OM14 is a mitochondrial receptor for cytosolic ribosomes that supports co-translational import into mitochondria

Chen Lesnik1, Yifat Cohen2, Avigail Atir-Lande1, Maya Schuldiner2 & Yoav Arava1

It is well established that import of proteins into mitochondria can occur after their complete synthesis by cytosolic ribosomes. Recently, an additional model was revived, proposing that some proteins are imported co-translationally. This model entails association of ribosomes with the mitochondrial outer membrane, shown to be mediated through the ribosome-associated chaperone nascent chain-associated complex (NAC). However, the mitochondrial receptor of this complex is unknown. Here, we identify the *Saccharomyces cerevisiae* outer membrane protein OM14 as a receptor for NAC. OM14Δ mitochondria have significantly lower amounts of associated NAC and ribosomes, and ribosomes from NACΔ cells have reduced levels of associated OM14. Importantly, mitochondrial import assays reveal a significant decrease in import efficiency into OM14Δ mitochondria, and OM14-dependent import necessitates NAC. Our results identify OM14 as the first mitochondrial receptor for ribosome-associated NAC and reveal its importance for import. These results provide a strong support for an additional, co-translational mode of import into mitochondria.
mitochondria contain several hundred proteins that are critical for their diverse functions. Most of these proteins are encoded in the nucleus, synthesized in the cytoplasm and imported into mitochondria through their import complexes (TOM and TIM complex). The import process of most mitochondrial proteins can occur following their complete synthesis in the cytosol (that is, post-translationally) and the mechanisms for such a process are well established (reviewed in refs 2–4). An additional model, in which proteins are imported while being translated (co-translationally) was proposed ~40 years ago following the detection of translationally active ribosomes near the mitochondrial outer membrane5,6. This model was abandoned for many years and was recently revived following diverse observations (reviewed in refs 7, 8). In particular, genome-wide microarray analyses revealed that many messenger RNAs are associated with the mitochondrial outer membrane, and advanced microscopic techniques provided important confirmation of these results9–12. These mRNAs were proposed to be translated locally (that is, near mitochondria), thereby positioning the emerging polypeptide chains in close proximity to the TOM complex and facilitating import. Indeed, treatments with various translation inhibitors or changes in various coding domains affected mRNA association, thereby supporting the hypothesis that mRNA association is linked to translation10,12–15. Furthermore, we have shown that deletion of Tom20, a protein receptor for incoming precursor proteins, affects mRNA association10. This result provides an important link between protein import and mRNA association, which can be explained by a co- translational mode of import. Yet, direct support for co-translational import is still scarce. Moreover, the proteins that may be involved in such a process are largely unknown.

The nascent chain-associated complex (NAC) is a ribosome- associated chaperone that is conserved from yeast to human16. It binds ribosomes in close proximity to the protein exit tunnel, and interacts with newly synthesized proteins as they exit the ribosome17. NAC was shown to support protein transport to various cellular destinations, including mitochondria18,19. The α-subunit of NAC (the Egd2 protein in the budding yeast S. cerevisiae) can dimerize either with a β1 (Egd1 in yeast) or β3 (Btt1 in yeast) subunit, thereby forming two different heterodimers20–22. One of the two NAC heterodimers was shown to associate preferentially with nascent chains of mitochondria-targeted ribosome–nascent-chain complexes (RNCS)22. In vitro studies have shown that NAC can promote protein import when preformed RNCS are mixed with purified mitochondria23,24 and deletion of NAC subunits in yeast cells reduced ribosomal association with mitochondria25. Furthermore, in vivo studies have shown that co-translational import of mitochondrial fumarase is lower upon NAC deletion26. Thus, NAC is a mediator of ribosomes’ association with mitochondria, and a critical player in co-translational import. Notably, association of NAC with mitochondria was shown to necessitate a mitochondrial receptor24, however, such a receptor has not yet been identified. Furthermore, the two trivial candidates (the protein receptors Tom20 and Tom70) were specifically excluded24.

In this work, we perform a genome-wide protein complementation screen for proteins that interact with either NAC subunit. We find the mitochondria outer membrane protein OM14 to be a positive partner. OM14 appeared to interact with NAC in all eight different types of screen that we performed. Co- immunoprecipitation analyses confirmed these results. Furthermore, the mitochondrial fraction from OM14-deleted cells had significantly reduced levels of associated NAC and ribosomes. Complementary to this result, ribosomes from NAC-deleted cells had reduced OM14 association. Through import assays into mitochondria, we show that OM14-deleted mitochondria have reduced co-translational import efficiency, and this role in import is exerted through NAC. Thus, OM14 is a receptor for ribosome-associated NAC, thereby coordinating localized translation and import into the mitochondria.

Results

NAC interacts with OM14. The NAC complex was previously shown to support association of ribosomes with mitochondria in a manner that necessitated a mitochondrial receptor24,25,27. To identify a possible receptor for NAC (and hence ribosomes) on the mitochondrial outer membrane, we performed a genome-wide protein complementation assay (PCA) by utilizing the split dihydrofolate reductase (DHFR) system28 (Fig. 1a). In this system, a haploid yeast strain expressing NAC subunit fused to one half of DHFR protein (bait) is mated into a library of ~6,000 strains, each expressing a different yeast protein fused to the other half of DHFR (prey). Interaction between the bait and a candidate prey brings the two DHFR halves to a close proximity and renders the cells resistant to methotrexate. We performed such a screen with either the α-subunit of NAC (Egd2) or the β-subunit (Egd1) as baits. Each subunit was expressed in either a or α mating type with either N’ or C’ halves of DHFR, respectively) and mated with a library of the reciprocal mating type. Colonies were then grown on either glucose or galactose as carbon source. This resulted in eight different screens (two baits × two mating types × two growth media). Diploids of each screen were allowed to grow for 3 days on methotrexate and colony size was measured with the Balony program29. The list of strains that passed our threshold of confidence (were larger than 150 pixels) is shown in Supplementary Table 1. The only mitochondrial membrane protein that appeared in the largest 100 colonies was OM14, an outer membrane protein with an unknown function30. The high ranking of OM14 occurred in each of the eight screens, corroborating the validity of the interaction (Supplementary Table 1). These results were re-confirmed by mating only these strains and their controls (Fig. 1b).

To validate the PCA results by an alternative approach, we performed co-immunoprecipitation analysis (co-IP). OM14 gene was tagged with HA through homologous recombination. Cells expressing an HA-tagged OM14 were lysed in the presence of a detergent (to disassociate OM14 from mitochondrial membranes), and immunopurified with anti-HA beads. Western analysis with antibodies recognizing the Egd2 subunit of NAC revealed significant amounts at the IP fraction (Fig. 1c,d). This co-purification is not due to non-specific binding to the beads because it does not present when a strain expressing untagged OM14 was used or when an unrelated protein (HXX1) was tested.

In summary, the systematic PCA analysis uncovered a novel physical interactor for the NAC on the surface of mitochondria. Such an interaction could mediate NAC targeting to the surface of mitochondria.

Importance for association of ribosomes with mitochondria.

We next wished to determine if the interaction between NAC and OM14 is important for the association of ribosomes with mitochondria. To this end, we isolated a crude mitochondrial fraction from cells either expressing or devoid of OM14, and examined the amounts of NAC associated with this fraction by western analysis. Indeed, there was a significant reduction in the amount of NAC that appeared in the mitochondrial fraction of cells deleted for OM14. This is not due to reduced amounts of mitochondrial OM14A sample, as the levels of another outer
membrane protein (Tom20) or a luminal protein (Hsp60) appear similar (Fig. 2a). We note that there are still detectable levels of NAC in OM14A mitochondria fraction. These may be due to some impurities (for example, ER components) in this fraction, or due to activity of other receptors that compensate for OM14 deletion. A marker for ribosome association (Rpl3) appeared almost unchanged (not shown), probably because our crude mitochondrial fraction contains also significant amount of ER-bound ribosomes, which are not affected from OM14 deletion.

To test for impact on ribosomal association with mitochondria more precisely, we established an association protocol that utilize highly purified mitochondria (Fig. 2b) and ribosomes (see Methods). Mitochondria were separated from ER by density centrifugations through a sucrose gradient (Fig. 2b). To ensure that the only NAC source is the ribosome-associated one, mitochondria samples were purified from cells deleted of NAC. We note that this purification protocol necessitates disassembly of ribosomes (rountinely imposed by the Zymolyase treatment or by the addition of ethylenediaminetetraacetic acid (EDTA))33, leading to the absence of ribosomes in the mitochondria fraction. Any attempt to maintain ribosomes with mitochondria hinders the separation of the ER from mitochondria33. Ribosome-striped mitochondria from either OM14+ or OM14A cells were incubated with ribosomes that were cleared from heavy complexes (for example, mitochondria or microsomes) through differential centrifugation. Mixed mitochondria and ribosomes were then centrifuged at 10,000 g (Fig. 2c), as under these conditions ribosomes are maintained in the supernatant, unless mitochondria are present (Fig. 2c, lanes 5 and 6). Quantitation of ribosomes' sedimentation (by the Rpl3 signal normalized to the Tom20 signal), reveals at least twofold higher ribosomal association with mitochondria that contain OM14 (Fig. 2c).

We reasoned that the twofold higher association might be even higher if ribosomes were loaded with a mitochondrial targeting sequence (MTS). We, therefore, purified ribosomes from an \textit{in vitro} translation reaction, that was performed in the presence of a transcript encoding a mitochondria protein (MDH1t). This transcript did not contain a stop codon, to improve ribosomes stalling on the transcript24,32 (see also Methods). Indeed, ribosomes association with OM14A mitochondria was negligible, and significantly higher in OM14+ mitochondria (Fig. 2d; \textit{P} value = 0.042, independent-samples one-sided \textit{t}-test). Thus, an outer membrane-assembled OM14 is capable of increasing ribosome association with isolated mitochondria.

To substantiate that OM14 association is indeed with ribosomes, we performed the complementary analysis, in which we isolated ribosomes and tested for OM14 association.
Yeast cells were lysed in the presence of detergent to disassemble mitochondria membranes and ribosomes were isolated by differential centrifugation. The association of HA-tagged OM14 was tested by western analysis (Fig. 3). We found that significant amounts of OM14 co-sedimented in the ribosomal pellet prepared from WT cells, unlike other mitochondrial membrane proteins such as Tom20. Importantly, when ribosomes were devoid of NAC (that is, isolated from NACΔ cells (egd1-Δegd2Δ)), significantly lower amounts of OM14 co-sedimented. This is not due to lower amounts of ribosomes in this preparation, as the levels of ribosomal marker (Rpl3) appear similar. Intriguingly, the levels of Tom20 appear to be highly decreased in NACΔ cells, while the levels of Rpl3 appear similar. This may suggest a compensation mechanism that is activated upon the absence of this ribosome-associated complex. Overall, these results confirm that NAC interacts specifically with OM14. More important, they reveal that NAC–OM14 interaction occurs while NAC is associated with ribosomes, consistent with NAC being almost exclusively ribosome-associated.

OM14 supports import to mitochondria. The functional role of OM14 is unknown. Considering its interaction with NAC, we sought to determine if it has a role in co-translational protein import to the mitochondria. We generated ribosome–nascent chain (RNC) complexes in a rabbit reticulocyte lysate by utilization of a truncated MDH1 construct (MDH1t) that induce ribosome stalling at the end of the mRNA. RNCs were isolated from the lysate by centrifugation through a sucrose cushion, and indeed RNA of both ribosomal subunits was detected in the pellet (Fig. 4a). To confirm the functionality of these RNCs, they were mixed with highly purified mitochondria (that is, depleted of ribosomes or ER marker) (Fig. 2b), and import of MDH1t was allowed (Fig. 4b); imported MDH1t appears as a shorter protein (due to cleavage of the leader sequence) when proteins are resolved on gel. This shorter band appears as a shorter protein (due to cleavage of the leader sequence) when proteins are resolved on gel. This shorter band does not appear when membrane potential is diminished by the addition of valinomycin (ΔΨ) and is protected from cleavage by proteinase K (see also Fig. 4c). About 60% of the protein appeared to co-sediment with mitochondria, half of it appeared as a shorter band in the gel. This reveals that the RNCs are functional and permit protein association and insertion into mitochondria, at least up to a point that allows cleavage by the mitochondrial peptidase. Importantly, when the same experiment was performed in the presence of EDTA, which disassembles ribosomes, a significant reduction in import was observed (Fig. 4b). This strongly suggests that import occurs while ribosomes are in complex with the nascent chain.
To test if OM14 contributes to co-translational import, we performed time-course import assay with RNCs and mitochondria purified from OM14A strain or its parental strain. These strains were also deleted of NAC, to ensure that the only source of NAC is from the RNCs. More than twofold decrease in import efficiency was observed in mitochondria deleted of OM14 (Fig. 4c–e) compared with mitochondria that contain OM14. The slope of the best-fit linear curve, which is a proxy to import rate, is more than twice lower in OM14A mitochondria compared with OM14+ cells (Fig. 4e). We note that OM14 is not essential for import, as even in its absence there is some import of MDH1. This is not surprising as OM14 is not a core import component, thus is more likely to have an auxiliary role. This is consistent with OM14 being non-essential for yeast viability, even under respiration-dependent conditions (Supplementary Fig. 1).

We further tested whether OM14 affects import of fully synthesized proteins (that is, post-translationally). For that, we synthesized MDH1 from its full open reading frame (ORF; MDH1f), leading to a complete synthesis of the protein, without ribosome stalling. As these import reactions do not rely on functional RNCs, their efficacy and quality is much higher (Fig. 4f). The mitochondrial preps that are used for the post-translational import assay are the same as the ones that are used for the co-translational assays, thus these results validate the integrity of the mitochondria and their import potency. Comparing the post-translational import of OM14+ to OM14A mitochondria from multiple experiments, revealed an insignificant small decrease in import (P value = 0.33, independent-samples one-sided t-test). Thus, OM14 impact appears to be specific for the co-translated MDH1. Intriguingly, when import reactions were performed with a commonly used chimeric Su9-DHFR protein (Supplementary Fig. 2), a more significant decrease was observed in OM14A mitochondria. Although this may suggest that OM14 have a differential impact on post-translational import of proteins, a larger repertoire of proteins, preferably native ones need to be tested. Overall, however, these data reveal that OM14 contributes to co-translational import to yeast mitochondria.

**OM14 role in import is exerted through NAC.** To validate that OM14 necessitates NAC to exert its function, we assembled import complexes that lack NAC, and then introduced NAC to the import assay. NAC-depleted RNCs were obtained by centrifugation of the RNCs through a sucrose cushion supplemented with high salt concentration (0.8 M KAc; ref. 34). Western analysis with anti-αNAC confirmed that NAC is depleted from the RNC pellet and maintained in the supernatant (Supplementary Fig. 3a). Consistent with previous studies, these NAC-depleted RNCs showed much lower import efficiency into mitochondria (Supplementary Fig. 3b–d).

We then added to the import reaction NAC from three different sources: First, an aliquot of the rabbit reticulocyte lysate high salt wash, which contains NAC, was added. As can be seen in Fig. 5a–c, import was much more significant if mitochondria contained OM14. Second, we purified ribosome-associated proteins (see Methods) either from WT or NACΔ yeast cells. The NAC-containing sample significantly improved import over the NACΔ extract (Supplementary Fig. 4a, lanes 1–6), consistent with its known role in improving import into mitochondria. This effect, however, was significantly reduced in mitochondria that lack OM14 (Fig. 5d–f and Supplementary Fig. 4a). Third, the NAC complex was purified to a high level from bacteria (Supplementary Fig. 4b), and added to the import reaction (Fig. 5g and Supplementary Fig. 4c,d). The bacterially purified complex improved import efficiency to OM14+ mitochondria (Supplementary Fig. 4d lanes 2–7). However, the contribution of NAC to import was much lower when OM14 was absent (Fig. 5g and Supplementary Fig. 4c,d). We note that the latter data are presented only in a qualitative manner as low signals hindered reliable quantification of the impact of the bacterially purified NAC. Nevertheless, in at least three experimental repeats, the same pattern was observed (Supplementary Fig. 4c,d). Thus, the overall data from the three sources of NAC strongly suggest that OM14 exerts its function on mitochondrial import through the NAC complex.

**Discussion**

Our data provide two critical features in establishing that co-translational import into mitochondria occurs (Fig. 6): first, we identify a novel receptor for NAC (and hence ribosomes) on the mitochondrial outer membrane. Second, we assign a functional significance to this receptor, namely supporting co-translational import into mitochondria.

A receptor for NAC on the mitochondrial outer membrane was proposed 15 years ago, yet was never identified. Our data clearly show that OM14 is critical for NAC association with mitochondria and may serve as its primary receptor. The molecular details of OM14–NAC interaction are yet to be resolved. Interaction may be direct, presumably through the cytoplasmic domains of OM14 (amino acids 1–38 and 90–104; ref. 30) and one of the NAC subunits. Alternatively, OM14 may necessitate an additional protein to stabilize its interaction with NAC, in a mode that resembles the SRP receptor, which is a heterodimer of a small, membrane-embedded protein (SRβ) and a large, mostly cytosolic protein (SRα), which interacts with SRP38. We find the latter more likely, and a screen for OM14-interacting proteins is underway.
The clear impact of OM14 deletion on protein import, and the contribution of NAC to this process, strongly suggests that the association between NAC and OM14 is important for co-translational import of proteins into mitochondria (Fig. 6). We suggest that translating ribosomes associate with mitochondria through interaction between NAC and OM14. As NAC interacts with the nascent chains of many proteins, the association with OM14 may be important for initiating specificity for those that are destined to mitochondria. Once association is established, the growing peptide interacts with the import machinery (the TOM complex) and is imported into mitochondria. At least one TOM component (Tom20) was previously shown to be involved in mRNA association with mitochondria, in a translation-dependent manner10. Furthermore, the increased levels of Tom20 in NACΔ cells (Fig. 3) may indicate a compensation mechanism, which maintains sufficient import efficiency. Import rates in a strain deleted of both Tom20 and OM14 are yet to be determined.

It is difficult to appreciate the relative contributions of post-translational and co-translational protein import to mitochondria. Under standard experimental conditions, when steady-state protein levels are measured, these two processes seem redundant (for example, knockout of key factors does not affect cellular growth). Considering the importance of mitochondrial function (for example, knockout of key factors does not affect cellular growth). Considering the importance of mitochondrial function for example, knockout of key factors does not affect cellular growth. Nevertheless, we speculate that co-translational import is the preferred mode under most natural conditions as it enables efficient and rapid import. Furthermore, it minimizes the chances of protein ectopic expression or the need for an elaborate net of chaperones.

The complete repertoire of proteins that are imported co-translationally is yet unknown. Genome-wide studies of mRNAs association with mitochondria identified many candidates10,13,14, yet direct impact on mitochondrial activity was rarely demonstrated39,40. This strongly suggests that both co- and post-translational import mechanisms apply to most proteins, and post-translational import maintains mitochondrial activity when co-translational import is affected. The fact that the same protein can be imported by either way complicates determination of the contribution to the import of each mode. It may, therefore, be necessary to intervene with the post-translational pathway to expose the in vivo importance of co-translational import. Nevertheless, we cannot exclude the possibility that co-translational import is applicable only to a subset of proteins. Indeed some specificity in NAC activity was inferred from a genome-wide association study12. Thus, NAC may recognize and interact with a subset of emerging peptides. Once NAC interacts with a proper nascent chain, it will be in a better position for interaction with OM14, thereby stabilizing it with the outer membrane.

In this work, we identified a long-sought-after receptor for the ribosome-associated complex NAC. Importantly, we found a
physiological role for this receptor, namely import of proteins
into mitochondria. The data presented herein are the first to
show that localized translation (mediated through NAC–OM14 in-
teraction) supports protein import into mitochondria. The amount
of proteins that are targeted by this mode and its implication
in vivo are yet to be determined. Nevertheless, our data directly
link protein synthesis and mitochondria import, thereby provid-
ing a strong support to the idea of co-translational import to
mitochondria.

Methods
Yeast growth and strains. For mitochondrial fractionation and ribosomes iso-
lution, cells were grown in YP-glycerol medium (1% yeast extract, 1% peptone and
2% glycerol). Strains are listed in Table 1.

Antibodies. The following antibodies were used: polyclonal anti-EGD2
(Hb1) (1:20,000 dilution) (gift from Professor M. Collart)41, polyclonal anti-EGD2
(1:20,000 dilution) (gift from Professor B. Beatrice42), monoclonal anti-HA (1:500
dilution) (gift from Professor A. Aronheim43, polyclonal anti-Tom20 (1:10,000
dilution) (gift from Professor D. Rapaport44) and polyclonal anti Rpl3 (1:5,000 dilution) (gift from Professor J. Warren45).

DHFR-based protein fragment complementation assay. The protein–protein
interactions screen was done using the yeast DHFR PCA library according to the
published protocol28 in 1,536 format. In brief, MATa strains with the ORFs of
either Egd1 or Egd2 fused to F[1,2] were mated to the entire MATalpha collection of ORFs tagged with F[3]. The complementary mating was also performed, in
which MATalpha strains with the ORFs Egd1 or Egd2 fused to F[3] were mated to
the entire MATa collection of ORFs tagged with F[1,2]. The resulting diploids were
subsequently selected for growth in the presence of metronexate for positive
DHFR PCA reconstitution for 5 days in 30 °C. The analysis of each plate was done
by taking image of the entire plate, which was saved in JPG format at a resolution
of 300 d.p.i. Using the freely available Baloney software47, the area for each colony
was extracted and a threshold of a positive interaction was set to be > 150.

Cell fractionation for import analyses. Crude or highly purified mitochondria
were isolated as described36, with several modifications: Yeast strains were grown
in YP-glycerol medium to OD600 1.5–2.0, pelleted, washed and treated with
Zymolase for 30 min at 30 °C with gentle shaking. Spheroplasts were resuspended
with 3 ml of ice-cold homogenization buffer (0.6 M Sorbitol, 10 mM Tris HCl pH 7.4,
1 mM EDTA, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.2% bovine
serum albumin (BSA), and homogenized by 15 strokes using Dounce homogenizer
equipped with tight-fitting pestle. Unbroken cells and nuclei were removed by
centrifugation at 6 min at 4 °C at 1,500 g. The supernatant was then centrifuged
for 10 min at 4 °C at 100,000 g and the crude mitochondrial pellet was frozen in
liquid nitrogen. To obtain highly purified mitochondria, the pellet was washed in
SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS pH 7.2), resuspended
with SEM buffer and layered over four-step sucrose gradient: 1.5 ml 60%, 4 ml 32%,
1.5 ml 23% and 1.5 ml 15% sucrose in EM buffer (10 mM MOPS pH 7.2, 1 mM

Figure 5 | OM14 necessitates NAC to exert its role. (a–c) NAC from
rabbit reticulocyte lysates (RRL): MDH1t RNCs depleted of NAC were
mixed with mitochondria purified from either OM14+ or OM14Δ cells. The
import reactions were supplemented with NAC-containing high-salt
supernatant (HS) from RRL, and at the indicated time points a sample
was collected. The arrow indicates the imported protein. Import assays were
repeated four times, each time with a new mitochondria prep and a new
RNC prep. Graphs (b), histogram (c) and statistics were calculated as
described for Fig. 4. (d–f) NAC from yeast: Import assays entailing NAC-
depleted RNCs and OM14+ or OM14Δ mitochondria were supplemented with ribosome-associated factors, prepared from NAC+ yeast strain.
Graphs (e), histogram (f) and statistics were calculated as described for
Fig. 4. (g) NAC purified from bacteria: Import assays entailing OM14+ or
OM14Δ mitochondria and MDH1t RNCS depleted of NAC, were
supplemented with NAC complex that was expressed and purified to a
high degree from bacteria (pur. NAC). Samples were collected at the indicated
time points, resolved on PAGE and exposed to phosphorimager for 3 days.
Panel is from a single image (provided as Supplementary Fig. 4e), which
was cropped between lanes 4 and 5 to remove irrelevant lanes.

Figure 6 | Two modes for protein import into mitochondria. Depicted to
the left is the well-established post-translational import mode, in which
chaperones (circles) assist fully translated proteins (black line) in their
transport through the TOM complex into the mitochondria92. Our results
corroborate an additional mode, described on the right. In this model,
OM14 interacts with the heterodimeric NAC, while it is associated with
translating ribosomes. This interaction brings the emerging protein to
proximity with the TOM complex thereby enhancing import. These modes
are not mutually exclusive and likely to be redundant under many
experimental conditions.
EDTA). The gradient was centrifuged at 134,000g at 4°C for 1 h and purified mitochondria were recovered from the 60%/32% interface. Mitochondria were centrifuged again at 10,000g at 4°C for 10 min, resuspended in SEM buffer and snap frozen in liquid nitrogen and stored at −80°C. Mitochondria were thawed at 25°C bath immediately before use and kept on ice.

**Ribosomes purification.** Ribosome purification is based on the method described in ref. 46. Specifically, yeast strains were grown in 250 ml YP-glycerol medium to OD600 1.0–1.5. Cells were centrifuged at room temperature for 10 min at 3,000g and the pellet was washed in double distilled water. Cells were resuspended in 1.38 M sucrose, 2 mM DTT) and cleared of any membrane compartments or large complexes (0.4–0.6 mm) were added. Cells were lysed by two pulses of 1.5 min each in a Beater and the lysate was clarified. One percent of the sample was set aside as ‘FT’ (Flow Through). The column was first washed with buffer B and then twice with washing buffer (100 mM NaCl, 50 mM Tris HCl, 20 mM HepesKOH pH 7.4, 2 mM DTT, 2 mM Mg(Ac)2, 0.8 M KAc, 1 leupeptin, 1.8 mg·ml⁻¹ leupeptin, 7 µg·ml⁻¹ Pepstatin A, 20 units per ml DNase, 1 µg·ml⁻¹ aprotinin and 10 µg·ml⁻¹ tropin inhibitor) and glass beads (0.4–0.6 mm) were added. Cells were lysed by two pulses of 1.5 min each in Bead Beater and the lysate was clarified by centrifugation in a Sorval SS-34 rotor at 12,000 r.p.m. for 30 min at 4°C. The supernatant was loaded on 2.5 ml of sucrose cushion (Ribo buffer supplemented with 500 mM KCl, 1 M sucrose and 2 mM DTT) and centrifuged using Beckman Type 70Ti ultracentrifuge rotor at 60,000 r.p.m. for 106 min at 4°C. The ribosomal pellet was resuspended in 300 µl storage buffer (Ribo buffer supplemented with 250 mM sucrose and 2 mM DTT) and immediately frozen in liquid nitrogen.

Factors that are associated with yeast ribosomes (for Fig. 5d–f) were isolated from ribosomes by loading isolated ribosomes on a high-salt sucrose cushion (25% sucrose, 20 mM HepesKOH pH 7.4, 2.5 mM Mg(OAc)2), 1 mg·ml⁻¹ heparin, 2 mM dithiothreitol (DTT), 1 mM MgCl₂, 2.5 µg·ml⁻¹ leupeptin, 0.01 mg·ml⁻¹ Pepstatin A, 1 mM PMSF, 1% Triton X-100 and glass beads (0.4–0.6 mm) were added. Cells were lysed by two pulses of 1.5 min each in Bead Beater and the lysate was clarified by centrifugation in a Sorval SS-34 rotor at 12,000 r.p.m. for 30 min at 4°C. The supernatant was loaded on 2.5 ml of sucrose cushion (Ribo buffer supplemented with 500 mM KCl, 1 M sucrose and 2 mM DTT) and centrifuged using Beckman Type 70Ti ultracentrifuge rotor at 60,000 r.p.m. for 106 min at 4°C. The ribosomal pellet was resuspended in 300 µl storage buffer (Ribo buffer supplemented with 250 mM sucrose and 2 mM DTT) and immediately frozen in liquid nitrogen.

**Co-immunoprecipitation.** Cells were grown in 200 ml YP-glycerol medium to OD600 0.5–1.0, centrifuged at 4°C for 4 min at 3,000g and the pellet was washed twice in buffer A (20 mM Tris HCl pH 8, 140 mM KCl, 1.8 mM MgCl₂, 0.1% NP-40). Cells were resuspended in 5 µl buffer B (buffer A supplemented with 0.5 mM DTT, 1 mM PMSF, 10 µg·ml⁻¹ leupeptin, 7 µg·ml⁻¹ Pepstatin A, 20 units per ml DNase, 1 µg·ml⁻¹ aprotinin and 10 µg·ml⁻¹ tropin inhibitor) and glass beads (0.4–0.6 mm) were added. Cells were lysed by two pulses of 1.5 min each in Bead Beater and the lysate was clarified. One percent of the sample was set aside as ‘Total’ and the rest of the sample was loaded twice on anti-HA coupled sepharose columns and 1% was set aside as ‘FT’ (Flow Through). The column was washed first with buffer B and then twice with washing buffer (100 mM NaCl, 50 mM Tris HCl pH 8, 5% glycerol). Elution sample was collected by adding 0.1 M of acetic acid pH 3. The acetic acid was dissipated and pellets were resuspended in 100 mM Tris HCl pH 8 in 1 x SDS loading dye for western blot analysis.

**Assessing association of ribosomes with mitochondria.** Yeast ribosomes were purified as described in the above section (ribosome purification) including a final step of centrifugation in a low-salt sucrose cushion (25% sucrose, 20 mM HepesKOH pH 7.4, 2 mM DTT, 2 mM Mg(OAc)₂, 0.1 M KAc, 2.5 µg·ml⁻¹ leupeptin, 1.8 µg·ml⁻¹ Pepstatin A, 5 mM PMSF). The ribosomal pellet was resuspended in Ribo storage buffer (0.1 M KOAc, 20 mM HepesKOH pH 7.4, 2.5 mM Mg(OAc)₂, 250 mM sucrose, 2 mM DTT) and cleared of any membrane compartments or large ribosomal complexes by five centrifugations at 100,000g for 5 min. The supernatant from the last centrifugation step was immediately frozen in liquid nitrogen. To test the association of ribosomes with highly purified mitochondria, 25 µg of highly purified mitochondria (prepared as described above) were incubated with 10 µg of ribosomes. Incubation was performed for 5 min at room temperature in mitotic association buffer (MA buffer: 0.6 M sorbitol, 50 mM HepesKOH pH 7.4, 50 mM KCl, 10 mM MgCl₂, 2 mM KH₂PO₄, 1 mg·ml⁻¹ BSA, 0.75 mg·ml⁻¹ methionine and fresh 250 mM creatine phosphate and 5 mg·ml⁻¹ creatine kinase, 2 mM ATP, 8 mM NaN₃ in a total volume of 25 µl). Samples were then centrifuged for 5 min at 10,000g. Supernatant and pellet were subjected to western analysis.

For assessing the association of RNCs containing mitochondrial protein precursor (Fig. 2d), RNCs were generated as described in the section below (Generating ribosome–nascent chains), and 40 µl of RNCs were loaded on 200 µl low-salt cushion sucrose cushion (25% sucrose, 20 mM HepesKOH pH 7.4, 2.5 mM DTT, 2 mM Mg(OAc)₂, 0.1 M KAc, 2.5 µg·ml⁻¹ leupeptin, 1.8 µg·ml⁻¹ Pepstatin A, 5 mM PMSF), centrifuged using Beckman MicroUltra Centrifuge Rotor TLA-120.2 MicroFixed at 95,000 r.p.m. for 30 min at 4°C and the RNCs pellet was suspended with 60 µl of MA buffer without BSA. Immediately after isolation, 10 µl of RNCs were incubated with 25 µg of mitochondria for 5 min at room temperature in MA buffer. Samples were then centrifuged for 5 min at 10,000g and the supernatant and pellet were subjected to western analysis.

**Generating ribosome–nascent chains.** Plasmid or PCR product containing a truncated MDH1 gene was introduced into T₃/T₅ SP6 Quick Coupled Transcription/Translation System (Promega L2080). Usually, 1 µg of DNA was mixed with 40 µl of master mix and 2 µl of EasyTag Methionine L-[¹⁵⁴]Nε-N-709A500UC. The reaction was incubated for 1.5 h at 30°C. The lysate was loaded on sucrose cushion (25% sucrose, 20 mM HepesKOH pH 7.4, 2.5 mM DTT, 2 mM Mg(OAc)₂, 0.1 M KAc, 2.5 µg·ml⁻¹ leupeptin, 1.8 µg·ml⁻¹ Pepstatin A, 5 mM PMSF) and centrifuged using Beckman MicroUltra Centrifuge Rotor TLA-120.2 MicroFixed at 95,000 r.p.m. for 30 min at 4°C. For high-salt wash (Supplementary Fig. 3), the KC ac concentration was increased to 0.8 M. The RNCs pellet was washed and resuspended with 60 µl of import buffer (0.6 M sorbitol, 50 mM HepesKOH pH 7.4, 50 mM KCl, 10 mM MgCl₂, 2 mM KH₂PO₄, 1 mg·ml⁻¹ BSA, 0.75 mg·ml⁻¹ methionine and fresh 250 mM creatine phosphate and 5 mg·ml⁻¹ creatine kinase).

**Co-translational import assay.** Import assay was performed essentially as described in ref. 47. Briefly, RNCs were mixed with 25 µg of mitochondria in import buffer supplemented with 2 mM ATP and 8 mM NaN₃ in a total volume of 300 µl. At each time point (2, 5 or 10 min), 100 µl aliquots were transferred to a new ice-cold tube, 1 µg·ml⁻¹ of valinomycin was added and the sample was kept on ice. Half of each time point sample was treated with 2.5 µg protease K for 15 min on ice (protease K was inactivated by the addition of PMSF to a final concentration of 2 mM). Samples were centrifuged at 10,000g for 3 min at 4°C. Mitochondria pellets were washed with SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS pH 7.2) and resuspended with import buffer without BSA and analysed by polyacrylamide gel electrophoresis (PAGE). Radioactive signals were detected by phosphorimager and quantified with ImageQuant.

**Purification of yeast NAC complex from bacteria.** Purification of yeast NAC complex (EGD2–EGD1) from bacteria was performed essentially as was previously described in ref. 48. Briefly, the dual expression plasmid pOPE-HEG2-EGD1 (pAP96) was introduced into Escherichia coli strain MH1 together with a laq-Iq repressor (pA917) (plasmid and bacteria are a gift from Prof. Elke Deuring).
Cells were grown at 30 °C and NAC expression was induced with IPTG for 3 h. Cell lysate was subjected to initial purification on Ni²⁺-column (cat. 1018-25, Adar Biotech), utilizing the His6 tag present at the amino (N) terminus of EGD2. Eluted material was further purified by gel filtration size-exclusion column (Superdex 200, GE Healthcare) in buffer that contains 20 mM Tris pH 7, 500 mM NaCl and 5 mM MgCl₂. Both subunits were eluted at a fraction that corresponds to their combined mass (that is, as a formed complex).

### Statistical analyses
Statistical analyses were performed with SPSS. For each strain in each experiment, normality of the results was verified using either Shapiro–Wilks or Kolmogrov–Smirnov tests. Variances were calculated and accordingly P values were determined by independent-samples one-sided t-test.

### References
1. Wiedemann, N., van der Laan, M. & Pfanner, N. SnapShot: import and sorting of mitochondrial proteins. Cell 138, 808–808.e1 (2009).
2. Neupert, W. Protein import into mitochondria. Annu. Rev. Biochem. 66, 863–917 (1997).
3. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T. & Pfanner, N. Importing mitochondrial proteins: machineries and mechanisms. Cell 138, 628–644 (2009).
4. Rapaport, D. How does the TOM complex mediate insertion of precursor proteins into the mitochondrial outer membrane? J. Cell Biol. 171, 419–423 (2005).
5. Kelkem, R. E., Allison, V. F. & Butow, R. A. Cytoplasmic type 80 S ribosomes associated with yeast mitochondria. II. Evidence for the association of cytoplasmic ribosomes with the outer mitochondrial membrane in situ. J. Biol. Chem. 249, 3297–3303 (1974).
6. Kelkem, R. E. & Butow, R. A. Cytoplasmic type 80 S ribosomes associated with yeast mitochondria. J. Changes in the amount of bound ribosomes in response to changes in metabolic state. J. Biol. Chem. 249, 3304–3310 (1974).
7. Ahmed, A. U. & Fisher, P. R. Import of nuclear-encoded mitochondrial proteins: a cotranslational perspective. Int. Rev. Cell. Mol. Biol. 273, 49–68 (2009).
8. Lithgow, T. Targeting of proteins to mitochondria. FEBS Lett. 477, 22–26 (2000).
9. Marc, P. et al. Genome-wide analysis of mRNAs targeted to yeast mitochondria. EMBO Rep. 3, 159–164 (2002).
10. Elyahu, E. et al. tom20 mediates localization of mRNAs to mitochondria in a translation-dependent manner. Mol. Cell. Biol. 30, 284–294 (2010).
11. Garcia, M. et al. Mitochondria-associated yeast mRNAs and the biogenesis of molecular complexes. Mol. Cell. Biol. 18, 362–367 (2007).
12. Gadir, N., Haim-Vilmovsky, L., Kraut-Cohen, J. & Gerst, J. E. Localization of mRNAs coding for mitochondrial proteins in the yeast Saccharomyces cerevisiae. RNA 17, 1551–1565 (2011).
13. Saint-Georges, Y. et al. Yeast mitochondrial biogenesis: a role for the PUF RNA-binding protein Puf4p in mRNA localization. PLoS ONE 8, e2293 (2008).
14. Elyahu, E., Lesnik, C. & Arava, Y. The protein chaperone Saal affects mRNA localization to the mitochondrial matrix. FEBS Lett. 586, 64–69 (2012).
15. Garcia, M., Delaveau, T., Goussett, S. & Jacq, C. Mitochondrial presequence and open reading frame mediate asymmetric localization of messenger RNA. EMBO Rep. 11, 285–291 (2010).
16. Rospert, S., Dubaques, Y. & Gauthchi, M. Nascent-polypeptide-associated complex. Cell. Mol. Life Sci. 59, 1632–1639 (2002).
17. Wang, S., Sakai, H. & Wiedmann, M. NAC covers ribosome-associated nascent chains thereby forming a protective environment for regions of nascent chains just emerging from the peptidyl transferase center. J. Cell Biol. 130, 519–528 (1995).
18. Wiedmann, B., Sakai, H., Davis, T. A. & Wiedmann, M. A protein complex required for signal-sequence-specific sorting and translocation. Nature 370, 434–440 (1994).
19. George, R., Beddoe, T., Landi, K. & Lithgow, T. The yeast nascent polypeptide-associated complex initiates protein targeting to mitochondria in vivo. Proc. Natl Acad. Sci. USA 95, 2296–2301 (1998).
20. Reimann, B. et al. Initial characterization of the nascent polypeptide-associated complex in yeast. Yeast 15, 397–407 (1999).
21. Beatrix, B., Sakai, H. & Wiedmann, M. The alpha and beta subunit of the nascent polypeptide-associated complex have distinct functions. J. Biol. Chem. 275, 37838–37845 (2000).
22. del Alamo, M. et al. Defining the specificity of cotranslationally acting chaperones by systematic analysis of mRNAs associated with ribosome-nascent chain complexes. PLoS Biol. 9, e1001100 (2011).
23. Wiedmann, B. & Prehn, S. The nascent polypeptide-associated complex (NAC) of yeast functions in the targeting process of ribosomes to the ER membrane. FEBS Lett. 458, 51–54 (1999).
24. Funfschilling, U. & Rospert, S. Nascent polypeptide-associated complex stimulates protein import into yeast mitochondria. Mol. Biol. Cell 10, 3289–3299 (1999).
appreciated. This work was funded by an ISF grants number 1193/09 and 1096/13. Y.C. was supported by a Karen Siem Fellowship and Y.C. and M.S. are supported by an ERC StG 260395.

Author contributions
C.L. and Y.A. conceived the project; C.L. performed the Co-IP, ribosomal and mitochondrial association assays and the import assays; Y.C. and M.S. performed and analysed the DHFR PCA screen and the in vivo imaging; A.A.-L. performed some of the biochemical purifications; C.L., M.S. and Y.A. wrote the paper. All the authors discussed and analysed the data.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Lesnik, C. et al. OM14 is a mitochondrial receptor for cytosolic ribosomes that supports co-translational import into mitochondria. Nat. Commun. 5:5711 doi: 10.1038/ncomms6711 (2014).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/
Corrigendum: OM14 is a mitochondrial receptor for cytosolic ribosomes that supports co-translational import into mitochondria

Chen Lesnik, Yifat Cohen, Avigail Atir-Lande, Maya Schuldiner & Yoav Arava

Nature Communications 5:5711 doi: 10.1038/ncomms6711 (2014); Published 9 Dec 2014; Updated 30 Mar 2015

This Article was originally published without the accompanying Supplementary Table 1. This has now been corrected in the HTML version of the Article; the PDF was correct from the time of publication.