunits (RIC3 and RIC5) had no marked effect on protein synthesis in either compartment.

To determine whether the identified factors were sufficient for import, *in vitro* reconstitution was attempted using purified proteins expressed in *Escherichia coli*. Initially, the eight nuclear-encoded subunits expressed in *E. coli* were separately re-folded, then mixed together to allow assembly to occur. A major band (R8) of approximately 500 kDa was observed by using BN-PAGE (Fig 5A). ATP-dependent tRNA import directed by the assembled complex into liposomes was observed (Fig 5B). Furthermore, import of tRNA^Tyr, a type I tRNA, was inhibited by low concentrations of tRNA^Ile, a type II tRNA (Fig 5B). Such an antagonistic action of type II tRNAs on type I tRNA import

has been previously observed (Bhattacharyya *et al*, 2002, 2003). Therefore, the reconstituted complex has the regulatory properties of native RIC. Furthermore, the specific activity of liposome-bound R8 was approximately 75% that of the RIC in intact mitochondria (Fig 5C; supplementary information online).

Next, complexes lacking individual recombinant subunits were assembled. Omission of RIC1, RIC4A, RIC6, RIC8B and RIC9 resulted in the abolition of import of type I tRNA^Tyr; in the absence of RIC3 and RIC5 there was a small reduction in activity, whereas lack of RIC8A led to a slight stimulation (Fig 5D). In the presence of the type II tRNA^Ile effector, import by the complete, as well as RIC3- and RIC5-deficient complexes, was inhibited, but import by the RIC8A-deficient complex persisted, indicating the loss of allosteric inhibition as previously reported (Chatterjee *et al*, 2006). tRNA^Ile alone was not imported by any of the complexes, but import was induced by the type I tRNA^Tyr effector in complete as well as RIC3- and RIC5-deficient complexes; tRNA^Tyr was ineffective in the absence of RIC1, which positively regulates type II import, or RIC8A, the type II receptor (Fig 5D).

This experiment confirmed the *in vivo* observations showing the requirement of RIC1, RIC4A, RIC6, RIC8A, RIC8B and RIC9. Furthermore, a complex reconstituted with only these six subunits (R6; Fig 5A) was functional *in vitro* (Fig 5B), showing them to be sufficient for tRNA import. The import efficiencies of R6 and R8 were nearly identical (Fig 5B).

Our previous work identified RIC1 and RIC8A as allosterically interacting receptors for distinct tRNA subsets (Chatterjee *et al*, 2006; Goswami *et al*, 2006). The current work defines the essential subunits and provides leads for future investigations into their individual roles in import.

A significant number of multifunctional, 'moonlighting', proteins have been recently identified in many systems (for a review, see Jeffery, 2003). The convergence of so many respiratory proteins
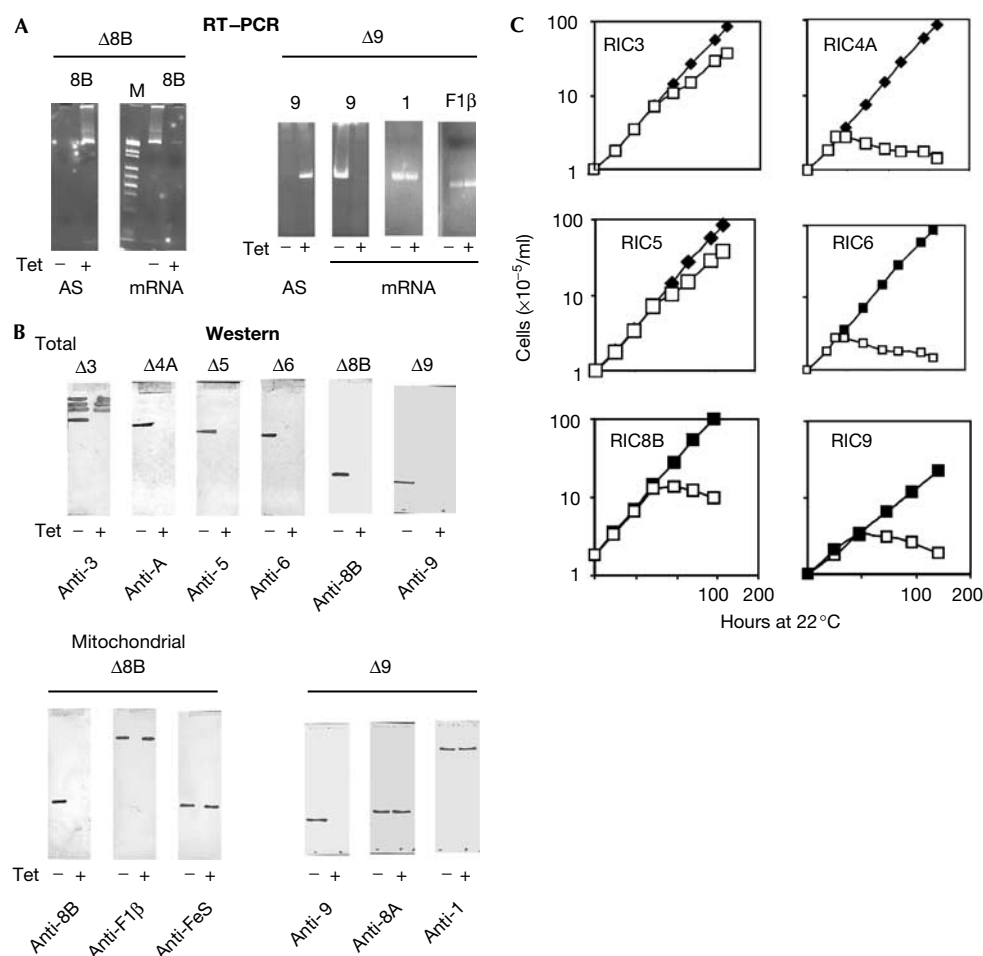
Fig 3 | Knockdown of RNA import complex subunits. *Leishmania tropica* 13-90 hosts were transfected with the appropriate antisense vector, and transformed clones were cultured in the absence (−) or presence (+) of tetracycline. Each knockdown line is represented as Δx, where x is the RIC subunit number. In (A) and (B), tetracycline induction times were 72, 36, 72, 36, 48 and 48 h—that is, the initial point of cell growth cessation (C) for subunits 3, 4A, 5, 6, 8B and 9, respectively. (A) Messenger RNA (mRNA) and antisense (AS) RNA were assayed by reverse transcription–PCR (RT–PCR) using primers specific for the subunit numbered at the top of each panel. Two typical examples (knockdown of RIC8B and RIC9) are shown. (B) Western blots of total (upper panel) or mitochondrial (lower panel) proteins of indicated knockdown lines with antibodies against the indicated subunits. (C) Growth of knockdown lines in the absence (filled squares) or presence (open squares) of tetracycline. $F_1\beta$, F_1F_o-ATP synthase β-subunit; FeS, complex II iron-sulphur protein; M, molecular weight markers; RIC, RNA import complex; Tet, tetracycline.

moonlighting for a single function is unprecedented. Moonlighting proteins have been identified as tRNA import factors in different systems (see above; Tarassov *et al*, 1995a,b; Entelis *et al*, 2006; Salinas *et al*, 2006). The preponderance of multifunctional proteins might be a reflection of the mechanism of emergence of a new cellular function (tRNA import) during evolution.

METHODS

Preparation and analysis of mitochondrial complexes. Mitochondrial fractions were isolated from *L. tropica* promastigotes as described previously (Bhattacharyya *et al*, 2000). Mitochondria or mitoplasts were extracted using sodium dodecyl maltoside, and complexes were separated by using BN-PAGE, excised and subjected to two-dimensional SDS–PAGE (Goswami *et al*, 2006). Chromatography of maltoside extracts of mitochondria (5×10^6

cell-equivalent) used an RNA affinity column as described previously (Bhattacharyya *et al*, 2003).

Protein radiolabelling. Promastigotes were cultured in RPMI1640 medium (Invitrogen, Bethesda, MD, USA) in the presence of a methionine-deficient amino-acid mixture (prepared using the SelectAmine kit, Invitrogen) supplemented with ^{35}S-methionine (1,000 Ci/mmol, 0.4 mCi/ml) for 4 h at 22 °C. [^{35}S]-labelled bands were visualized by fluorography.

Peptide analysis and gene retrieval. Tryptic peptides of the RIC subunit bands were sequenced by mass spectrometry at the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia (VA, USA). Nuclear-encoded ORFs were retrieved from the *L. major* database (http://www.genedb.org/genedb/leish/index.jsp). Details of peptide analysis and shortlisting are provided in the supplementary information online. Mitochondrial ORFs from

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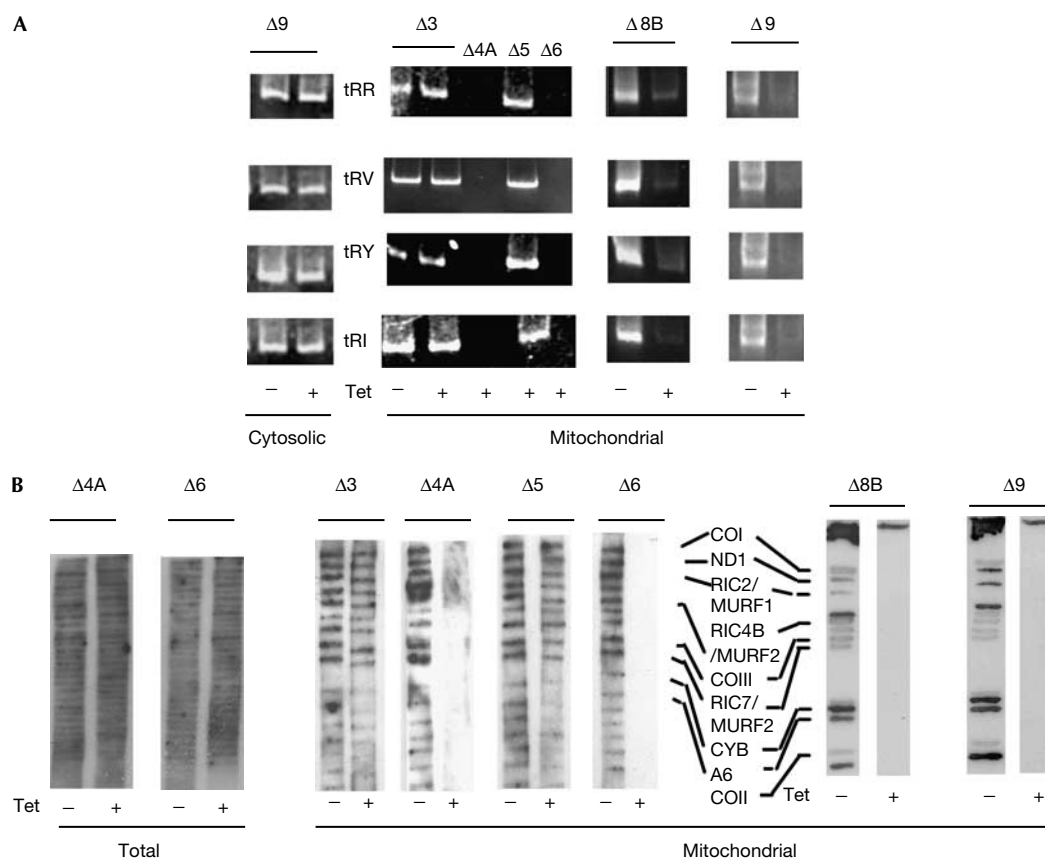
Fig 4 | Effect of subunit knockdown on mitochondrial transfer RNAs and translation. (**A**) Reverse transcription–PCR assays of tRNAs in the cytosolic (10^2 cell-equivalent) or mitochondrial (10^3 cell-equivalent) compartments of knockdown cells. (**B**) Total or mitochondrial proteins in uninduced or knockdown lines [^{35}S]methionine labelled in presence of cycloheximide, resolved by SDS–polyacrylamide gel electrophoresis and fluorographed. CO, cytochrome oxidase; CYB, cytochrome *b*; MURF, maxicircle unidentified reading frame; ND, NADH dehydrogenase subunit; RIC, RNA import complex; Tet, tetracycline; tRR, tRNA^{Arg}; tRV, tRNA^{Val}; tRY, tRNA^{Tyr}; tRI, tRNA^{Ile}.

L. tarentolae were obtained from Larry Simpson's U-insertion/deletion edited sequence database (http://dna.kdna.ucla.edu/trypanosome/database.html).

Sequence analysis and homology modelling. The *L. tropica* protein sequences were found to be identical to corresponding sequences in *L. major*. Selected ORFs were re-subjected to BLAST analysis. Sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw/index.html). Homology models of shared subunits were constructed by SWISS MODEL (www.expasy.ch/swissmod/SWISS-MODEL.html) using the bovine homologue as a template.

Cloning, expression and purification of recombinant protein. Each complete nuclear-encoded ORF was amplified from *L. tropica* strain UR6 genomic DNA using *Bam*HI (sense) and *Eco*RI (antisense) linker-primers (supplementary Table S3 online). Expression of glutathione-*S*-transferase-tagged proteins and purification of recombinant subunits were carried out as described previously (Goswami *et al*, 2006).

Gene knockdown. In a standardized protocol (details in Goswami *et al*, 2006), each gene was cloned in the antisense orientation downstream of a tetracycline-regulated T7 RNA polymerase promoter of the expression-cum-targeting vector pGET, and introduced into the expression host *L. tropica* 13–90. Transformed

clones were selected on semisolid agar with 2.5 µg/ml phleomycin. Clonal cultures were induced using 1 µg/ml tetracycline. Cell growth and morphology were monitored and knockdown cells collected at the earliest observed point of growth slowdown—that is, the point of inflection in the growth curve. Cytochrome oxidase and O_2 uptake assays were carried out as described previously (Goswami *et al*, 2006).

RNA analysis. Gene-specific sense or antisense RNA was amplified from cytosolic RNA (10^5 cell-equivalent) by reverse transcription–PCR using appropriate primer pairs (supplementary Table S3 online), as described previously (Goswami *et al*, 2006). Cytosolic and mitochondrial tRNAs (from 10–10^3 cell-equivalent RNA) were similarly amplified.

Reconstitution. Each recombinant protein (100 ng of gel-eluted band in 10 µl of 0.05 M Tris–HCl (pH 8.0), 0.2 M NaCl, 0.1 mM EDTA, 5 mM dithiothreitol (DTT) and 0.2% SDS) was separately diluted fivefold in buffer DB (0.2 M Tris–HCl (pH 7.5), 5 mM MgAc$_2$, 1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride and 10% glycerol), re-folded on ice for 2 h, and concentrated in a Microcon 10 unit to 30 µl. Indicated combinations of re-folded subunits (15 µl or 50 ng) were combined and adjusted to 120 µl with DB, incubated for 2 h on ice and microconcentrated to a
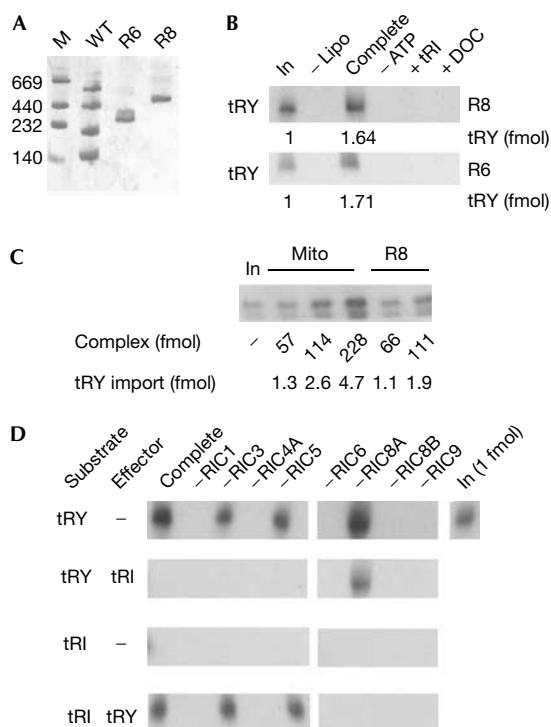
Fig 5 | Reconstitution of functional import complexes with recombinant RNA import complex subunits. (**A**) BN-polyacrylamide gel electrophoresis analysis of complexes reconstituted with six essential (R6, containing subunits 1, 4A, 6, 8A, 8B and 9) or eight (R8, containing the six essential plus subunits 3 and 5) nuclear-encoded subunits. Molecular mass markers (M) of sizes (kDa) indicated on the left. Minor bands in the lanes marked R6 and R8 are possibly dimeric forms. (**B**) R6- or R8-induced import of tRNA^{Tyr} into liposomes (Lipo). Sodium deoxycholate (0.5%; DOC) added post-import. (**C**) Comparison of import by indicated amounts of liposome-bound R8 and mitochondrial (Mito) RIC. (**D**) Single-omission experiment. Complexes were reconstituted with eight subunits (R8 = complete) or with single indicated subunits omitted, and assayed for import into liposomes of substrate with high specific activity (100 fmol) in the absence or presence of effector with low specific activity (10 fmol). In, input substrate, 1 fmol; RIC, RNA import complex; tRI, tRNA^{Ile}; tRY, tRNA^{Tyr}; WT, mitochondrial complexes from normal cells.

volume of 45 μl. Assembled complexes (10 ng/subunit in 10 μl) were assayed for import of ^{32}P-labelled tRNAs into liposomes by RNase protection (Bhattacharyya *et al*, 2003).

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

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