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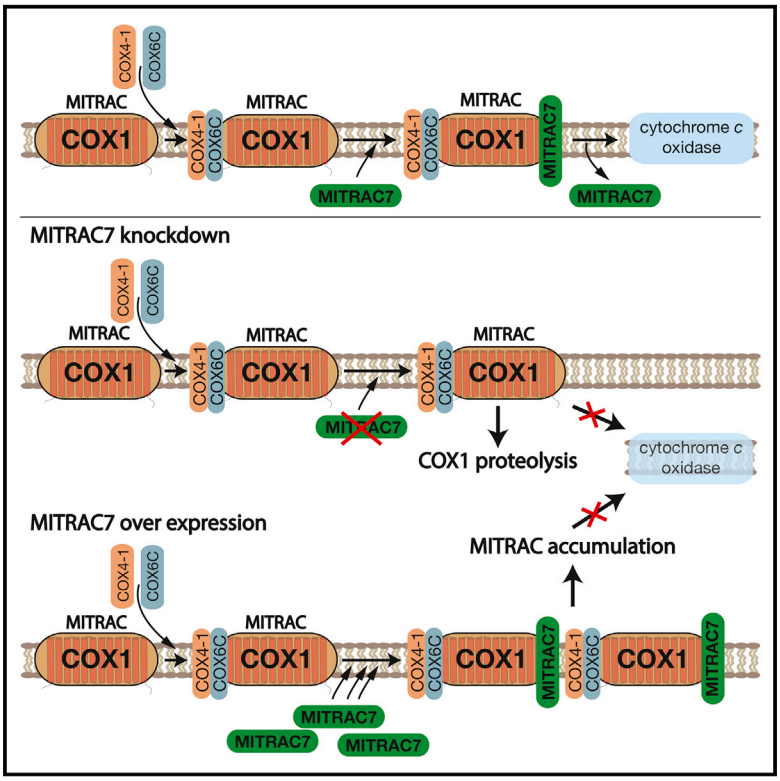
MITRAC7 Acts as a COX1-Specific Chaperone and Reveals a Checkpoint during Cytochrome *c* Oxidase Assembly

Graphical Abstract



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In Brief

Dennerlein et al. show that MITRAC7 is a COX1-specific chaperone that binds newly synthesized COX1 to stabilize the molecule during assembly. Although a lack of MITRAC7 causes turnover of newly synthesized COX1, overabundance of MITRAC7 traps COX1 in the MITRAC assembly intermediate. Hence, MITRAC7 reveals a checkpoint of the cytochrome *c* oxidase assembly process.

Highlights

- MITRAC7 is a component of early COX1 assembly intermediates
- Overexpression of MITRAC7 causes accumulation of complex IV assembly intermediates
- Lack of MITRAC7 causes turn over of complex IV assembly intermediates
- A quality control checkpoint exists for COX1 assembly intermediates

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MITRAC7 Acts as a COX1-Specific Chaperone and Reveals a Checkpoint during Cytochrome *c* Oxidase Assembly

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SUMMARY

Cytochrome *c* oxidase, the terminal enzyme of the respiratory chain, is assembled from mitochondria- and nuclear-encoded subunits. The MITRAC complex represents the central assembly intermediate during this process as it receives imported subunits and regulates mitochondrial translation of COX1 mRNA. The molecular processes that promote and regulate the progression of assembly downstream of MITRAC are still unknown. Here, we identify MITRAC7 as a constituent of a late form of MITRAC and as a COX1-specific chaperone. MITRAC7 is required for cytochrome *c* oxidase biogenesis. Surprisingly, loss of MITRAC7 or an increase in its amount causes selective cytochrome *c* oxidase deficiency in human cells. We demonstrate that increased MITRAC7 levels stabilize and trap COX1 in MITRAC, blocking progression in the assembly process. In contrast, MITRAC7 deficiency leads to turnover of newly synthesized COX1. Accordingly, MITRAC7 affects the biogenesis pathway by stabilizing newly synthesized COX1 in assembly intermediates, concomitantly preventing turnover.

INTRODUCTION

Mitochondrial oxidative phosphorylation provides the majority of ATP required to sustain the energetic requirements of eukaryotic cells. The respiratory chain complexes generate the proton gradient that drives the F_1F_o ATP-synthase that produces ATP from ADP and P_i. There are 13 core subunits of the oxidative phosphorylation system that are encoded within mtDNA and translated by membrane-associated ribosomes (Chacinska et al., 2009; Hällberg and Larsson, 2014; Mick et al., 2011; Ott and Herrmann, 2010). To form functional enzyme complexes, mitochondrial-encoded proteins associate with imported nuclear-encoded subunits in the inner mitochondrial membrane. This assembly process is highly regulated and a plethora of so-called assembly factors are required to stabilize maturing intermediates in the inner membrane, or to catalyze the insertion of cofactors, which are essential for the functionality of the complexes (Carr and Winge, 2003). However, for none of the known assembly factors, except for some enzymes involved in cofactor maturation, a molecular function or mechanism of action has been defined. Nevertheless, these assembly factors have been instrumental for defining distinct stages of the biogenesis process as well as regulatory processes. Malfunction of many of these assembly factors and the concomitant defects in the assembly process have been linked to severe human disorders that usually affect tissues with high energy demands, such as neurons, skeletal, and cardiac muscle (Bourens et al., 2013; Ghezzi and Zeviani, 2012; Shoubridge, 2001). While a loss of function of structural subunits can affect biogenesis of a given complex, the lack of an assembly factor can lead to a block in enzyme maturation. Concomitantly, intermediates of the assembly process have been frequently found to accumulate in patients with mitochondrial disorders (Ghezzi and Zeviani, 2012; Smeitink et al., 2006). While on the one hand a lack or reduction of any functional enzyme complex affects the overall performance of oxidative phosphorylation with systemic consequences for cellular metabolism, the accumulation of assembly intermediates may exacerbate the effect on the cell through the production of reactive oxygen species (Khalimonchuk et al., 2007; Shoubridge, 2001; Smeitink et al., 2006; Tatsuta and Langer, 2008). Hence, eukaryotic cells need to provide mechanisms that control respiratory chain biogenesis and protect mitochondria from damaging assembly intermediates.

In the case of cytochrome *c* oxidase (complex IV), the three core components, COX1, COX2, and COX3, are mitochondria-encoded. COX1 represents the nucleus for the biogenesis process to which imported subunits as well as COX2 and COX3,

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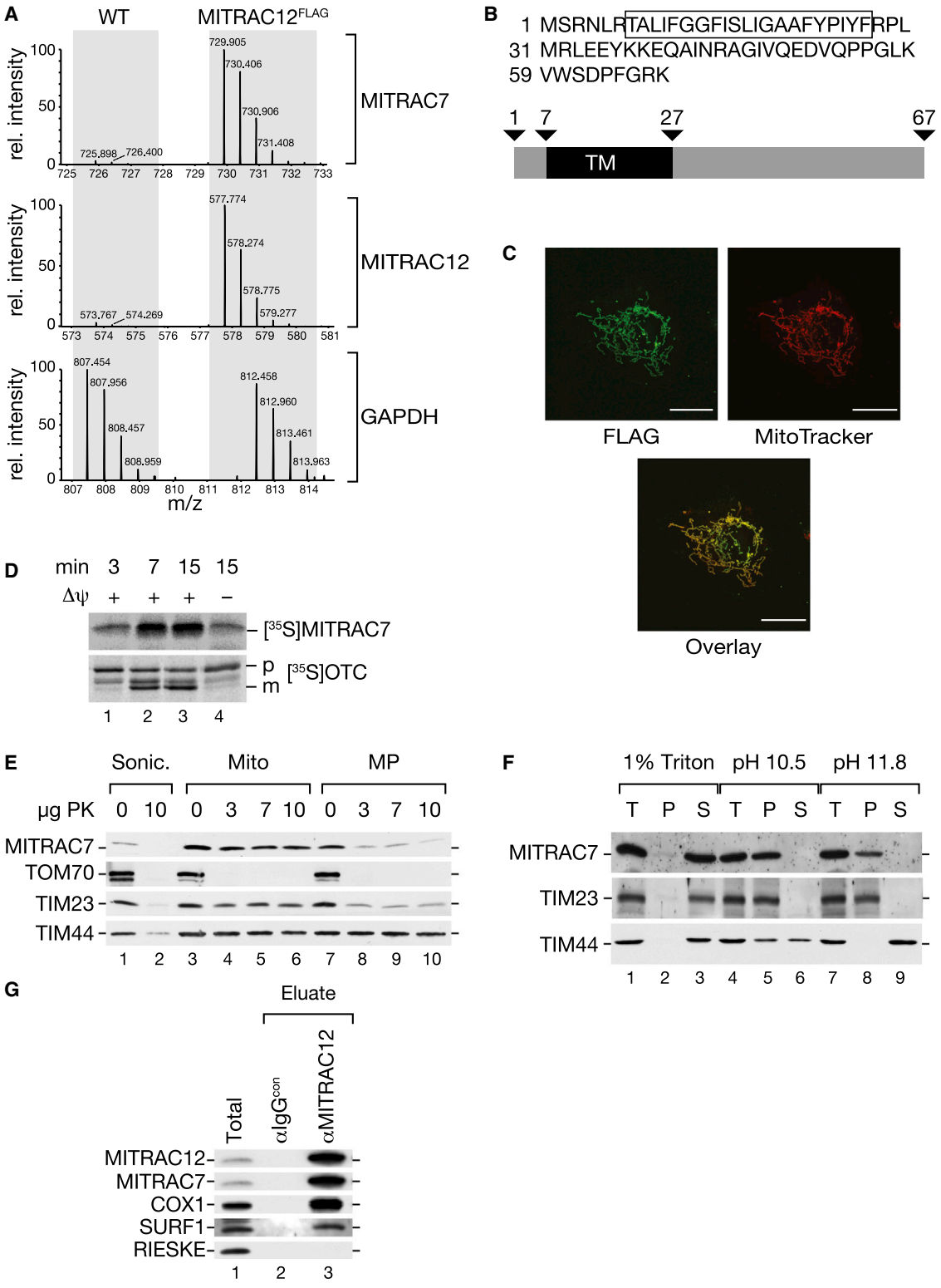

Figure 1. MITRAC7 Is a Mitochondrial Protein Associated with MITRAC

(A) Identification of MITRAC7. Sections of mass spectra of selected SILAC peptide pairs from MITRAC12 (bait), MITRAC7 (prey), and GAPDH (control) are shown.

(B) Deduced primary sequence of MITRAC7. The predicted transmembrane spans (TM) and protein organization (numbers indicate amino acid residues) are shown.

(legend continued on next page)

associate in a sequential manner. In yeast mitochondria, translation of *COX1* mRNA depends on specific translational activator proteins that interact with its 5′UTR (Barrientos et al., 2009; Hällberg and Larsson, 2014; Mick et al., 2012; Soto et al., 2012). The newly synthesized Cox1 protein engages with the early assembly factors Coa3 and Cox14 in the inner membrane of mitochondria, which stabilize the protein. Under conditions in which assembly of Cox1 becomes stalled, translation of *COX1* mRNA is shut down through inactivation of the translation-activator Mss51 (Barrientos et al., 2009; Mick et al., 2011; Soto et al., 2012). This feedback inhibition is thought to prevent an accumulation of assembly intermediates at the start of the biogenesis process (Barrientos et al., 2009; Mick et al., 2011). Similar translation regulating mechanisms have been found for *COB1* mRNA of the bc_1 complex (Hildenbeutel et al., 2014).

In contrast to yeast, human mitochondrial mRNAs lack 5′UTRs and functional homologs of the translation activators have not been identified. However, a regulatory cycle that links COX1 assembly to the translation of its mRNA is also present in human mitochondria, as suggested by several studies (Clemente et al., 2013; Mick et al., 2012; Szklarczyk et al., 2012; Weraarpachai et al., 2012). The newly synthesized COX1 protein rapidly incorporates into a multi-subunit assembly intermediate in the inner membrane termed mitochondrial translation regulation assembly intermediate of cytochrome *c* oxidase (MITRAC) complex. The two core components of MITRAC are MITRAC12 (COA3) and C12ORF62 (COX14). A lack of these proteins leads to cytochrome *c* oxidase deficiency and reduced COX1 translation (Mick et al., 2012; Ostergaard et al., 2015; Weraarpachai et al., 2012). Thus, at the early stage of cytochrome *c* oxidase biogenesis, a control mechanism is in place that allows mitochondria to adapt the synthesis of COX1 to cellular demands. However, the molecular mechanisms underlying this process are still unknown and it is clear that they will be significantly different from the processes in yeast.

The MITRAC complex receives early-assembling imported subunits of cytochrome *c* oxidase from the mitochondrial presequence translocase (Mick et al., 2012). This process requires the TIM21 protein, which shuttles between the TIM23 complex and MITRAC to deliver the imported subunits. Hence, the MITRAC complex in fact represents several crucial stages of distinct COX1 assembly intermediates during the early steps of cytochrome *c* oxidase biogenesis. Accordingly, COX1 biogenesis through MITRAC is a dynamic process. However, the distinct stages of this MITRAC-associated process remain to be defined.

Here, we report on the identification and functional analysis of MITRAC7. MITRAC7 represents an uncharacterized subunit of MITRAC that acts in the biogenesis pathway downstream of the translation feedback stage. Our analyses show that MITRAC7 is required for cytochrome *c* oxidase biogenesis. MITRAC7 promotes the progression of COX1 assembly after TIM21-dependent incorporation of the early subunit COX4-1. Our analyses suggest a second checkpoint in cytochrome *c* oxidase assembly downstream of COX1 translation regulation. While increased levels of MITRAC7 block COX1 assembly, stabilizing it as a late MITRAC intermediate, loss of MITRAC7 leads to turnover of COX1. We conclude that mitochondria monitor respiratory chain biogenesis to initiate stage-specific protective mechanisms during cytochrome *c* oxidase biogenesis. MITRAC7 is a chaperone-like assembly factor required to stabilize newly synthesized COX1 to prevent its premature turnover.

RESULTS

MITRAC7 Associates with MITRAC12 in the Inner Mitochondrial Membrane

The MITRAC complex represents a platform for the early steps of cytochrome *c* oxidase assembly, integrating the biogenesis of mitochondrial-derived COX1 with that of imported nuclear-encoded subunits. To identify and functionally define cytochrome *c* oxidase assembly factors in human mitochondria, we analyzed proteins in affinity purified MITRAC12-containing complexes by quantitative mass spectrometry using stable isotope labeling by amino acids in cell culture (SILAC) (Mick et al., 2012). Since assembly factors often represent small membrane proteins, we focused on proteins with a low molecular weight. Among proteins that copurified specifically with $MITRAC12^{FLAG}$ and displayed significant enrichment based on SILAC ratios, we identified the uncharacterized C4ORF52 protein that we later termed MITRAC7 (Figure 1A). Sequence similarity searches did not reveal potential homologs in lower eukaryotes. However, MITRAC7 is highly conserved in metazoa (Figure S1). MITRAC7 displays a single predicted transmembrane span, but lacks a predictable N-terminal presequence for translocation across the inner mitochondrial membrane (Figure 1B). To confirm the mitochondrial localization of MITRAC7, we transfected U2OS cells with a $MITRAC7^{FLAG}$-expressing plasmid. MITRAC7 was detected with monoclonal anti-FLAG antibodies and mitochondrial networks were visualized with MitoTracker red. Diffraction-limited confocal microscopy was performed and superimposition of green and red signals supports the mitochondrial localization of MITRAC7 (Figure 1C).

To assess processing and import, we synthesized [^{35}S] MITRAC7 in reticulocyte lysate. MITRAC7 could be imported into isolated mitochondria in a time-dependent manner to a protease-protected location. However, no processing of the imported protein was observed, supporting the prediction that it is not directed into mitochondria by a cleavable presequence (Figure 1D). Interestingly, a fraction of MITRAC7 was imported under conditions in which the membrane potential ($\Delta\psi$) had been

(C) Immunofluorescence microscopy of U2OS cells expressing $MITRAC7^{FLAG}$ using anti-FLAG antibodies and staining with MitoTracker red (scale bar represents 5 μm).
(D) [^{35}S]MITRAC7 and [^{35}S]OTC were imported into isolated mitochondria in the presence or absence of membrane potential ($\Delta\psi$). The samples were treated with PK, subjected to SDS-PAGE, and analyzed by digital autoradiography (precursor, p; mature protein, m).
(E) Submitochondrial localization of MITRAC7 analyzed by protease protection. PK was applied to mitochondria (Mito.), mitoplasts (hypotonically swollen mitochondria, MP), or to sonicated mitochondria (Sonic.).
(F) Membrane association of MITRAC7. The mitochondria were subjected to carbonate extraction or detergent lysis (total, T; pellet, P; and soluble faction, S).
(G) Immunoprecipitation using MITRAC12 and control antisera (αIgG^{con}) (Total, 1% and Eluate, 100%).

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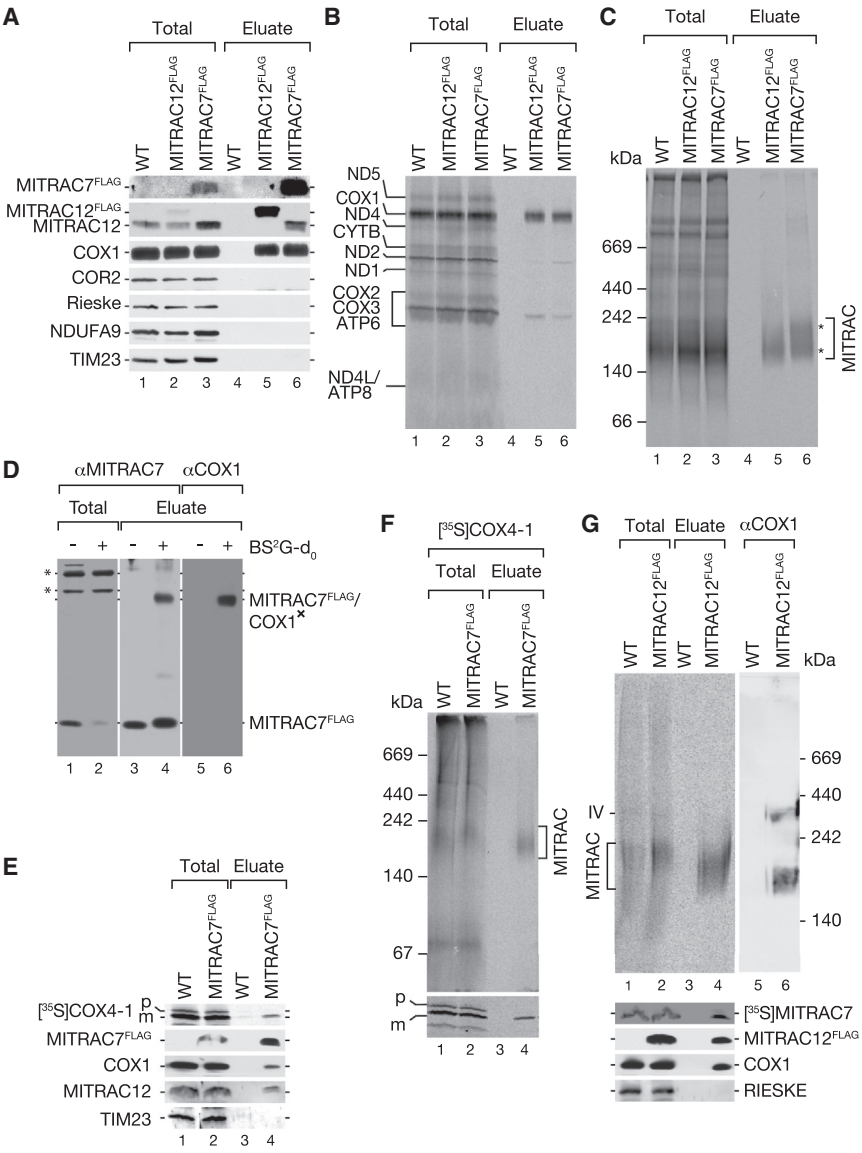

Figure 2. MITRAC7 Is a Constituent of the MITRAC Complex

(A) Immunoprecipitation from wild-type, MITRAC12^{FLAG}-, and MITRAC7^{FLAG}-containing mitochondria (Total, 1% and Eluate, 100%).

(B and C) Immunoprecipitation from whole cells as described in Figure 1A after 1 hr labeling of mitochondrial translation products with [^{35}S]methionine. The samples were analyzed by SDS- (B) or BN-PAGE (C) and digital autoradiography (Total, 2.5% and Eluate, 100%).

(D) MITRAC7^{FLAG}-containing mitochondria were treated with BS^2-d$_0$ crosslinker or left untreated and subjected to denaturing immunoprecipitations. The eluates were split and subjected to SDS-PAGE followed by western blotting (Total, 2% and Eluate, 100%, asterisk indicates unspecific signal).

(E) [^{35}S]COX4-1 was imported into mitochondria from wild-type or MITRAC7^{FLAG}-expressing cells. After import, immunoprecipitation was performed as in Figure 2A and samples were analyzed by SDS-PAGE and digital autoradiography (Total, 2% and Eluate, 100%); (precursor, p; mature, m)

(F) Import of [^{35}S]COX4-1 into isolated mitochondria containing MITRAC7^{FLAG}, followed by anti-FLAG immunoprecipitation under native conditions. The samples were separated either by SDS- (bottom) or BN-PAGE (top) and visualized by digital autoradiography (Total, 2% and Eluate, 100%).

(G) [^{35}S]MITRAC7 was imported into isolated mitochondria from MITRAC12^{FLAG}-containing cells and the experiment performed as in Figure 2E. The elution was split for BN-PAGE (60% for digital autoradiography, top left and 30% for western blotting, top right) and SDS-PAGE (10%, bottom) (complex VI, VI) (Total, 2%).

dissipated. In contrast, radiolabeled ornithine-transcarbamylase ([^{35}S]OTC) was imported in a $\Delta\psi$-dependent manner across the inner membrane (Figure 1D). The fact that small membrane proteins frequently display only limited dependency on $\Delta\psi$ is in agreement with previous findings (Mick et al., 2012; Wagner et al., 2009).

To address the submitochondrial localization of MITRAC7, we performed hypo-osmotic swelling and carbonate extraction experiments. The recovery of MITRAC7 in each sample was determined by western blot analysis using an antiserum directed against the C terminus of the protein. MITRAC7 was present in isolated mitochondria and only became accessible to protease treatment when the outer membrane was disrupted (Figure 1E). Since MITRAC7 was resistant to carbonate extraction (Figure 1F), we conclude that MITRAC7 is an inner mitochondrial membrane protein with its C terminus facing the intermembrane space (IMS).

To confirm the association between MITRAC12 and MITRAC7, we isolated MITRAC12-associated proteins by immunoprecipitation from digitonin solubilized mitochondria, using an antiserum directed against the C terminus of MITRAC12. Established MITRAC components, such as COX1 and SURF1, as well as MITRAC7, were efficiently copurified with MITRAC12 (Figure 1G). Thus, we conclude that MITRAC7 represents a MITRAC12-associated protein in the inner mitochondrial membrane.

MITRAC7 Interacts with Newly Synthesized COX1

To further investigate the association of MITRAC7 with cytochrome *c* oxidase assembly intermediates, we generated a stable cell line expressing MITRAC7 with a C-terminal FLAG-tag under control of an inducible promoter. Mitochondria from this cell line were isolated and subjected to immunoprecipitation experiments. For comparison, MITRAC12^{FLAG} was immunoprecipitated from a corresponding cell line (Mick et al., 2012). Both proteins were efficiently precipitated from detergent extracts and COX1 copurified in both samples. MITRAC12 was detected in the MITRAC7^{FLAG} elution, whereas subunits of complex III, complex I, and the TIM23 complex were not recovered (Figure 2A).

The copurification of COX1 with MITRAC7^{FLAG} prompted us to address whether MITRAC7 associates with newly synthesized

COX1. Therefore, we radiolabeled mitochondrial translation products prior to the isolation of MITRAC12^{FLAG}- and MITRAC7^{FLAG}-containing complexes. Similar to MITRAC12, newly synthesized COX1 was preferentially coisolated with MITRAC7 (Figure 2B). When samples were eluted under native conditions and subjected to separation by blue native (BN)-PAGE, complexes containing radiolabeled mitochondrial COX1 were visualized by digital autoradiography. As previously reported, isolation of MITRAC12^{FLAG} enriched the COX1-containing MITRAC complex (Mick et al., 2012) and a complex of similar size was detected in the MITRAC7^{FLAG} elution. However, compared to MITRAC12^{FLAG}, COX1 was also present in a slower migrating form of the MITRAC complex upon isolation with MITRAC7^{FLAG} (Figure 2C, lane 6). These observations demonstrate that MITRAC7 is a component of the MITRAC complex during early steps of COX1 assembly.

To investigate whether MITRAC7 directly interacts with COX1, we carried out crosslinking experiments. Therefore, mitochondria containing MITRAC7^{FLAG} were subjected to BS^2-d_0-mediated chemical crosslinking prior to MITRAC7^{FLAG} immunoprecipitation. As expected, a crosslink between MITRAC7 and COX1 was specifically recovered in immunoprecipitations from BS^2-d_0-treated samples, but not from the control (Figure 2D). Hence, we conclude that MITRAC7 directly contacts COX1.

To this end, we wanted to determine at which stage MITRAC7 participates in the assembly process and if MITRAC7 is present in MITRAC after incorporation of the first nuclear-encoded subunits. Therefore, we imported [^{35}S]COX4-1, an early-assembling subunit of cytochrome *c* oxidase, into MITRAC7^{FLAG}-containing mitochondria. As expected, [^{35}S]COX4-1 coisolated with MITRAC7^{FLAG}, indicating an association of MITRAC7 with early cytochrome *c* oxidase assembly intermediates (Figure 2E). This interpretation was further supported when isolations of MITRAC7^{FLAG} were analyzed by BN-PAGE. The [^{35}S]COX4-1-containing complexes migrated like the MITRAC complex, in the range of 200 kDa, and thus below the mature oxidase (Figure 2F). In agreement with this, when [^{35}S]MITRAC7 was imported into MITRAC12^{FLAG}-containing mitochondria prior to the isolation of MITRAC12^{FLAG}-containing complexes, [^{35}S]MITRAC7 was solely recovered in the MITRAC complex and not in mature cytochrome *c* oxidase. In contrast, in the same isolation, COX1 was detected with the MITRAC-complex and the mature cytochrome *c* oxidase (Figure 2G).

Interaction of MITRAC7 with COX1 Depends on Integration of Nuclear-Encoded Subunits into MITRAC

Our analyses indicated that the MITRAC complex represents a population of COX1-containing complexes, which differ to some extent in their protein composition (Mick et al., 2012; Figure 2C). Among these complexes, MITRAC7 appears to be present in a slower migrating form of MITRAC compared with MITRAC12. This interpretation was corroborated when mitochondrial protein complexes were separated in a first-dimension by BN-PAGE and subsequently subjected to 2D separation by SDS-PAGE (Figure S2A). MITRAC7 clearly comigrated with the MITRAC complex as expected, based on the previous experiments (see Figure 2C). However, the majority of MITRAC7 was present in the slower migrating MITRAC-form (Figure S2A).

Hence, we asked at which stage of the assembly process MITRAC7 acts. After initial association of COX1 with MITRAC12 and C12ORF62, nuclear-encoded cytochrome *c* oxidase subunits are integrated into MITRAC through TIM21. To assess whether MITRAC7 and TIM21 are present in MITRAC complexes simultaneously, we isolated complexes via MITRAC12^{FLAG} and MITRAC7^{FLAG}. While MITRAC12^{FLAG} efficiently coisolated TIM21, only low amounts of TIM21 were present in MITRAC7^{FLAG} samples (Figure 3A). Moreover, when MITRAC12^{FLAG}-, MITRAC7^{FLAG}-, or TIM21^{FLAG}-containing complexes were purified after radiolabelling of mitochondrial translation products and separation by BN-PAGE, different sets of complexes were apparent. MITRAC7^{FLAG} enriched two distinct COX1-containing complexes. Interestingly, TIM21^{FLAG} purified a complex that migrates between the two complexes apparent in the MITRAC7-^{FLAG} sample (Figure 3B). Thus, we conclude that MITRAC7 is a constituent of complexes largely devoid of TIM21.

To address whether MITRAC7 acts prior to the insertion of nuclear-encoded proteins, or downstream of TIM21, we analyzed if the interaction of MITRAC12 or TIM21 with COX1 depends on the presence of MITRAC7. Thus, we assessed the binding of MITRAC12 or TIM21 to newly synthesized COX1 in MITRAC7-depleted cells (see also Figure 4). Mitochondrial translation products were pulse labeled in MITRAC7 depleted cells and TIM21^{FLAG}- or MITRAC12^{FLAG}-containing complexes were purified. The association of newly synthesized COX1 with TIM21^{FLAG} and MITRAC12^{FLAG} (Figures 3C and 3D) remained unaltered, demonstrating that the interactions of TIM21 and MITRAC12 with newly synthesized COX1 occur independent of MITRAC7.

Based on this observation and the migration of MITRAC7 in a large MITRAC-complex, we hypothesized that MITRAC7 acts at a later stage of COX1 assembly rather than at the step of TIM21-dependent insertion of nuclear-encoded subunits. To test this directly, we established immunoprecipiation conditions for the endogenous MITRAC12 and MITRAC7 proteins. After radiolabelling of mitochondrial translation products, proteins copurifying with MITRAC12 and MITRAC7 were separated by SDS-PAGE. Among the mitochondrial-encoded proteins, COX1 was specifically enriched in MITRAC12 and MITRAC7 eluates (Figure 3E, left). This result is consistent with our previous analyses using the FLAG-tagged proteins (see Figure 2A). Western blot analyses showed that MITRAC12 and MITRAC7 were enriched in the eluates, coisolated each other efficiently, but did not immunoprecipitate abundant mitochondrial proteins such as the RIESKE Fe/S protein (Figure 3E, right).

Hence, we addressed the association of newly synthesized COX1 with MITRAC12 and MITRAC7 in the absence of TIM21. In general, TIM21-depleted cells displayed a subtle increase in the translation of all mitochondrial-encoded proteins (Figures S2B and S2C). While a lack of TIM21 did not affect the association of MITRAC12 with COX1 (Figures 3F and S2B), we observed a significant reduction in the amount of COX1 coisolating with MITRAC7 (Figures 3F and S2C). This finding prompted us to investigate whether the interaction of MITRAC7 with newly synthesized COX1 depends on the integration of early-assembling nuclear-encoded cytochrome *c* oxidase subunits. Therefore, we depleted COX4-1 and COX6C. Both proteins were efficiently reduced after small interfering (si)RNA treatment (Figures S2D

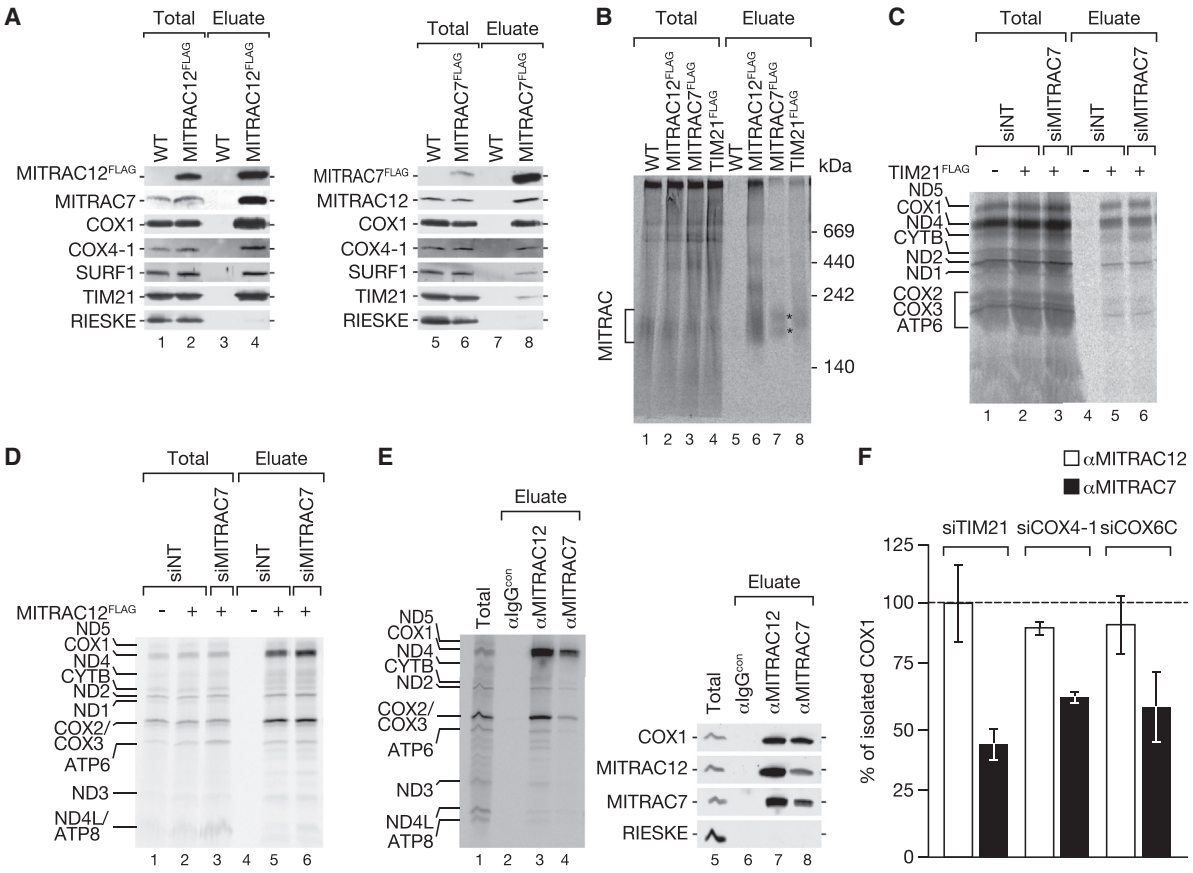

Figure 3. COX1-MITRAC7 Interaction Depends on Incorporation of Nuclear-Encoded COX-Subunits

(A) Mitochondria from wild-type, MITRAC12^{FLAG}-, and MITRAC7^{FLAG}-expressing cells were solubilized and subjected to anti-FLAG immunoprecipitation. The eluates were analyzed by SDS-PAGE and western blotting (Total, 1% and Eluate, 100%).

(B) Mitochondrial translation products were radiolabeled in wild-type, MITRAC12^{FLAG}-, and MITRAC7^{FLAG}-expressing cells. The anti-FLAG immunoprecipitations were performed as described in (A) and samples were analyzed by BN-PAGE and digital autoradiography (Total, 10% and Eluate, 100%).

(C and D) TIM21^{FLAG}- (C) or MITRAC12^{FLAG}- (D) expressing cells were treated with MITRAC7-specific or non-targeting (NT) control siRNA for 72 hr. After [^{35}S] methionine labeling of mitochondrial translation products, the whole cell lysates were subjected to anti-FLAG immunoprecipitation. The eluates were analyzed by SDS-PAGE and digital autoradiography (Total, 2% and Eluate, 100%)

(E) Antibodies against MITRAC12, MITRAC7, or control antisera (αIgG^{con}) were used for immunoisolation after [^{35}S]methionine labeling of mitochondrial translation products and analyzed by SDS-PAGE and digital autoradiography (Total, 7% and Eluate, 100%).

(F) Quantification of isolated COX1 from immunoprecipitations with anti-MITRAC12 or anti-MITRAC7 antibodies after TIM21, COX4-1, or COX6C depletion (mean ± SEM and n = 3).

and S2E). Upon COX4-1 and COX6C knockdown, the interaction of MITRAC7 with newly synthesized COX1 was significantly reduced. In contrast, the association of MITRAC12 with COX1 was only marginally affected (Figure 3F). In conclusion, the association of newly synthesized COX1 with MITRAC7 depends on the TIM21-mediated incorporation of nuclear-encoded subunits into MITRAC.

MITRAC7 Is Required for Cytochrome *c* Oxidase Assembly

To assess the function of MITRAC7, we generated two different siRNAs targeting MITRAC7 and transfected these into human embryonic kidney cell lines (HEK)293T cells. Reduction of MITRAC7 levels resulted in a severe growth phenotype compared to the non-targeting control (Figure 4A). To exclude off-target effects, we created a stable HEK293T-cell line expressing an siRNA1-resistant *MITRAC7^{FLAG}* transcript, encoding scMITRAC7^{FLAG}. Induction of scMITRAC7^{FLAG} fully rescued the growth phenotype after siRNA application (Figure 4B) despite the fact that scMITRAC7^{FLAG} was slightly less abundant than the endogenous MITRAC7 protein (Figure 4C). These results verified the specific targeting of MITRAC7 by siRNA1 and the functionality of the MITRAC7^{FLAG} construct. Since MITRAC7 and MITRAC12 are present in the same complex, we investigated the interdependence of both proteins by performing protein steady-state level analysis after depletion experiments. The stability of both proteins did not depend on the presence of the other protein (Figures S3A and S3B).

Next, we investigated the effect of MITRAC7 knockdown on cytochrome *c* oxidase biogenesis. Therefore, we analyzed

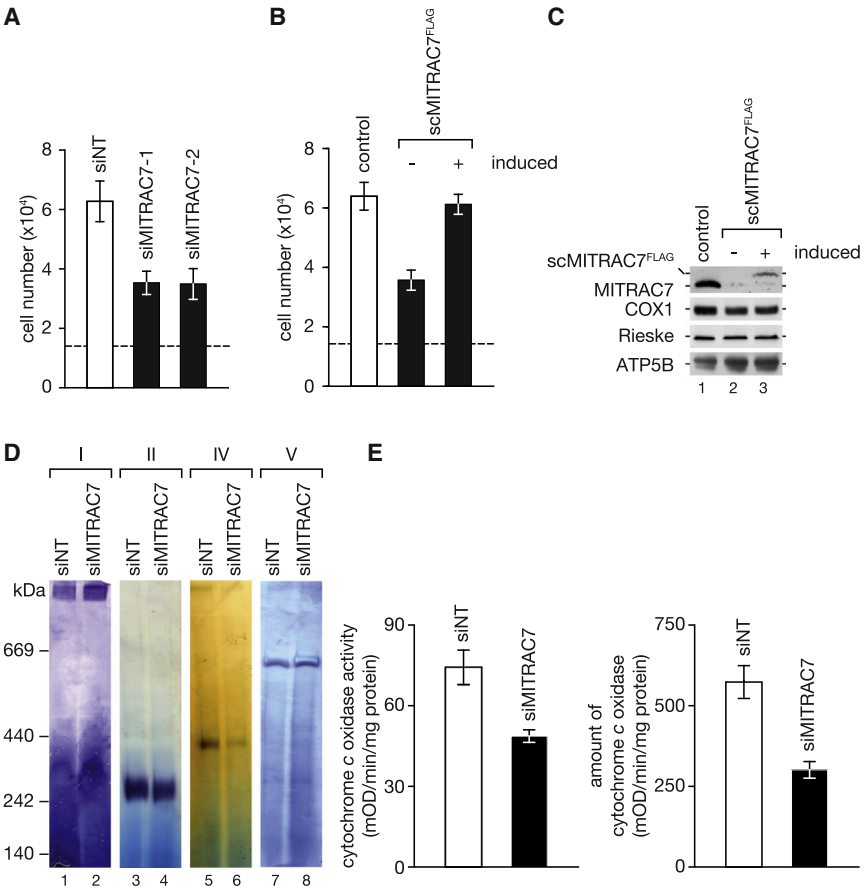

Figure 4. MITRAC7 Is Required for Cytochrome *c* Oxidase Biogenesis

(A) HEK293T cells were transfected with siRNAs against MITRAC7 or non-targeting (NT) control siRNA as indicated. The cells were grown for 72 hr and counted after harvesting (dotted-line marks starting amount of cells; 1.25×10^4) (mean ± SEM and n = 6).

(B and C) Wild-type HEK293T cells or cells expressing the siRNA-resistant scMITRAC7^{FLAG} were transfected, cultured, and counted as in Figure 4A (dotted-line marks starting amount of cells; 1.25×10^4) (mean ± SEM and n = 6) and further analyzed by SDS-PAGE and western blotting (C).

(D) Isolated mitochondria from cells treated with MITRAC7 siRNA1 as in Figure 4A were analyzed by BN-PAGE and in-gel activity assays for complexes I, II, IV, and V.

(E) Measurement of enzyme activity (left) and relative amount of cytochrome *c* oxidase (right); the siRNA treatment was performed as described in (A) (mean ± SEM and n = 6).

respiratory chain complexes after solubilization by BN-PAGE and performed in-gel activity staining for complexes I, II, IV, and V. While the activities of complex I, II, and V were not decreased upon knock down of MITRAC7, complex IV activity was drastically reduced (Figure 4D). For a quantitative assessment, we measured cytochrome *c* oxidase activity and quantified the amount of enzyme by ELISA. In MITRAC7 knockdown cells, the cytochrome *c* oxidase activity was reduced to ~65% compared to the non-targeting siRNA control (Figure 4E, left). This reduction in activity was fully explained by a drastic reduction of complex IV to ~45%, as compared to the control (Figure 4E, right). Accordingly, loss of MITRAC7 in HEK293T cells leads to a loss of cytochrome *c* oxidase.

Increasing MITRAC7 Levels Affect Cytochrome *c* Oxidase Biogenesis

Given that loss of MITRAC7 resulted in complex IV deficiency, we sought to investigate the phenotype of MITRAC7 overexpressing cells. To this end, MITRAC7^{FLAG} was expressed at a ~10-fold higher amount compared with the endogenous MITRAC7 level (Figure 5A). Cells with increased MITRAC7^{FLAG} levels displayed a clear growth phenotype at the tested time points (Figure 5A). To study the amount and activity of cytochrome *c* oxidase in mitochondria isolated from cells overexpressing MITRAC7^{FLAG}, compared with control cells, we performed in-gel activity assays. The activities of complex I and the F_1F_o ATP-synthase were not affected by MITRAC7^{FLAG} overexpression, but a subtle increase in complex II activity was detected. However, a drastic reduction of cytochrome *c* oxidase activity was apparent (Figure 5B). Given that in-gel activity staining is not quantitative, we overexpressed MITRAC7^{FLAG} and quantified the activity and amount of cytochrome *c* oxidase in cell extracts. Cytochrome *c* oxidase activity was severely reduced after 1 day of MITRAC7^{FLAG} induction and decreased even further after 3 days (Figure 5C, left). Similarly, the amount of complex IV was reduced compared to the control (Figure 5C, right), thereby explaining the reduction in cytochrome *c* oxidase activity. Thus, we conclude that a tightly balanced level of MITRAC7 is critical for cytochrome *c* oxidase assembly. Reduced, as well as increased, amounts of MITRAC7 impact the biogenesis of the enzyme complex.

Loss of MITRAC7 Destabilizes COX1 in Mitochondria

Defects or loss of the MITRAC complex constituents C12ORF62 or MITRAC12 selectively affect COX1 translation (Mick et al., 2012; Ostergaard et al., 2015; Weraarpachai et al., 2012). As MITRAC7 similarly binds newly synthesized COX1, we analyzed mitochondrial protein synthesis in cells overexpressing MITRAC7^{FLAG} or in which MITRAC7 levels were reduced by siRNA-mediated knockdown. Upon MITRAC7^{FLAG} overexpression, a reduction in the amount of newly synthesized COX1 was apparent when mitochondrial translation products were pulsed labeled with [^{35}S]methionine (Figure 6A). In contrast, subunits of other complexes were not affected, e.g., ND1/ND2. Quantifications confirmed these significantly reduced COX1 levels upon MITRAC7^{FLAG} overexpression (Figure 6A, right). In contrast, after [^{35}S]methionine pulse labeling, MITRAC7

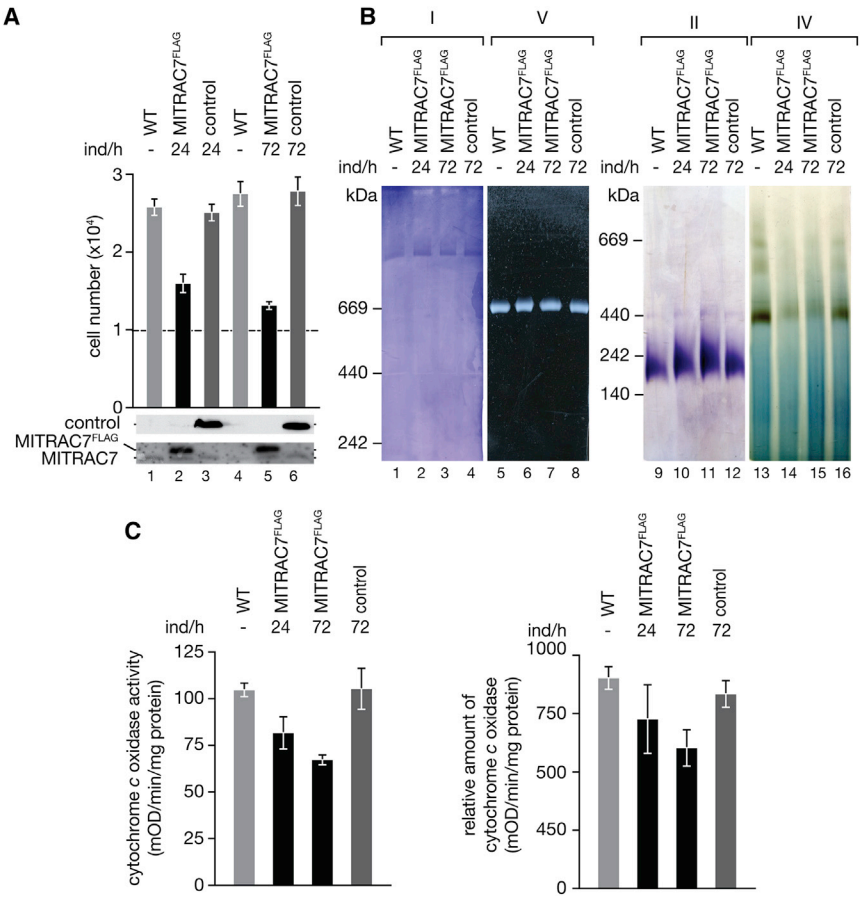

Figure 5. Cytochrome *c* Oxidase Biogenesis Is Affected by Increased MITRAC7 Levels

(A) HEK293T cells were counted after indicated induction times (ind/h) of MITRAC7^{FLAG} or an unrelated control mitochondrial protein (top, mean ± SEM and n = 6) or analyzed by SDS-PAGE and western blotting (bottom).

(B) In-gel activity assays for complexes I, II, IV, and V after mitochondria isolation from cells induced for expression of MITRAC7^{FLAG} or a control protein.

(C) Quantification of cytochrome *c* oxidase activity (right) and amount (left) as in Figure 4E after overexpression of MITRAC7 (mean ± SEM and n = 6).

depleted cells did not display a significant change in the amount of mitochondrial-encoded proteins (Figure 6B).

This experiment did not provide an explanation as to why MITRAC7 overexpression and knockdown similarly lead to cytochrome *c* oxidase deficiency. Therefore, we carried out pulse chase analyses to follow the fate of newly synthesized COX1 over the course of 12 hr. This time frame is usually sufficient for newly synthesized COX1 to be incorporated into the mature complex. Despite the reduced amount of COX1 observed under conditions of a short pulse (Figure 6A), in an extended chase, the newly synthesized COX1 accumulated over time when MITRAC7 was overexpressed (Figures 6C and S3C). In contrast, ND1/ND2 did not display significant changes in their amount. Surprisingly, in MITRAC7 depleted cells, that displayed no difference to the wild-type with regard to COX1 upon pulse labeling (Figure 6B), we observed less COX1 than in the control in an extended chase analysis, indicative of decreased COX1 stability. At the same time ND1/ND2 did not display significant changes in their stability (Figures 6C and S3D). Accordingly, although both loss of MITRAC7 and its overproduction caused cytochrome *c* oxidase deficiency, we observed opposing effects on the stability of newly synthesized COX1 in mitochondria. While an increased turnover of COX1 explains the defect in cytochrome *c* oxidase biogenesis for the knockdown condition, the increased stability of COX1 in MITRAC7 overexpressing cells could still not be directly linked to a loss of the cytochrome *c* oxidase.

We hypothesized that the newly synthesized COX1, which accumulates upon MITRAC7 overexpression, reflects an arrest of COX1 assembly at the level of MITRAC. To address this hypothesis, we labeled mitochondrial translation products in vivo and analyzed COX1-containing complexes after an extended chase. Complexes were separated in a first dimension by BN-PAGE and subsequently subjected to a 2D separation by SDS-PAGE. For comparison, we detected selected MITRAC components by western-blotting after digital autoradiography visualization of the [^{35}S]methionine-labeled protein (Figure 6D). As demonstrated by MITRAC12 and MITRAC7 detection, the MITRAC complex, in the control, migrated between 230 kDa and 400 kDa, whereas MITRAC7 overexpression resulted in a shift of MITRAC7 and MITRAC12 to a slower migrating form of the complex. Clearly, newly synthesized COX1 accumulated in MITRAC of mitochondria with increased MITRAC7 levels and COX1 did not assemble into mature cytochrome *c* oxidase compared to control cells (Figure 6D). This observation is in agreement with the presence of MITRAC7 in slower migrating MITRAC complexes and explains the increased COX1 stability upon MTRAC7^{FLAG} overexpression. To address the presence of newly synthesized COX1 in MITRAC directly, we immunoprecipitated MITRAC12 after pulse chase labeling of mitochondrial-encoded proteins in MITRAC7 overexpressing and control cells. Eluates were subjected to SDS-PAGE and copurified, radiolabeled proteins were visualized by digital autoradiography (Figure 6E). The coisolation of newly synthesized COX1 with MITRAC12 was drastically increased when MITRAC7 was overexpressed (Figure 6E). Hence, an increase in the amount of MITRAC7 leads to COX1 accumulation in MITRAC. Taken together, with the observed lack of mature cytochrome *c* oxidase, we conclude that increased levels of MITRAC7 stall COX1 during the assembly process.

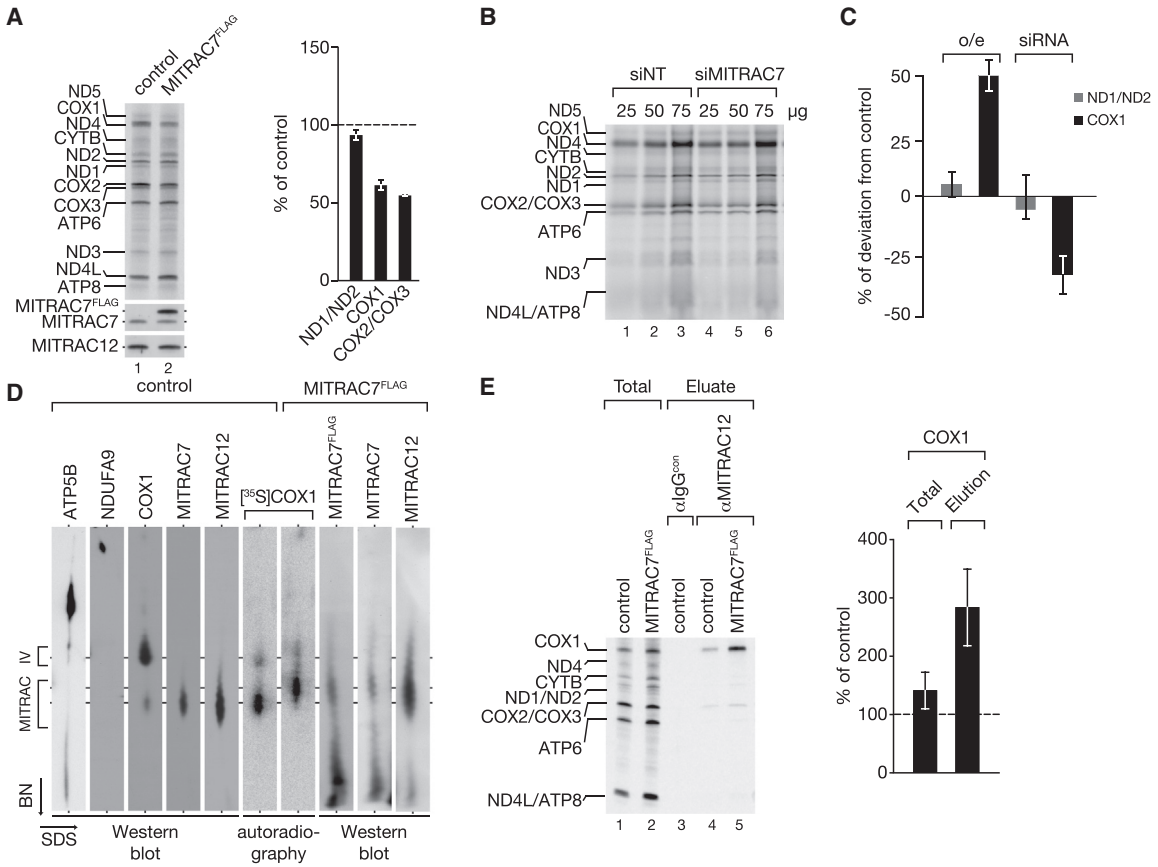

Figure 6. MITRAC7 Stabilizes Newly Synthesized COX1

(A) After 72 hr of MITRAC7^{FLAG} induction in HEK293T cells, mitochondrial translation products were [^{35}S]methionine pulse labeled for 1 hr. The cell extracts were subjected to SDS-PAGE followed by western blotting and were analyzed by digital autoradiography (left). The values represent quantifications of the indicated mitochondrial translation products normalized to ATP6 (mean ± SEM and n = 3).

(B) Labeling of mitochondrial translation products after siRNA mediated MITRAC7 depletion. The cell extracts were subjected to SDS-PAGE followed by western blotting and digital autoradiography.

(C) [^{35}S]methionine labeling of mitochondrial translation products as in Figures 6A and 6B. Subsequently, the medium was replaced and cells were further cultured in standard medium (chase; 12 hr). The cell extracts were analyzed by SDS-PAGE (Figures S3C and S3D). The values represented were normalized to ATP6 (mean ± SEM and n = 3).

(D) Pulse chase [^{35}S]methionine-labeling of mitochondrial translation products after MITRAC7^{FLAG} overexpression as described in Figure 6C. The proteins from whole cells were isolated under non-denaturing conditions and complexes were separated by BN-PAGE, followed by a second dimension SDS-PAGE and western blot analysis. The proteins were detected by digital autoradiography (COX1) or by using indicated antibodies (complex IV, IV).

(E) [^{35}S]methionine labeling of mitochondrial translation products (pulse) followed by chase after MITRAC7^{FLAG} expression as described in Figure 6D. The whole cell extracts were subjected to immunoprecipitation using anti-MITRAC12 or control antiserum (αIgG^{con}). The eluates were analyzed by digital autoradiography after SDS-PAGE (Total, 5% and Eluate, 100%) and quantified using ImageQuant software.

DISCUSSION

The MITRAC complex defines an early stage in assembly of the cytochrome *c* oxidase (Clemente et al., 2013; Mick et al., 2012; Ostergaard et al., 2015; Peralta et al., 2012; Szklarczyk et al., 2012; Weraarpachai et al., 2012). MITRAC receives early-assembling nuclear-encoded subunits, which are imported into mitochondria along the presequence import pathway. At the same time, MITRAC constituents have been implicated in the regulation of COX1 translation on mitochondrial ribosomes, but may at the same time contribute to the stabilization of the newly synthesized COX1 protein. It is conceivable that both of these functions are tightly linked. A translational regulation by MITRAC constituents plays an important role for the fidelity of the assembly process (Mick et al., 2012; Weraarpachai et al., 2012). Upon an early block in assembly and the concomitant accumulation of COX1, the supply of newly synthesized COX1 is stalled to prevent further accumulation of MITRAC complexes. Accordingly, this stage of translational regulation represents an early regulatory step in cytochrome *c* oxidase assembly, adapting COX1 availability to the influx of early-assembling imported subunits. Moreover, this regulatory stage allows cells to provide a readily available pool of COX1 in the inner membrane as a seed for assembly. While in yeast mitochondria a translational feedback mechanism for Cox1 expression is mediated by mRNA-specific translation activators, the molecular details of the feedback

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regulation process in metazoa are only beginning to be approachable through the identification of MITRAC12 and C12ORF62 (for review see Soto et al., 2012; Dennerlein and Rehling, 2015). An experimental challenge in defining proteins that participate in translation regulation is the fact that a loss of COX1 can be due to reduced synthesis, as well as to an increased turnover. Hence, detailed experimental analyses are required to differentiate between these two possibilities. However, obviously translation regulation in yeast and metazoan have to be mechanistically very different. In yeast *coa3Δ* and *cox14Δ* mutants the half time of Cox1 is in the range of a few minutes, while the synthesis of Cox1 is increased by several-fold through hyperactivation of Mss51. Hence, Coa3 and Cox14 do not affect translation directly, but do so indirectly through the translational activator Mss51 (for review see Mick et al., 2011). Despite low sequence similarity between these yeast proteins and MITRAC12 and C12ORF62, respectively, the human proteins fulfill opposite function in human mitochondria (Mick et al., 2012; Ostergaard et al., 2015; Weraarpachai et al., 2012).

We find that MITRAC represents a population of complexes that are ill defined in composition and function. Our analyses identify MITRAC7 as a so far uncharacterized constituent of a late MITRAC complex. MITRAC7 is required for assembly of cytochrome *c* oxidase. Interestingly, by unbalancing MITRAC7 availability in mitochondria, the assembly process of cytochrome *c* oxidase is affected in different ways. While a loss of MITRAC7 leads to turn over of COX1, overabundance of MITRAC7 stalls assembly and stabilizes newly synthesized COX1. Under both conditions, the translation of COX1 mRNA remains unaffected, indicating that the stage of the assembly process at which MITRAC7 acts has passed beyond the stage of translational feedback regulation of COX1. Our findings reveal a checkpoint in the assembly pathway of COX1. Lack of MITRAC7 is accompanied by proteolytic removal of COX1. The degradation of COX1-containing assembly intermediates upon loss of an assembly factor is not a general phenomenon that occurs at every stage of an improper cytochrome *c* oxidase biogenesis process. In fact, a number of patients with mutations in cytochrome *c* oxidase assembly factors have been found to accumulate intermediates of the assembly pathway, while others do not display significant amounts of immature complexes (Antonicka et al., 2003a, 2003b; Bourens et al., 2014; Ghezzi and Zeviani, 2012; Huigsloot et al., 2011; Leary et al., 2004; Lim et al., 2014; Oláhová et al., 2015; Ostergaard et al., 2015; Stiburek et al., 2009, 2005; Tiranti et al., 1999; Weraarpachai et al., 2009; Williams et al., 2004). Hence, the assembly pathway appears to progress through distinct intermediates that are subject to quality control processes at distinct stages. Despite a physical interaction between MITRAC7 and COX1, our analyses do not demonstrate a direct role for MITRAC7 in quality control. However, our analyses assign a chaperone-like function to MITRAC7, it stabilizes COX1 in the assembly process. It is likely that MITRAC-mediated stabilization of COX1 is required until additional subunits engage with the COX1-containing intermediate.

We show that MITRAC7 acts at a stage of the assembly process at which first nuclear-encoded subunits of the cytochrome *c* oxidase have already been integrated into the maturing COX1-containing MITRAC complex. Overexpression of MITRAC7 stalls the COX1 assembly process and leads to the accumulation of a slower migrating form of MITRAC. Moreover, when MITRAC7-containing complexes are isolated, they contain COX4-1. The knockdown of MITRAC7 did not affect the association of TIM21 with newly synthesized mitochondria-encoded proteins, which are present in early assembly intermediates of complexes I and IV (Mick et al., 2012). Upon knock down of TIM21, COX4-1, or COX6C, the association between newly synthesized COX1 and MITRAC7 is drastically reduced, while the interaction between COX1 and the early acting MITRAC12 remains unaffected. Hence, we conclude that MITRAC7 is dispensable for early stages of COX1 assembly, but acts after the association of COX1 with COX4-1 and COX6C. At this point, MITRAC7 is required for progression of the assembly process. Once MITRAC7 function is lacking, assembly cannot continue and the accumulating transient assembly intermediate is obviously recognized by a yet undefined mechanism and concomitantly removed. Under conditions of MITRAC7 over abundance, MITRAC7 associates with COX1 and apparently protects the COX1-containing assembly intermediate in agreement with a stabilizing chaperone-like function.

In summary, our analyses suggest that two distinct checkpoints during the assembly process of the cytochrome *c* oxidase exist. The presence of control elements, that evaluate the abundance of intermediates, initiate processes to adjust the balance within the cytochrome *c* oxidase assembly process, or that stabilize transient intermediates, are essential to monitor and coordinate formation of enzyme complexes derived from subunits of dual genetic origin. In this process, MITRAC7 represents a chaperone-like assembly factor that stabilizes COX1 in a late MITRAC form, timing the catch to receive additional subunits.

EXPERIMENTAL PROCEDURES

Cell Culture

HEK293-Flp-In™ T-REx™ 293 or HEK293T were cultured in DMEM, supplemented with 10% (volume/volume [v/v]) fetal bovine serum (FBS) (Biochrom), 2 mM L-glutamine and 50 μg/ml uridine at 37°C under a 5% CO_2 humidified atmosphere. A HEK293T cell line, with inducible expression of the C-terminal FLAG-tagged MITRAC7 (NM_001145432.1) protein, was generated as described previously (Mick et al., 2012).

Immunofluorescence

Immortalized human osteosarcoma cells (U2OS) were incubated with MitoTracker red under cell culture conditions for 5 min. The cells were fixed with 4% paraformaldehyde (PFA) for 20 min at 37°C and permeabilized with 0.2% Triton. The cells were blocked with 1% BSA and incubated with anti-FLAG antibodies at room temperature (RT). The secondary antibody (anti-mouse M488) was applied for 1 hr at RT. Light microscopy was performed using a Leica SP5 confocal microscope (Leica Microsystems) equipped with a 1.40 NA oil immersion objective (Leica HCX PL APO lambda blue 63× /1.40–0.60 Oil UV). The image stacks were taken and maximum projections of the stacks were generated.

siRNA Constructs and Transfection

The following sequences and concentrations were used: TIM21 (5′-CCCAG GAAGUGGUGAAUAU-3′; 8.25 nM), MITRAC12 (5′-CGCAGUUGUUACGAG GUUA-3′, 33 nM), MITRAC7 (5′-GAAGGAACAAGCUAUAAAU-3′, 33 nM), COX4-1 (5′- GUCGAGUUGUAUCGCAUUA-3′, 33 nM), and COX6C (5′-GG CUGGUAUCUUUCAGAGU-3′, 33 nM). Approximately 2.5 × 10^5 cells were seeded in a 25 cm^2 flask and transfected as described previously (Ovcharenko

et al., 2005) using Lipofectamine™ RNAiMAX (Invitrogen). The cells were cultured for 72 hr prior to analyses.

Protein Localization and Protease Protection Assays

Carbonate extraction and mitochondrial swelling experiments were essentially performed as previously described (Mick et al., 2012). Briefly, isolated mitochondria were suspended in 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH7.2), 50 mM NaCl containing either 1% Triton X-100 or 0.1 M carbonate at pH 10.5 or 11.8. Samples were centrifuged at 55,000 rpm and 4°C for 45 min in a TLA-55 rotor (Beckman Coulter). To analyze submitochondrial localization, samples were either suspended in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS [pH 7.2]), to osmotically stabilize mitochondria, or in EM buffer (1 mM EDTA, and 10 mM MOPS [pH 7.2]), to rupture the outer mitochondrial membrane, followed by the addition of proteinase K (PK). As a positive control, mitochondria were lysed with 1% Triton X-100 in the presence of PK. The reaction was stopped after 10 min by addition of PMSF (2 mM final concentration), followed by trichloroacetic acid (TCA) precipitation.

In Vitro Protein Import into Isolated Mitochondria

Protein coding sequences were cloned downstream of the SP6 promoter into pGEM3Z (Promega) or RNA was synthesized, using the mMESSAGE mMACHINE SP6 Kit and MEGAclear Kit (Ambion) using SP6 promotor-containing PCR products as templates. TNT SP6 Quick Coupled Transcription/Translation system (Promega) or alternatively Flexi Rabbit Reticulocyte Lysate System (Promega) were used to synthesize in vitro [^{35}S]methionine-labeled precursor proteins. In vitro protein import into isolated human mitochondria was performed as described previously (Lazarou et al., 2009). Samples were separated either by SDS- or BN-PAGE and radiolabeled proteins were visualized using Storage Phosphor Screens and a Storm 820 scanner from GE Healthcare. For quantifications ImageQuant TL software was used (GE Healthcare).

In Vivo [^{35}S]methionine Labeling of Mitochondrial Translation Products

Labeling was performed in 25 cm^2 flasks as described previously (Chomyn, 1996). To inhibit cytosolic translation, cells were either treated with 100 μg/ml emetine (Invitrogen) during pulse experiments, or with 100 μg/ml anisomycin in pulse chase experiments. Mitochondrial translation products were labeled with 0.2 mCi/ml [^{35}S]methionine for 1 hr. In chase experiments, the radioactive medium was removed, 10 ml fresh growth medium added, followed by incubation at 37°C under 5% CO_2 atmosphere for the indicated time points. The cells were recovered in 1 mM EDTA/PBS. The samples were further analyzed either by SDS- or BN-PAGE.

BN-PAGE Analysis

BN-PAGE analysis was performed as previously described (Mick et al., 2012). Briefly, mitochondrial protein-complexes were extracted by lysing mitochondria in 1% digitonin, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 10% (weight/volume [w/v]) glycerol, and 1 mM PMSF buffer. Non-solubilized material was removed by centrifugation (20,000 xg, 15 min, 4°C). Mitochondrial complexes were separated on 4%–13% or 4%–14% polyacrylamide gradient gels as described previously (Wittig et al., 2006). In gel activity, assays were performed according to published procedures (Wittig et al., 2006).

Cytochrome c Oxidase Activity and Quantitation Assay

Complex IV Human Specific Activity Microplate Assay Kit (Mitosciences, Abcam) was used to determine specific activity and relative amount of cytochrome *c* oxidase according to the manufacturer's instructions. Total 15 μg of cell lysate was loaded per well. The oxidization of cytochrome *c* and the decrease of absorbance at 550 nm were measured for cytochrome *c* oxidase activity. To determine the relative COX amount, the increase of absorbance at 405 nm after incubation with a specific cytochrome *c* oxidase antibody, conjugated to alkaline phosphatase, was measured.

Affinity Purification Procedures

Isolated mitochondria or whole cell extracts (0.5–2 mg) were resuspended (final 1 mg/ml) in lysis-buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1 mM EDTA, 10% (w/v) glycerol, 1 mM PMSF, and 1% (w/v) digitonin) and incubated for 30 min at 4°C under mild agitation. To remove non-solubilized material, the lysate was centrifuged at 20,000 xg, 4°C for 15 min. Supernatants were incubated with anti-FLAG-agarose (Sigma-Aldrich) or ProteinA-Sepharose (GE Healthcare) conjugated with specific or control antibodies. Affinity resins were washed prior to elution with FLAG-peptides or by pH shift.

In Vitro Crosslinking

MITRAC7^{FLAG} containing mitochondria (500 μg) were incubated with BS^2-d$_0$ crosslinker (final 2.5 μM) in crosslinking buffer (20 mM HEPES [pH 7.4] and 100 mM NaCl) for 30 min on ice. The crosslinking reaction was quenched by the addition of glycine (pH 8.0) to a final concentration of 250 mM and incubation on ice for 15 min. Mitochondria were pelleted, resuspended in 200 μl lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1 mM EDTA, 10% [w/v] glycerol, 1 mM PMSF; 1% [w/v] SDS, and 1% Triton X-100), incubated at RT for 30 min, and 1.2 ml lysis buffer without SDS and Triton X-100 was added. To remove non-solubilized material, the lysate was centrifuged at 20,000 xg, 4°C for 15 min and immunoprecipitation was performed as described above.

Miscellaneous

Standard methods were used for SDS-PAGE and western-blotting of proteins to PVDF membranes (Millipore). Primary antibodies were raised in rabbits and HRP-coupled secondary antibodies applied to antigen-antibody complexes and detected by enhanced chemiluminescence on X-ray films.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.009.

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Cell Reports

Supplemental Information

MITRAC7 Acts as a COX1-Specific Chaperone and Reveals a Checkpoint during Cytochrome *c* Oxidase Assembly

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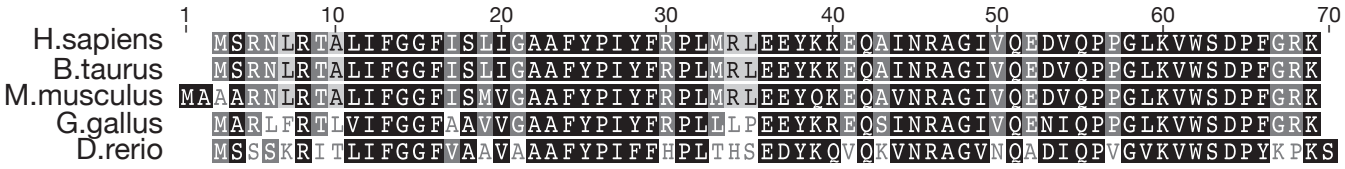

Figure S1. MITRAC7 is conserved in metazoa. Related to Figure 1.
Alignment of MITRAC7 homologous proteins of selected metazoa species using ClustalW2. Black boxes, 100% similar amino acids; dark grey, 80–100% similarity; light gray, 60– 80% similarity between species.

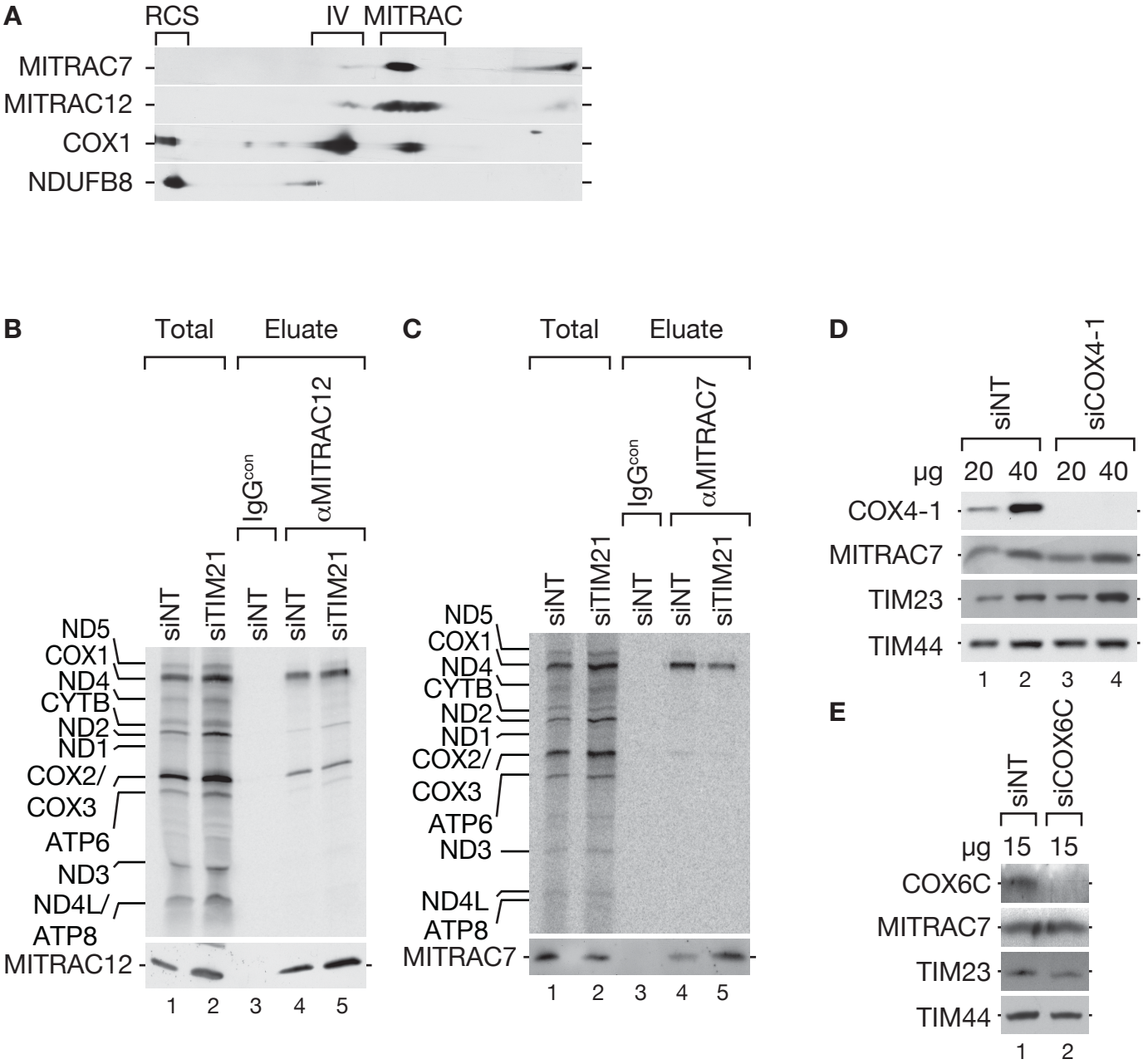

Figure S2. MITRAC7 comigrates with a slower MITRAC fraction and the association of MITRAC7 with COX1 depends on TIM21, COX4-1 and COX6C function.
Related to Figure 3.
(A) HEK293T mitochondria were analyzed by 2D-BN-/SDS-PAGE and Western-blotting. Membranes were probed with anti-MITRAC7, anti-MITRAC12, anti-COX1 and anti-NDUFB8 antibodies. RCS, respiratory-chain supercomplexes; IV, complex IV.
(B) and (C) HEK293T cells were treated with TIM21-specific or non-targeting control siRNA for 72 hr and mitochondrial translation products were labeled with [^{35}S]methionine. Whole cell lysates were subjected to immunoisolation with control (IgG), anti-MITRAC12 (B) or anti-MITRAC7 (C) antisera (Total, 5%; Eluate, 100%).
COX4-1 (D) or COX6C (E) were depleted with siRNA-applications, whole cell lysates analysed by Western-blotting and membranes probed with indictaed antibodies.

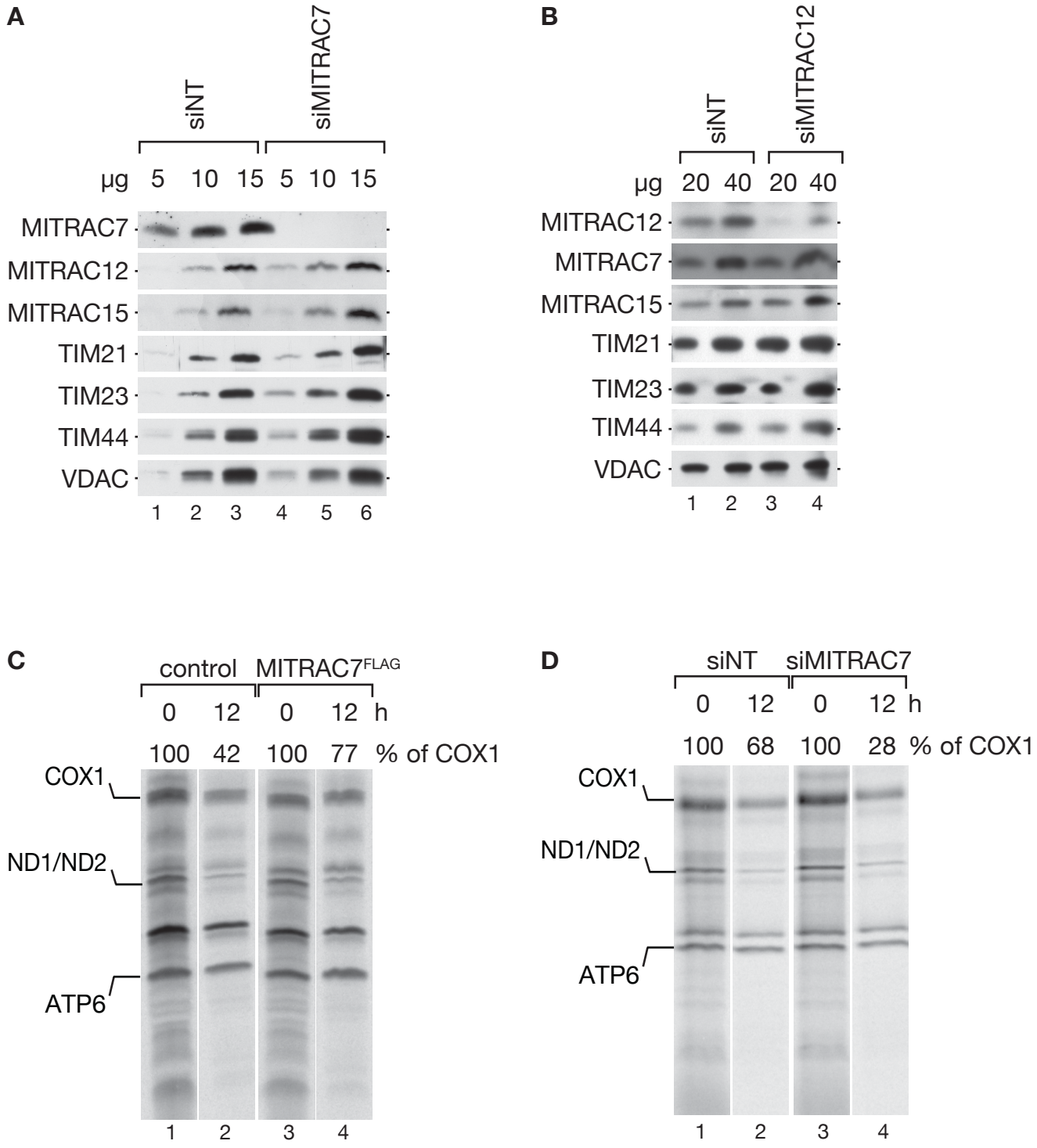

Figure S3. MITRAC7 and MITRAC12 stability do not depend on each other. Related to Figure 4 and 6.

After depletion of MITRAC7 or MITRAC12 in HEK293T cells, mitochondria (A) or cell extracts (B) were analyzed by Western-blotting. Membranes were probed with indicated antibodies.

(C) and (D) Mitochondrial translation products were pulse-labeled with [35S]methionine for 1hr either after MITRAC7 over expression (C) or depletion (D) and further cultured in the absence of [35S]methionine (chase) for 12 hr. Whole cell lysates (50 µg) were subjected to digital autoradiography.