LINC00116 codes for a mitochondrial peptide linking respiration and lipid metabolism

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Genes coding for small peptides have been frequently misannotated as long noncoding RNA (lncRNA) genes. Here we have demonstrated that one such transcript is translated into a 56-amino-acid-long peptide conserved in chordates, corroborating the work published while this manuscript was under review. The Mtln peptide could be detected in mitochondria of mouse cell lines and tissues. In line with its mitochondrial localization, lack of the Mtln decreases the activity of mitochondrial respiratory chain complex I. Unlike the integral components and assembly factors of NADH:ubiquinone oxidoreductase, Mtln does not alter its enzymatic activity directly. Interaction of Mtln with NADH-dependent cytochrome b5 reductase stimulates complex I functioning most likely by providing a favorable lipid composition of the membrane. Study of Mtln illuminates the importance of small peptides, whose genes might frequently be misannotated as lncRNAs, for the control of vitally important cellular processes.

**Significance**

Short peptides are encoded in genomes of all organisms and have important functions. Due to the small size of such open reading frames, they are frequently overlooked by automatic genome annotation. We investigated the gene that was misannotated as long noncoding RNA LINC00116 and demonstrated that this gene codes for a 56-amino-acid-long peptide, Mtln, which is localized in mitochondria. Inactivation of the Mtln coding gene leads to reduction of oxygen consumption attributed to respiratory complex I activity and perturbs lipid composition of the cell. This influence is mediated by Mtln interaction with NADH-dependent cytochrome b5 reductase. Disruption of the mitochondrial localization of the latter phenocopies Mtln inactivation.

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Results

Analysis of 1500011k16Rik Coding Potential. Many IncRNAs contain putative ORFs occurring by chance and not translated into a functional peptide entity. The murine 1500011k16Rik transcript contains a 56-amino acid ORF with a predicted single pass transmembrane segment (27), but not any detectable domain relative. Several sequence characteristics are indicative of coding potential. Analysis of nucleotide conservation of the gene homologous to 1500011k16Rik (Fig. 1A), among 60 vertebrate species (28), revealed that a region of putative ORF is the most, and almost only, conserved region of the gene. Analysis of the aggregated ribosome profiling data (29) revealed substantial ribosome coverage of the putative ORF (SI Appendix, Fig. S1A). Alignment of the putative products of ORF translation revealed a high degree of conservation at the amino acid level (Fig. 1B) with a high ratio of synonymous over non synonymous codon substitutions and absence of premature in-frame stop codons (SI Appendix, Fig. S1 A and B). As a result, we concluded that the transcript 1500011k16Rik is most likely to be translated into a 56-amino-acid-long peptide, which we will refer to as Mitoregulin (Mtln) (25) for consistency in the scientific literature.

The 1500011k16Rik Encoded Peptide Is Expressed and Localized in Mitochondria. To confirm the presence of Mtln in the cultured murine cells, we applied CRISPR-Cas9 technology to inactivate 1500011k16Rik gene coding for Mtln in NIH 3T3 and NS0 cell lines (SI Appendix, Fig. S2) and to append the Mtln coding region by the C-terminal fluorescent protein mCherry tag in NIH 3T3 cell lines. The presence of Mtln-mCherry fusion protein, expressed at natural expression level from an endogenous Mtln promoter, could be detected in the cells by immunoblotting (SI Appendix, Fig. S3A) and immunocytochemistry (SI Appendix, Fig. S3B). To further determine exact peptide localization, we ectopically expressed Mtln-mCherry fusion and observed its colocalization with mitochondria using an organelle-specific dye, MitoTracker Green FM (Fig. 2A). Cell fractionation revealed that the Mtln peptide is always copurified with mitochondria similarly to the mitochondrial Tom20 protein (Fig. 2B, Left and SI Appendix, Fig. S3C). Our results perfectly match the conclusions of two independent groups, which showed that Mtln localizes in the inner membrane of mitochondria (25, 26). To exclude the possibility of any influence of the mCherry tag on the localization of Mtln, we raised polyclonal rabbit antibodies to Mtln and detected an endogenous peptide presence in mitochondria of the NIH 3T3 cell lines, but not in the generated cell lines with an inactivated Mtln gene (Fig. 2B, Left). To check that the Mtln peptide is present not only in the NIH 3T3 cell line but also other cell lines and living organisms, we performed immunoblotting analysis of Mtln’s presence in the NS0 murine myeloma cell line (Fig. 2B, Right) using the NS0 cell line with inactivated 1500011k16Rik gene coding for Mtln as a control and mouse tissues (Fig. 2C). Analysis revealed a high level of Mtln in the NS0 cell line and ubiquitous expression of Mtln in different mouse tissues.

Respiratory Complex I Electron Transfer Activity Is Impaired in Knockout Cell Lines. To test the influence of Mtln on the respiration of mitochondria, we compared NIH 3T3 and NS0 cells with their derivatives with inactivated 1500011k16Rik gene coding for Mtln (SI Appendix, Fig. S2A). Distribution of cells with high and low mitochondrial membrane potential was tested by the ratiometric fluorescent dye JC-1 and FACS analysis. The level of total reactive oxygen species (ROS) production in wild-type and mutant cells was estimated by FACS analysis with the help of DCFDA. Mitochondria isolated from the NIH 3T3 cell lines, but not in the generated cell lines with an inactivated Mtln gene (Fig. 2B, Left). To check that the Mtln peptide is present not only in the NIH 3T3 cell line but also other cell lines and living organisms, we performed immunoblotting analysis of Mtln’s presence in the NS0 murine myeloma cell line (Fig. 2B, Right) using the NS0 cell line with inactivated 1500011k16Rik gene coding for Mtln as a control and mouse tissues (Fig. 2C). Analysis revealed a high level of Mtln in the NS0 cell line and ubiquitous expression of Mtln in different mouse tissues.

To test if mitochondrial Mtln peptide function is essential for the activity of any respiratory chain components, we compared respiratory efficiency of knockout cells with a parental cell line carrying functional Mtln. Oxygen consumption rates were measured to examine electron flow through respiratory complexes I–IV (Fig. 3A), showing that isolated mitochondria revealed a significant decrease of complex I activity in the Mtln knockout and parental cell lines, attributed to complex I, is maximal activity of any respiratory chain components, we compared respiratory efficiency of knockout cells with a parental cell line carrying functional Mtln. Oxygen consumption rates were measured to examine electron flow through respiratory complexes I–IV (Fig. 3A), showing that isolated mitochondria revealed a significant decrease of complex I activity in the Mtln knockout and parental cell lines, attributed to complex I, is maximal 

At the same time, measurements of oxygen consumption rates for isolated mitochondria revealed a significant decrease of complex I activity for both cell lines and a tendency to decrease for complex III (Fig. 3B and SI Appendix, Fig. S5B). The difference between the oxygen consumption rates by mitochondria isolated from the Mtln knockout and parental cell lines, attributed to complex I, is maximal upon the stimulation by ADP or uncoupler carbonyl cyanide-(trifluoromethoxy)phenylhydrazone (FCCP). Nonstimulated respiration on NAD-linked substrates was not different for the mitochondria from mutant and parental cell lines likely due to the lack of energy-consuming processes. Complex I-associated respiration deficiency observed for mitochondria purified from Mtln knockout cell lines demonstrated that only perturbation of the mitochondria, but not of any other cellular compartment upon Mtln inactivation, is sufficient for the phenotypic manifestation. To check for a genetic complementation, we reintroduced either a copy of the Mtln gene or luciferase into the knockout cell lines under a more potent synthetic promoter. Ectopic expression of Mtln, but not luciferase, restored complex I activity in both knockout cell lines (SI Appendix, Fig. S4C).

NAHD:ubiquinone oxidoreductase is a large multisubunit complex which requires several additional assembly factors.
Mtln Deletion Does Not Change the Cytosolic NADH/NAD\(^+\) Balance. Reduced contribution of NADH:ubiquinone oxidoreductase into mitochondrial respiration might be explained by a lower rate of NADH regeneration. To evaluate the influence of Mtln knockout on NADH/NAD\(^+\) balance, we applied SoNar fluorescent protein sensor (31). We found that in the steady-state condition, the wild-type measurements in mutant cells (Fig. 4B) revealed that loss of Cyb5r3 localization in mitochondria altered the activity of respiratory complex I, similar to inactivation of its interacting peptide Mtln.

Further, to determine the functional relationship between Mtln and Cyb5r3, we generated Cyb5r3 Gly2-to-Ala mutant cells with Mtln deletion and overexpression (SI Appendix, Figs. S2B and S8A). To check whether Mtln absence or overexpression would affect the Cyb5r3 mutant cell’s phenotype, we compared oxygen consumption rates attributed to mitochondrial respiratory complexes (SI Appendix, Fig. S8B). We observed that inhibition of complex I activity caused by Cyb5r3 mutation could not be rescued or aggravated by the Mtln deletion or overexpression. Thereby, we concluded that it is likely that Mtln fulfills its function only via its interaction with Cyb5r3, the latter being the major functional component of the complex.

**Mtln Interacts with Cytochrome b5 Reductase 3.** Since Mtln inactivation does not affect complex I activity directly, we immunopurified complex I from the wild-type and Mtln knockout cells and measured its ability to oxidize NADH. Surprisingly, NADH dehydrogenase activity of the purified respiratory complex I appeared to be independent of Mtln (SI Appendix, Figs. S5C and D). Additionally, complex I-III activity, measured as NADH-dependent cytochrome C reduction in the presence of potassium cyanide (KCN), turned out to be independent of Mtln presence (SI Appendix, Fig. S6). This result led us to the hypothesis that Mtln manifests its influence on the function of respiratory complex I indirectly.

Our results are consistent with a recently published study (25), demonstrating that Mtln deletion does not affect assembly of Complex I, but impedes association of Complex I into respiratory chain supercomplexes.

**Disruption of Cyb5r3 Mitochondrial Localization Phenocopies Mtln Inactivation.** Multiple localizations were reported for Cyb5r3, making its functional analysis somewhat complicated. Apart from the mitochondrial outer membrane, this enzyme was found in microsomal and cytoplasmic membranes. Luckily, it is known that Cyb5r3 must be N-myristoylated at Gly2 residue to get incorporated in the mitochondrial outer membrane (30). To check whether the Mtln peptide functions via its interaction with Cyb5r3, we decided to disrupt mitochondrial localization of the latter. We generated mutant NIH 3T3 cells with Gly2-to-Ala substitution (ΔCyb5r3\(_{Gly2}\)), which appeared to be viable (SI Appendix, Fig. S7D). This mutation results in the decrease of Cyb5r3 protein levels in mitochondria (SI Appendix, Fig. S7E, Left). Oxygen consumption rate
and Mtln knockout cells do not demonstrate any significant difference in the cytoplasmic NADH/NAD+ redox balance (SI Appendix, Fig. S9A). In addition, we decided to assess the dynamics of redox balance changes in the cytosol affected by different metabolic substrates (SI Appendix, Fig. S9 B and C). The test revealed a lack of difference in the NADH/NAD+ dynamic between parental and Mtln-depleted cells. We can conclude that the cytosolic redox balance is not affected by Mtln ablation.

**How Does Mtln Influence Cyb5r3?** Frequently, protein complex formation is necessary for the stabilization of components against proteolysis. However, it is not the case for Cyb5r3 interaction with Mtln, as revealed by immunoblotting of Cyb5r3 in Mtln knockout cells (SI Appendix, Fig. S10A). To evaluate any possible influence of Mtln on the localization of Cyb5r3, we used cell lines ectopically expressing the Cyb5r3-eGFP fusion protein. Localization of the Cyb5r3 fusion protein appeared to be mitochondrial in both the parental NIH 3T3 cell line and its derivative devoid of the Mtln peptide (SI Appendix, Fig. S10B). Moreover, we do not observe any difference in Mtln level or localization upon ΔCyb5r3mito mutation (SI Appendix, Fig. S10 C and D).

Cyb5r3 catalyzes redox reactions with several different substrates, while it uses only NADH as a donor of an electron being one of the main consumers of the cytosolic NADH (32). To test an influence of Mtln on the ability of Cyb5r3 to oxidize NADH, we measured its NADH dehydrogenase activity in a reaction with K[Fe(CN)6] as a spurious electron acceptor (SI Appendix, Fig. S10E). An ability of Cyb5r3 to extract an electron from NADH was found to be independent of the Mtln presence.

Two main processes that require the membrane-bound form of Cyb5r3 are Δ fatty acid desaturation (33) and cholesterol biosynthesis (34). For the evaluation of Mtln’s influence on Cyb5r3 activity in lipid metabolism, we performed LC-MS analysis of cell lines NIH 3T3 and N50 deficient in Mtln. We quantified more than 1,000 lipids in each cell line (Materials and Methods). Quantities of hundreds of lipids were significantly and reproducibly (SI Appendix, Fig. S11A) changed upon Mtln depletion in two independently produced NIH 3T3-derived knockout cell lines. Most glycerolipids are overrepresented while most glycerophospholipids are underrepresented upon Mtln knockout in both NIH 3T3 and N50 cell lines (Fig. 5A).

While phosphatidylcholines (PCs) are the most abundant lipids in our dataset, their amounts are strongly reduced upon Mtln knockout, resulting in overall reduction of the PC fraction by about 20% (Fig. 5 B and C). The PCs whose quantity is most affected by Mtln knockout are one to three unsaturated with relatively long chains (Fig. SC). Contrary to PCs, concentrations of most TAGs are elevated upon Mtln knockout and most affected species are either short or polyunsaturated with the highest effect observed for TAGs 52:6, 54:7, and 56:8 (Fig. 5 B and D and SI Appendix, Fig. S11B). MS-MS analysis of these TAGs revealed that all of them contain an acyl group of docosahexaenoic acid.

To check whether an influence of Mtln on cellular lipid composition is mediated by its interaction with Cyb5r3 we analyzed lipid composition of the cells possessing only the mutant Cyb5r3 form lacking mitochondrial localization. Lipidome analysis demonstrated a good correlation between lipid composition changes of Mtln knockout and ΔCyb5r3mito mutant cells (Fig. 6). Moreover, we observe decrease in some PC fractions (SI Appendix, Fig. S12A) and increase in polyunsaturated TAGs (SI Appendix, Fig. S12B) for both mutant cell lines. These tendencies are reproduced but not aggravated in the Cyb5r3 mutant cells with additional Mtln deletion (SI Appendix, Figs. S13 A and B and S14). Analysis of lipid composition of ΔCyb5r3mito mutant cells overexpressing the Mtln gene does not demonstrate a complete suppression of lipidome changes (SI Appendix, Figs. S13C and S14).

**Discussion**

An assortment of short peptides encoded in genomes of higher organisms, such as mouse and human, could compose a nearly overlooked layer of regulatory molecules (11, 35, 36). In this work, we have appended the list of small functional peptide entities by a remarkable member, the Mtln mitochondrial peptide, in line with the results of other groups (25, 26) published while this work was under review. Mtln is highly conserved across vertebrates and, as it appears from our results, functions in the cell metabolism.

The majority of small peptides manifest their functions by binding to a partner protein or being a constituent of multiprotein complexes (10), as was shown for sarcolin and phospholamban (37, 38), humanin (18), Toddler/ELABELA (39, 40), and DWORF (41). Only a few small peptides have separate enzymatic functions, such as 4-oxalocrotonate tautomerase, whose monomer contains
only 62 amino acids (42). In line with this trend, we demonstrated that the Mtln peptide interacts with NADH:cytochrome b5 oxidoreductase 3 (Cyb5r3). Disruption of mitochondrial localization of the latter leads to the same phenotype as Mtln depletion in regard to the respiratory complex I attributed oxygen consumption and lipid composition change. Thus, Mtln may function by stimulation of Cyb5r3 activity in mitochondria necessary for maintenance of lipid homeostasis. Recent study demonstrated that Mtln may also interact with other mitochondrial proteins (26), such as HADHA and HADHB involved in fatty acid β-oxidation. Although particular Mtln interaction partners identified in our study and in the work of Makarewich et al. (26) are different, they both are attributed to related processes, which may indicate an involvement of Mtln in fatty acid metabolism with tissue or cell-type peculiarities.

This activity, as we demonstrated, is needed for respiratory complex I functioning in the context of the mitochondrial OXPHOS system, but not for NADH oxidase activity of isolated complex I or complexes I+II. This observation is in line with that of Stein et al. (25), demonstrating an influence of Mtln deficiency on the formation of respiratory CHH supercomplexes, containing CI, but not onto the assembly of CI and association of CI and CIII complexes.

Multiple functions were ascribed to the Cyb5r3. Apart from the erythrocyte-specific methemoglobin reductase activity of the soluble Cyb5r3 isoform (43), which is unlikely to be relevant for our study, membrane-anchored Cyb5r3 is implicated in the desaturation of fatty acids (33), cholesterol biosynthesis (34), and the amidoxime system involved in lipogenesis (44–46) and drug metabolism.

Scenarios where Mtln may be involved in localization or stabilization of Cyb5r3 against partial or complete proteolysis were ruled out, since neither localization nor the protein level of Cyb5r3 are affected by Mtln, leaving the possibility that Mtln influences Cyb5r3 activity related to lipid metabolism. Lipid composition is known to be crucial for mitochondrial functioning and in particular to respiratory complex I activity (47–50). Relevance of Mtln for Cyb5r3 functioning in the lipid metabolism was deduced from the lipidomic analysis of Mtln knockout cells. We found that most TAGs are overrepresented and most phospholipids are underrepresented upon knockout. The PCs are affected more than other types of lipids. The fact that disruption of mitochondrial Cyb5r3 localization leads to the lipidome change that correlate well with that caused by Mtln gene inactivation supports the idea that Mtln functions via its association with Cyb5r3. We speculate that the decrease in the phosphatidylcholine content observed in both the cell line devoid of Cyb5r3 mitochondrial localization and Mtln knockout might be the cause of the observed decrease in respiratory complex I activity. Congruent results (50) demonstrated that removal of choline from the rat diet results in accumulation of triglycerides in liver and impaired respiratory function, particularly in regard to complex I-linked, NADH-dependent respiration. Also, the importance of PCs for complex I activity is corroborated by the facts that addition of 1-alpha-glycerolphosphorylcholine (GPC) increased the efficacy of complex I-linked mitochondrial oxygen consumption (51) and catalytic activity of complex I is determined by the amounts of PC and phosphatidylethanolamine (PE) (52). According to published data, the decrease in mitochondrial PCs impairs oxygen consumption rate, affects formation of the mitochondrial supercomplexes, and leads to a disorganized cristae structure (53, 54). Recent studies revealed that deletion of Mtln results in disrupted cristae structure (26) and alters respiratory supercomplex formation (25). Supercomplexes reside on the cristae membrane (55), of which the positively curved leaflet consists of predominantly phosphatidylcholines (~80%) (56). It is tempting to speculate that supercomplex formation can be affected by the shape of the cristae, which in turn depends on lipid composition. These observations imply that the possible mechanism of complex I inactivation in Mtln-deficient cells might be governed by the inactivation of mitochondria-related functioning of Cyb5r3, which, in turn, changes the lipid compositions of the mitochondrial membrane. The last event leads to a change in the shape of the cristae, which disrupts the distribution of supercomplexes and the interaction of their components, hence slowing down the inactive/active transition of complex I (57, 58) resulting in its inactivation.

Materials and Methods

Constructs for gene inactivation and genome editing were created on the basis of pX458 plasmid (59). Knockin constructs were created on the basis of Sleeping Beauty transposon vectors (60, 61). Monitoring of ROS production in adherent and suspension cells was performed according to the protocol proposed by Wojtala et al. (62). Oxygen consumption rates were measured as described (63). Mitochondria from cultured cells were isolated by differential centrifugation. Cyb5r3 enzymatic activity was determined in isolated mitochondria by monitoring NADH-dependent ferricyanide reduction (64). Metabolite extraction for lipid analysis was performed as described (65). Untargeted lipidome profiling was performed in positive ionization mode (66). The animal work was approved by the Lomonosov Moscow State University ethics committee.

More details on the study methodology are provided in SI Appendix. ACKNOWLEDGMENTS. We thank Alex Lebedeff for his help in editing the manuscript; Ksenia Smirnova for her help in growing cells; Nikolai Anikanov and Elena Stekolschikova for lipid extraction and mass-spectrometry measurements for double knockout cell lines; and Dr. Yi Yang for providing the pcDNA3.1-SoNar and pcDNA3.1-iNapC vectors. A.C. thanks the Boehringer Ingelheim Fond for a travel grant to attend a European Molecular Biology Laboratory course. We also thank the Skolkovo Institute of Science and Technology for funding for the publication charges and open access fee. This work was supported by the Russian Foundation for Basic Research (RFBR) Grants 17-04-01904 and 18-29-07005 (to P.S. and A.C.); Russian Science Foundation (RSF) Grant 17-75-30027 (to P.S.) for work related to genome editing; and the Moscow State University Scientific School (O.D.). Experiments on mice were supported by Russian Ministry of Science Grant 14.W03.31.0012. P.V. was supported by the President’s Scholarship (SP-4132.2018.4). The work of I.A. was supported by a scholarship of NADHD is responsible for the work of A.C. The work was supported by RFBR Grant 17-15-01175 (to D.B.) and RFBR Grant 18-54-74003 (to V.B.)
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