Cell. Mol. Life Sci. (2017) 74:631–646
DOI 10.1007/s00018-016-2342-7

Cellular and Molecular Life Sciences

REVIEW

Interplay of mitochondrial metabolism and microRNAs

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Received: 26 April 2016 / Revised: 7 July 2016 / Accepted: 12 August 2016 / Published online: 25 August 2016
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Abstract Mitochondria are important organelles in cellular metabolism. Several crucial metabolic pathways such as the energy producing electron transport chain or the tricarboxylic acid cycle are hosted inside the mitochondria. The proper function of mitochondria depends on the import of proteins, which are encoded in the nucleus and synthesized in the cytosol. Micro-ribonucleic acids (miRNAs) are short non-coding ribonucleic acid (RNA) molecules with the ability to prevent messenger RNA (mRNA)-translation or to induce the degradation of mRNA-transcripts. Although miRNAs are mainly located in the cytosol or the nucleus, a subset of ~150 different miRNAs, called mitomiRs, has also been found localized to mitochondrial fractions of cells and tissues together with the subunits of the RNA-induced silencing complex (RISC); the protein complex through which miRNAs normally act to prevent translation of their mRNA-targets. The focus of this review is on miRNAs and mitomiRs with influence on mitochondrial metabolism and their possible pathophysiological impact.

Keywords Mitochondria · miRNA · Metabolism · mitomiR

Electronic supplementary material The online version of this article (doi:10.1007/s00018-016-2342-7) contains supplementary material, which is available to authorized users.

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Introduction

Mitochondria are often referred to as the powerhouse of cells, because they are the main site of cellular energy production generating the energy-rich molecule adenosine triphosphate (ATP) by oxidative phosphorylation. Besides the role in energy homeostasis, mitochondria are involved in various other processes like heme- and steroid hormone synthesis, apoptosis and Ca^{2+}-signaling [1]. The central cellular status makes this organelle an important compartment also under pathological conditions. Mitochondria have been linked to a variety of diseases like cancer [2], type 2 diabetes (T2D) [3], the metabolic syndrome [4], cardiac dysfunction and aging [5].

MicroRNAs (miRNAs) constitute a class of small, non-coding ribonucleic acid molecules. They were discovered only 20 years ago [6]. Since then, they have emerged as abundant post-transcriptional regulatory factors, adding new layers to the complex network of gene regulation [1]. By transcript degradation or translational inhibition of messenger RNAs (mRNA), miRNAs can affect fluxes through metabolic pathways. Although the mitochondrion has its own genome and translation machinery, proper function is highly depending on import of proteins and enzymes synthesized in the cytoplasm. The miRNA-mediated down-regulation of such proteins consequently affects the mitochondrial metabolism.

MiRNA molecules are abundant in the cytosol and nucleus. However, mitochondria from human and mouse tissue have recently been shown to contain miRNAs [7–10]. The significance of these findings is incompletely understood [7], but it is an appealing possibility that due to their localization these miRNAs have a special impact on mitochondrial pathways.

Here, the focus is on miRNAs, for which there is experimental evidence that they influence metabolic steps inside the mitochondrial organelle, either by directly regulating the translation of enzymes or indirectly by tuning peripheral events like the expression of transporters and other regulatory proteins. Moreover, we further place emphasis on miRNAs that are also present in the mitochondrial compartment.

In general, the main experimental approaches to detect and analyse miRNAs are either sequencing-based [11], hybridization-based (e.g., microarray [9], in situ hybridization [8]) or qPCR-based [12]. Different techniques have specific strengths and weaknesses [13], although only sequencing allows identification of unknown miRNA species [11]. More sophisticated experimental setups are possible by introducing a prior cross linking step followed by immuno-precipitation (CLIP), e.g., miR-CLIP [14], PAR-CLIP [15]. With the exception of in situ hybridization, the detection of mitomiRs require a preceding mitochondrial isolation either by differential centrifugation [9] or an antibody-based enrichment for mitochondria [8, 16] followed by RNase treatment to remove cytosolic RNA contamination on the surface of mitochondrial membranes. To study the effects of miRNAs and their putative target genes, a number of methods may be used including reporter-gene analysis for evaluation of target sites [17], miRNA over-expression or inhibition using synthetic oligonucleotides as either miRNA mimetics or antagonists [18]. Plasmid based systems also exist for constitutive or inducible miRNA over-expression or inhibition in primary or clonal cell systems [19]. Moreover, it is also possible to modify miRNA expression transiently in vivo by administration of stable miRNA antagonists [18] or genetically by the use of modified mouse alleles [20].

Literature search strategy

Literature describing mitomiRs or the regulation of mitochondrial metabolism by miRNAs was searched in the databases PubMed, Scopus and Web of Science. Initially a list (Supplement S1) of search terms was conducted that included general terms (e.g., 'miRNA', 'mitochondria') as well as explicit enzymes of mitochondrial pathways (e.g., 'citrate synthase') and taking into consideration different abbreviations and ways of spelling. Terms were then combined in various combinations with AND, OR and star(*)-operators to create search queries. All English journal articles or review articles describing in vitro or in vivo human or rodent model systems were further examined.

The mitochondrial organelle

Mitochondria are small cellular organelles of 0.5–1 μM in diameter [21] separated from the cytosolic compartment by two membranes (Fig. 1a). The inner mitochondrial membrane (IMM) surrounds the mitochondrial matrix, while the outer membrane (OMM) forms a barrier against the cytosol. In between the outer and inner membrane lies the intermembrane space (IMS).

The inner membrane shows a tightly and highly folded topology of cristae providing a huge surface for the components of the electron transport chain (ETC). In addition to the process of oxidative phosphorylation, numerous conversions of metabolites take place inside the mitochondrial compartment, i.e., steps of ketogenesis, amino acid metabolism, steroid hormone synthesis, heme synthesis or the tricarboxylic acid (TCA) cycle.

The biochemical reactions inside mitochondria, including the catalyzing enzymes and the transport of the reactants between mitochondria and the cytosol, are here referred to as 'mitochondrial metabolism'.

The mitochondrial organelle is thought to originate from prokaryotic cells. According to the endosymbiotic theory, bacteria were engulfed by eukaryotic cells and formed a symbiotic conglomerate with them over time. The theory is supported by the finding that mitochondria have their own circular genomic DNA and replicate mostly independent from the surrounding cell. The mitochondrial genome is about 16 kb long and consists of a so called heavy and a light strand. The heavy strand harbors most of the coding sequences, while fewer are encoded on the light strand (Fig. 1b). The mitochondrial genome encodes 37 transcripts: 13 of them encode protein subunits of the ETC [22]. Furthermore, there are 2 rRNAs and 22 tRNAs [23] and embedded in the rRNA genes are coding sequences for short peptides [24, 25]. Most of the proteins present in mitochondria are nuclear encoded. The import of these proteins involves transporter proteins like translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM) for which several isoforms exist.

Mitochondria are vital for bioenergetic and biosynthetic processes. Despite the common depiction, showing single mitochondria (as in Fig. 1a), they form a complex network within the cells [26]. The role of mitochondria as the main energy supplier of a cell can be compromised under pathological conditions. In general, the inability of mitochondria to meet the ATP demand of a cell is often termed 'mitochondrial dysfunction' and has been intensively studied in the context of pathological states.

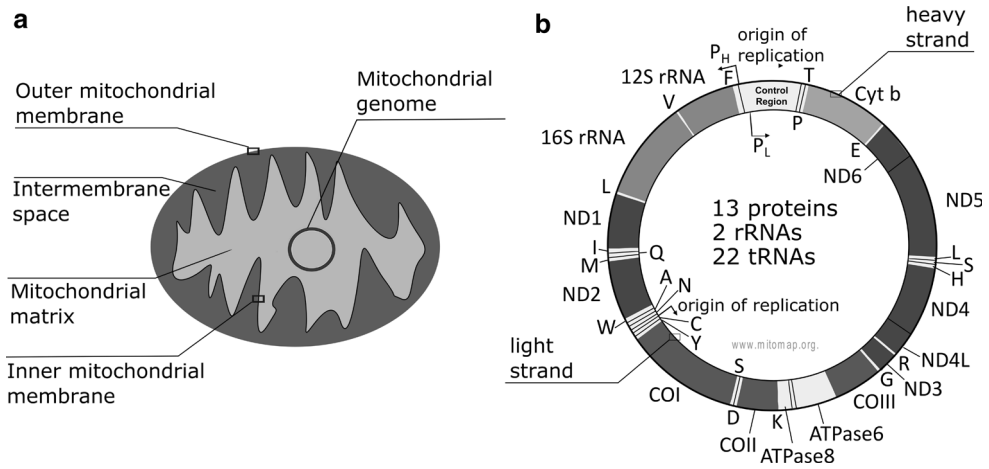

Fig. 1 The mitochondrial organelle. **a** The mitochondrial matrix is separated from the cytosolic compartment by the outer and the inner mitochondrial membrane. Between both membrane layers is the intermembrane space. Although most mitochondrial proteins are nuclear encoded and imported across the membranes, mitochondria have their own genome. **b** The mitochondrial genome consists of the heavy (*outer circle*) and light (*inner circle*) strand that encode for protein subunits of the NADH:ubiquinone oxidoreductase (ND), ferrocytochrome c:oxygen oxidoreductase (CO) and F1F0-ATP synthase. Furthermore, it contains sequences for rRNAs (12S, 16S) and tRNAs (indicated by *single letter code* of accepted amino acids). The initiation sites of transcription are denoted PL and PH. **b** was assembled using MITOMAP (www.mitomap.org)

MicroRNAs

In 1993, Lee, Feinbaum and Ambros discovered lin-4 [6] and although first thought of as a curiosity in nematodes, miRNAs turned out to be a new class of short regulatory RNA molecules. Today, these RNA molecules have become an intensively studied research subject. Currently, more than 1900 mature miRNAs have been described in mice and more than 2500 in humans (MirBase Version 21).

In general, miRNAs are encoded in the nuclear genome [27–29], although it has been suggested that a few miRNAs (i.e., miR-4485-3p, miR-1973, miR-4284) could have a mitochondrial origin [7, 11, 30]. The biosynthesis of miRNAs starts with transcription of nuclear DNA into a primary transcript (pri-miRNA) (Fig. 2). The majority of miRNAs is transcribed by RNA polymerase II. The pri-miRNA is processed in the nucleus by the nucleases DROSHA and DGCR8/PASHA, resulting in a single hairpin-folded molecule [the precursor-miRNA (pre-miRNA)], which is exported to the cytosol in an exportin-5 dependent process.

The pre-miRNA is further cleaved in the cytosol by DICER, a nuclease which generates a miRNA:miRNA* duplex structure of imperfect base-pairing. Typically, the miRNA strand with the lower thermodynamic stability is bound by the AGO2 protein and is now called mature miRNA. At that stage, miRNAs are 20–22 bp long [31]. The other duplex strand (indicated with *) is degraded. However, a number of exceptions are known where both strands have a biological function and exist in equimolar amounts [32].

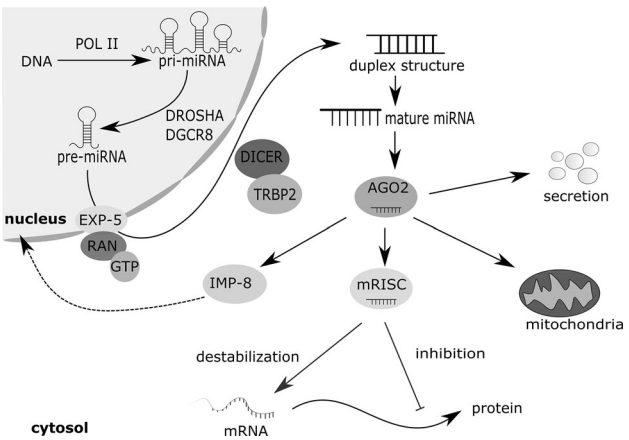

Fig. 2 MiRNA biosynthesis. MiRNAs are transcribed from the nuclear genome by POL II. The pri-miRNA construct is processed by the nuclease complex DROSHA/DGCR8 to the pre-miRNA. After export into the cytoplasm, the pre-miRNA is further cleaved by DICER and associates with the AGO2 protein. After incorporation into RISC, miRNAs can regulate the translation of mRNAs. Mature miRNAs can be transported into different compartments, like the nucleus, mitochondria or the extracellular environment. *AGO2* argonaute-2, *DGCR8* DiGeorge critical region 8 *EXP-5* exportin-5, *GTP* guanosine triphosphate, *IMP-8* importin-8, *mRISC* RNA-induced silencing complex loaded with mature miRNA, *mRNA* messenger ribonucleic acid, *POLII* DNA polymerase II, *RAN* GTP-binding nuclear protein RAN, *RISC* RNA-induced silencing complex, *TRBP2* RISC-loading complex subunit TARBP2

AGO2 facilitates the incorporation of the bound miRNA into the RNA-induced silencing complex (RISC), which afterwards is then termed mRISC. In addition, AGO2-bound miRNAs are distributed to other cellular

compartments, such as the nucleus [33] and mitochondria [16] or they are secreted from cells in exosomes [34] (Fig. 2).

The action of miRNAs in mRISC is well studied and resembles the RNA interference pathway. After incorporation into the RISC complex, miRNAs can bind mRNAs by imperfect base-pairing primarily in the 3′-untranslated region (UTR). Bound mRNAs are either degraded or their translation is inhibited [29, 35]. Since fully complementary base-pairing is not necessary for target recognition, one miRNA can modulate several mRNAs, and vice versa a single mRNA species may simultaneously be targeted by multiple miRNAs. It has been suggested that nearly one third of all protein-encoding genes are regulated by miRNAs [36]. By their diverse modes of action, miRNAs add a new dimension to the complex network of post-transcriptional regulation.

Mitochondrial miRNAs

The subset of ~150 miRNAs that have been detected in mitochondrial specimens are currently summarized under the term 'mitomiR' (Table 1) [7]. This is, however, a purely descriptive term. Both the function and other distinctive characteristic of mitomiR members are not well described. The overlap of miRNAs described in different articles is shown in Fig. 4a. The majority of mitomiRs has been identified in only one or two publications (Fig. 4a). Only seven miRNAs (i.e., let-7b-5p, let-7g-5p, 107, 125b-5p, 181a-5p, 221-5p and 494-3p) have been detected in mitochondria by three or more studies. So far the most frequently identified mitomiR is miR-494-3p.

A number of functions of mitomiRs are possible. Three possible roles are illustrated in Fig. 3. Since cytosolic miRNAs inhibit protein translation, a likely function of mitomiRs is to suppress the mRNA translation of mitochondrial proteins, thus regulating the mitochondrial proteome (Fig. 3a). Indeed, a miRNA-dependent regulation has been shown for several proteins of the ETC and is discussed in more detail in other sections of this review.

In this context, it is worth mentioning that mitochondrial DNA evolves faster than the nuclear genome [37]. Assuming a regulatory role in mitochondrial biology, mitomiR-sequences would be expected to have a low degree of conservation across species. Indeed, only a few mitomiRs (i.e., hsa-miR-494, hsa-miR-328) show high conservation in metazoan species, while the majority is only conserved in primates or even specific to humans [16]. Differences in the level of conservation were also found in a recent publication. From six newly discovered mitomiRs, only one was conserved from rat and mouse to human [37]. This supports a model in which a few mitomiRs are involved in the regulation of conserved mitochondrial features, while the rest may concern processes that were adapted during evolution.

A RISC-like complex inside mitochondria?

In the cytosol, the translational inhibition or transcript degradation by miRNAs requires their maturation and incorporation into the RISC complex. Whether proteins of miRNA biogenesis, RISC or RISC-like components are present inside mitochondria is an ongoing debate. Published indications for the mitochondrial presence of DICER and the RISC-components AGO2 and FXR1 (fragile X mental retardation 1) are summarized in Table 2.

A study in interfibrillar (IFM) and subsarcolemmal (SSM) mouse heart mitochondria indicates the presence of AGO2 and FXR1 in both [10]. Interestingly, upon STZ-induced hyperglycemia both proteins were re-distributed. AGO2 was nearly decreased to half in both mitochondrial subpopulations, while FXR1 was reduced only in SSM, and more than fourfold increased in IFM.

The possible mitochondrial localization of FXR1 is also corroborated by the MitoCarta2.0 database, while this is not the case for AGO2. Other RISC-components listed as possibly mitochondrial in MitoCarta2.0 are TRBP and SND1 but not GW182; the latter in accordance with published observations [10]. The case of DICER seems similarly complex. Even though it is listed as potentially mitochondrial in MitoCarta2.0, this could not be experimentally confirmed [10].

Taken together, the contradicting reports show that the subcellular abundance of RISC and RISC-like complex components is an understudied research area. Factors contributing to the conflicting results could be tissue-specific expression or the ability of at least some RISC-constituents to shuttle between different subcellular compartments. Yet, although the potential mitochondrial presence of the above mentioned proteins has been indicated, functional RISC-activity in mitochondria still needs to be shown.

Non-canonical functions of mitomiRs?

The classical view that miRNAs act exclusively through RISC incorporation has recently been challenged. MiRNAs as well as their precursor forms are able to interact with different proteins [38–40]. LIN28 and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), both RNA-binding proteins, recognize precursors of the let-7 family [39, 40]. The KH-type splicing regulatory protein (KSRP) binds pre-miR-1-2, a muscle specific RNA [40]. Incorporation of its mature form into RISC can be prevented by

Table 1 MiRNAs with reported mitochondrial localization (mitomiRs)

MiRNA	Tissue	Method	References
let-7f-5p; 101-5p; 122-5p; 181b-5p; 181d-5p; 188-5p; 29a-3p; 29c-3p; 361-5p; 432; 494-3p; 680; 690; 705; 711; 721; 762	Mouse liver	miRNA microarray	[9]
103-3p; 106a-5p; 107; 125a-5p; 125b-5p; 127-3p; 133a-5p; 133b; 134-5p; 149-5p; 151-5p; 181a-5p; 193b-5p; 1976; 199a-5p; 19b-5p; 20a-5p; 210-5p; 221-5p; 23a-5p; 23b-5p; 24-5p; 324-3p; 324-5p; 34a-5p; 365b-5p; 423-3p; 484; 486-5p; 490-3p; 501-3p; 503-5p; 532-3p; 542-5p; 574-3p; 598; 675-5p; 92a-5p; 93-5p; let-7b-5p; let-7g-5p	Human skeletal muscle	RT-qPCR, miRNA assay	[8]
1-3p	Human skeletal muscle	Functional study	[134]
130a-3p; 130b-3p; 140-5p; 320-3p; 494-3p; 671	Rat liver	miRNA microarray	[12]
107; 1275; 181a-5p; 1973[a]; 221-5p; 320a; 494-3p; let-7b-5p; let-7g-5p	Hela cells	NGS	[11]
103-3p; 146a-5p; 16-5p	143B	NGS	[57]
107; 181a-5p; 221-5p; 320a; let-7b-5p; let-7g-5p	HEK293	NGS	[11]
181c-5p	Rat cardiac myocytes	Functional study	[64]
1973[a]; 1275; 494-3p; 513a-5p; 1246; 328-5p; 1908-5p; 1972; 638	Hela	miRNA microarray	[16]
let-7d-5p; let-7b-5p; let-7c-5p; let-7f-5p; mghv-M1-7-3p; 1187; 1224-5p; 125a-3p; 125b-5p; 126-3p; 130a-5p; 133a-3p; 133a-5p; 133b; 135a-1-3p; 139-3p; 1-3p; 144-3p; 149-3p; 149-5p; 188-5p; 1894-3p; 1895; 1897-5p; 1904; 1934-3p; 1982-5p; 211-3p; 2137; 21a-5p; 22-3p; 23a-3p; 23b-3p; 24-3p; 26a-5p; 27a-3p; 27b-3p; 2861; 29a-3p; 29b-3p; 29c-3p; 3072-3p; 3081-5p; 3082-5p; 3085-3p; 3092-3p; 3095-3p; 3098-5p; 30a-5p; 30c-1-3p; 30d-5p; 30e-5p; 3102-5p; 3102-5p.2-5p; 3470a; 378a-5p; 451a; 466b-3p; 466i-5p; 483-5p; 486b; 494-3p; 497-5p; 574-5p; 652-5p; 671-5p; 680; 705; 709; 712-5p; 721; 877-3p; 99a-5p	Mouse heart (subsarcolemmal and interfibrillar)	miRNA microarray	[10]
21a-5p; 125b-5p; 128-3p; 151-3p; 203-3p; 212-3p; 22-3p; 23a-3p; 27a-3p; 27b-3p; 320-3p; 1932; 1199-5p; 5108; 375-3p; 3963; 341-3p; 342-3p; 148a-3p; 200c-3p; let7a-5p; 300-3p; let-7d-5p; 181b-5p; 5112; 203-3p; 423-3p; 378-5p; 2137; 5131; 26a-5p; 5119	Mouse heart (subsarcolemmal and interfibrillar)	RNA clip, NGS	[10]

NGS next generation sequencing

[a] MiRNA matches with mitosRNA-segments

interaction with TAR DNA-binding protein 43 (TDP-43) [38]. These findings point to a potential role for miRNAs as binding partners or mediators for molecular interactions. This could also to some extent explain the reported observation that most AGO2-bound miRNAs do not participate actively in repression of mRNA translation [41]. For some miRNAs, aptamer-like characteristics might be part of the regulation and biological effects, as it has been suggested for miR-21, miR-93 and miR-296 [42].

In this context, it should also be considered that the environmental conditions differ between the mitochondrial matrix and the cytosol. Acetyl-CoA is a highly abundant metabolite inside mitochondria and acts, because of its thioester bond, as potent acetylation reagent. Due to these conditions, non-enzymatic acetylation is a common event in mitochondria [43]. In line with this, the majority of mitochondrial proteins carry acetyl-groups.

It stands to reason that non-enzymatic acetylation of RNA molecules, including miRNAs, is a possible modification inside mitochondria (Fig. 3b). Possible acetylation sites of a miRNA molecule include the RNA bases (adenine, guanine, cytosine [44]) (structures 2–4, box in Fig. 3b) and the ribose backbone (structure 1, box in Fig. 3b). The latter has also been a proposed target for the pharmaceutical acetylating reagent aspirin [45]. Covalent attachment of an acetyl-group would potentially alter a miRNA's recognition pattern for mRNAs. Alternatively it may prevent spontaneous bond cleavage and thus increase the half-life time, if occurring on the 2-OH-group of ribose that is required for the cleavage reaction. Furthermore, post-transcriptional modifications can lead to structural changes [46] and modified interactions with other RNA molecules or proteins [47]. In addition to acetylation, other RNA modifications might be possible as well, e.g., methylation [48, 49] or succinylation [43].

In conclusion, post-transcriptional modifications of miRNAs could be one consequence following their mitochondrial entry. The mitochondrial subpopulation of one miRNA might, therefore, show altered features compared to their cytosolic counterparts. It is possible that mitomiRs function

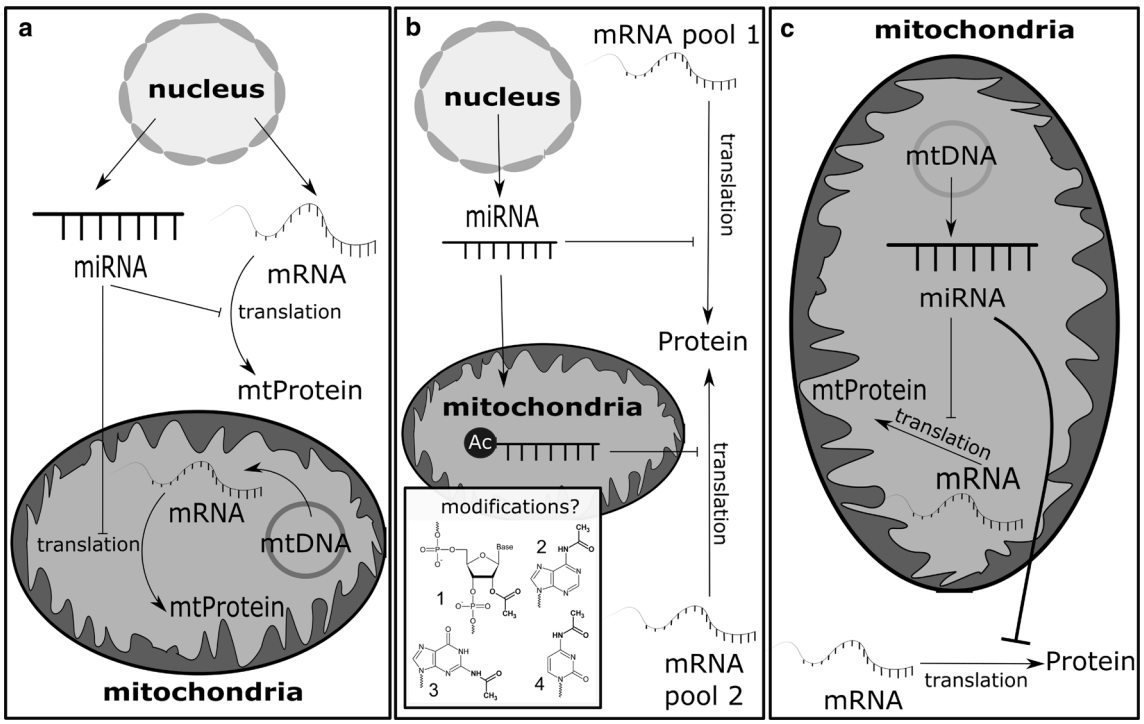

Fig. 3 Possible functions of mitomiRs. **a** MiRNAs encoded in the nucleus could suppress translation of mitochondrial proteins either in the cytosol or after re-location into the mitochondrial matrix. **b** After translocation from the cytosol to the mitochondrial matrix, small chemical molecules like acetyl-groups, may covalently be attached to the miRNA molecule. Due to modified ribose (structure *2*, acetyl-ribose) or modified bases (structure *2*, acetyl-adenine; structure *3*, acetyl-guanine, structure *4*, acetyl-cytosine), the miRNA then targets a different set of mRNAs. **c** MiRNA could be transcribed from the mitochondrial genome. After maturation inside the mitochondria, the miRNAs would inhibit mRNA translation in the mitochondrial and cytosolic compartment. *Ac* acetyl-group, *mRNA* messenger RNA, *miRNA* micro-ribonucleic acid, *mtDNA* mitochondrial DNA, *mtProtein* mitochondrial protein

Table 2 Reported mitochondrial presence of DICER and RISC-components

Protein	Used specimens	References
AGO2	Mouse cardiomyocytes	[10]
AGO2	MIN6 cells	[143]
AGO2	Mouse cardiomyocytes, C2C12 cells	[134]
AGO2	HeLa cells	[16]
AGO2	Rat hippocampus	[58]
FXR1	Mouse cardiomyocytes	[10]
DICER	Rat hippocampus	[58]

by additional or other mechanisms than repression of mRNA translation. Clearly, more research is needed to elucidate the origin, function and characteristics of mitomiRs.

Transport of mitomiRs

The numerous reports on miRNA in mitochondria (Table 1) point also to another puzzling aspect: How do miRNA translocate into the organelle? Given their size and charged nature, miRNAs are unlikely to cross membranes on their own. In general, RNA transport into human mitochondria is not well studied. Besides miRNA, only few RNA-species are imported into human mitochondria, e.g., 5S rRNA and RNase P [50]. Mitochondrial import of miRNAs constitutes, therefore, a clear knowledge gap.

A few suggestions have been published regarding potential import players, one of them being AGO2 [16]. Due to its RNA-binding properties and its dual localization in cytosol [51] and mitochondria [16], AGO2 could be involved in miRNA-trafficking.

Another study showed a role of the exoribonuclease polyribonucleotide nucleotidyltransferase (PNPT1/PNPASE) in mitochondrial RNA import [52]. PNPASE might therefore, possibly be part of an alternative, AGO2-independent mitochondrial miRNA-uptake pathway [16].

Furthermore, a potential mechanism could involve the voltage-dependent anion-selective channel protein (VDAC) [7]. At least in plants VDAC has, together with TOM20 and TOM40, a function in mitochondrial RNA-import [53, 54]. However, since RNA-transport pathways exhibit a high amount of species-specificity [55], these findings might be of limited relevance to mammalian and human mitochondria.

Import of miRNA would primarily be relevant for miRNA that are encoded outside the mitochondrial organelle. Although all currently reported mitomiRs originate from the nuclear genome, it has been speculated whether a few might in fact stem from mitochondrial DNA [7] (Fig. 3c). Of note, precursor forms of miRNAs have been also been observed in the organelle [8].

Intriguingly, recent studies showed that the mitochondrial genome encodes multiple non-coding RNAs, which are named mitosRNAs [30, 56, 57]. The size of these mitochondrial RNA molecules ranges from 12 to 137 nt. In humans, most mitosRNAs are 20–29 nt long, although length varies between different species [56]. Depending on the strand-of-origin, the mitosRNA molecules possess different nucleotide-patterns in the 5′- and 3′-end. For humans, the expression of at least some mitosRNAs is tissue-dependent. While *Dicer* knock-out clearly affected the mitosRNA generation, it did not completely abolish it [56]. Since RNAi components such as DICER and AGO2 were absent from mitochondria of HEK293T cells, it was suggested, that mitosRNAs are products of yet unidentified ribonucleases [56]. Other reports, however, demonstrate that DICER [58] and AGO2 [10, 16] are present in mitochondria and that some mitosRNAs are able to interact with DICER [30].

Thus, it appears that some previously reported miRNAs or mitomiRs could be mitosRNAs. Several mitosRNA clusters align well with human miRNAs, i.e., miR-4485-3p, miR-1973 (Table 1) or miR-4284 [11, 30]. So far mitosRNAs are in average longer than miRNAs and do not show the classical suppression of translation that is associated with miRNAs. Over-expression of the mitosRNAs L-A_3-sen and L-P+_6-sen was accompanied by a doubling in the expression level of their mitochondrial host genes TrnA and TrnP [56], suggesting that mitosRNAs could act in a RISC-independent way. Clearly, more studies need to be done to understand the role of mitosRNAs and to investigate potential overlaps between mitosRNAs and miRNAs.

MiRNAs and mitochondrial metabolism

Electron transport chain

The electron transport chain located in the IMS consists of several protein complexes (including cytochrome c oxidase) that generate a proton gradient, which drives ATP synthesis through ATP synthase. The majority of the coding sequences for ETC components reside in the nucleus, while only 13 subunits are encoded by the mitochondrial genome.

There are several ways by which miRNAs influence ETC protein expression from both nuclear and mitochondrial genes. First, miRNAs can directly target relevant transcripts (Fig. 4). Studies in neural cell culture systems revealed that miR-338-5p is able to influence the expression of cytochrome c oxidase (COX) IV and subunits of the ATP synthase complex with a >50 % reduction of protein levels [59, 60]. MiR-338-5p is mainly expressed in the brain, but also in pancreatic β-cells [61]. The ATP synthase is a reported target for two miRNAs. Its nuclear encoded subunit ATP5B is regulated by miR-101-3p, as shown by knock-down experiments in Hela cells yielding a ∼70 % reduction at the protein level [62]. The miR-101-3p sequence is conserved across humans, rats and mice and mature miR-101-3p has also been identified in mitochondria from mice liver [9]. In addition, ATP5B has been found to be regulated by miR-127-5p, which is able to decrease the amount of ATP5B protein by 50 % [63]. This miRNA is highly expressed in human liver, especially in fetal liver cells. The expression level in other cells and cell lines is modest. In concordance with these findings, the ATP synthase protein is barely detectable in liver tissue from fetal samples while increased protein levels are present in adult liver [63]. However, this does not exclude additional regulation by other miRNAs.

A study in neonatal rat myocytes revealed that miR-181c-5p alters mitochondrial gene expression: Following maturation, miR-181c-5p enters the mitochondrial matrix and represses mitochondrial COXI, a subunit of the ETC complex IV, by ∼30 % [64].

The ETC requires iron-sulfur clusters as co-factors for complete function. These clusters have a short half-life. Therefore COX10, ETC complexes I and III, are dependent on continued synthesis for proper function. Mitochondria constitute the principal location of the mammalian iron-sulfur cluster (FeS) assembly [65]. The vital role of iron-sulfur cluster assembly enzyme (ISCU) 1/2 in FeS-biosynthesis is well described [66]. Several studies have reported a large impact (reduction of protein levels by >50 %) of miR-210-5p on ISCU expression, which consequently affects FeS-cluster containing proteins [67–71]. MiR-210-5p causes a translational inhibition, which mainly affects the cytoplasmic ISCU2 isoform [72]. The miR-210-5p mediated repression of ISCU causes a blockage of the assembly pathway [73] and affects the overall ETC activity.

Due to its up-regulation under low oxygen concentrations, miR-210-5p has been termed a ‘hypoxamir’ [70] (also ‘hypoximir’ [74]). In general, hypoxia causes widespread changes in miRNA expression profiles of cells and tissues [75]. The transcription factor hypoxia-inducible factor (HIF) 1-α is a master regulator of the hypoxic response [75–77] working through hypoxia-responsive

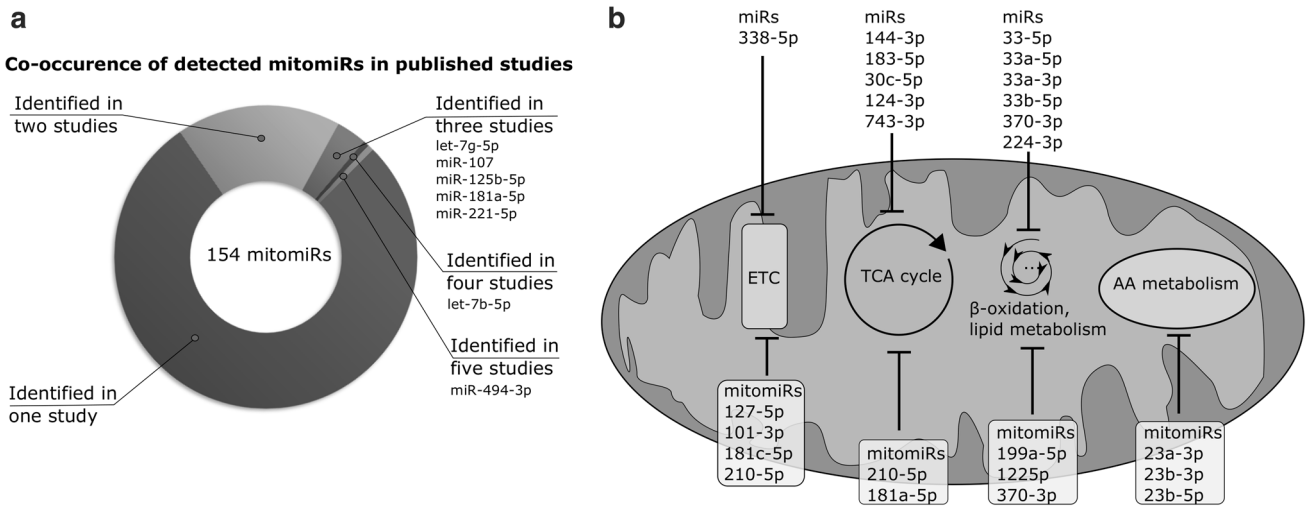

Fig. 4 **a** Number of studies (from Table 1) that independently identify the same mitomiRs. The overlap of mitomiRs that are reported from different articles is small. However, a subset of seven miRNAs has been identified with high confidence by at least three studies. **b** Mitochondria host important metabolic pathways of carbohydrate, lipid and amino acid metabolism. Shown are miRNAs and mitochondrial miRNAs (*in boxes*) that inhibit enzymes of the indicated pathways. *ETC* electron transport chain, *TCA* tricarboxylic acid, *AA* amino acid, *miR* micro-ribonucleic acid

elements (HREs) in promoters or enhancers. The miR-210 gene is a direct HIF-1α target [78], while many other hypoxia-induced miRNAs are not directly regulated by HIF-1α, but could be regulated via hypoxia-induced inflammatory responses [75]. Besides hypoxia, miR-210-5p is also up-regulated by as much as fourfold by reactive oxygen species (ROS) [72] or iron deficiency [69]. Although mir-210-5p has been identified in human muscle mitochondria, it is not known how or if its localization is influenced by hypoxia. On the other hand, in mammalian cell culture systems, the hypoxia-induced up-regulation of miR-210-5p was accompanied by a two to fourfold enhanced release of this miRNA into the extracellular medium [79]. The released miR-210-5p could influence target gene expression in recipient cells [79]. Theoretically, this miRNA might act as a form of mitochondrial messenger between cells.

In conclusion, miRNAs manipulate ETC activity by several mechanisms. The mRNAs of protein components can be directly targeted. A more indirect regulation occurs, if the biosynthesis of crucial co-factors, like iron-sulfur clusters, is affected.

The tricarboxylic acid cycle

Glycolysis generates pyruvate as a final product, which is converted to acetyl-CoA by pyruvate dehydrogenase (PDH), a multi-enzyme complex of several subunits. Inside the mitochondrial matrix, acetyl-CoA enters the TCA cycle. This results in production of redox-equivalents, which enter the ETC and drive the electrochemical proton gradient. In human colorectal cancer cells, miR-26a-5p inhibits nearly 40 % of PDH subunit X protein expression [80] followed by a reduced conversion of pyruvate into acetyl-CoA and a disconnection of the glycolytic flux from the TCA cycle.

The initial step of the TCA is catalyzed by citrate synthase (CS) fusing acetyl-CoA with oxalic acid. A number of miRNAs, i.e., miR-152-3p, miR-148a-3p, miR-148b-3p, miR-299-5p, miR-19a-3p, miR-19b-3p, miR-122-5p, miR-421, miR-494-3p, are suggested to regulate the expression of this enzyme [81]. Two thereof, miR-122-5p and miR-494-3p, have also been observed in mitochondria from rodent liver [9, 12]. Citrate is isomerized to isocitrate by aconitase, which requires an FeS-cluster for its activity. The previously discussed negative effects of miR-210-5p on iron-sulfur cluster assembly pathways, therefore, also negatively affect isocitrate production [72, 82]. The extensive effect of miR-210-5p exemplifies the principle of how a single miRNA can influence multiple mitochondrial processes. While there are currently no experimentally validated miRNAs targeting α-ketoglutarate dehydrogenase, the succinyl-CoA ligase (SUCL) mRNA was shown to be decreased by 75 % by miR-124-3p transfection in HepG2 cells [83].

Succinyl-CoA dehydrogenase (SDH) produces fumarate from succinyl-CoA and this step is impeded by miR-210-5p, which can reduce SDH-activity up to 50 % [84]. Thus, multiple actions of miR-210-5p contribute to the phenotype of mitochondrial dysfunction and loss of membrane potential. Collectively, miR-210-5p shifts energy metabolism towards anaerobic glycolysis, and therefore, contributes to

the Warburg effect. To the best of our knowledge, there are so far no published experimentally based reports on miRNAs targeting the final steps of the TCA in human peripheral tissue.

In conclusion, miRNAs are capable of regulating the TCA cycle, a central knot in metabolism (Fig. 4). MiR-210-5p can act negatively at several points of the TCA, having a major impact on TCA cycle metabolism. Other miRNAs could be more sophisticated switches, which decrease specific reactions of the TCA cycle possibly redirecting metabolic fluxes to other pathways.

Amino acid metabolism

Amino acids are important compounds in nutrition and metabolism giving rise to numerous metabolites. Several pathways of amino acid metabolism are placed inside the mitochondrial organelle. A major focus of the literature is on glutaminase (GLS), which converts the abundant amino acid glutamine to glutamate by deamidation. Glutamate can further be transformed to α-ketoglutarate and enter the TCA cycle. Since glutamine-conversion is a main pathway of energy production in cancer cells, research on glutaminase-regulation by miRNAs has strong medical relevance.

Glutaminase expression is normally limited by miR-23a-3p and miR-23b-3p. Both miRs bind to GLS-mRNA and suppress its translation. The oncogenic transcription factor c-myc, however, represses the expression of both miRNAs. In this way, c-myc indirectly increases mitochondrial glutaminase expression [85]. Other factors, like glutamine or the p65 subunit of NFκB, may also regulate miR-23a-3p expression [86]. The conversion of serine to glycine is catalyzed by the serine hydroxy-methyl transferase (SHMT). The mitochondrial isoform SHMT2 has been shown to be a direct target of miR-193b in MCF-7 cells, where it showed a ∼20 % reduction in proteomics data [87] (Fig. 4). This miRNA has also been traced to mitochondria from human skeletal muscle [8].

The influence of miRNAs on other main metabolic pathways like the urea cycle, remain to be investigated in detail. There could be additional targets in amino acid degradation, such as the branched-chain alpha ketoacid dehydrogenase [88]. Further research on miRNAs that participate in amino acid metabolism seems highly relevant.

Lipid metabolism

Lipids are essential parts of the human diet and important molecules in energy metabolism. The lipid content of diet impacts expression of miRNAs in offspring [89]. This could be relevant in a disease-related context. MiRNAs emerge as contributing factors in lipotoxicity [90], a phenomenon linked to T2D and cardiac problems. Moreover, miRNA and mitomiR expression can be affected by fatty acids (FA): miR-107-3p is two to eightfold increased by lipid treatment [91], and possible targets of this miRNA are *DICER1* and the *CLOCK* gene. The significance of the mitochondrial presence of miR-107-3p remains to be determined [8, 11, 92, 93].

A key pathway of fatty acids in the mitochondria is β-oxidation. Liver-specific disruption of *Dicer1* is followed by a 40–80 % decrease in levels of FA degrading enzymes, lipid accumulation and glycogen depletion in mice [94]. Thus, miRNAs contribute to regulation of β-oxidation (Fig. 4).

MiR-33-5p [95] and miR-370-3p [96] can diminish FA degradation by 30 % [97] via inhibition of carnitine-palmitoyl transferase A (CPT1A), which is important for generation of activated FA acyl-carnithines. MiR-33a-5p is generated from the primary mRNA transcript of the sterol regulatory element binding factor 2 (*SREBF2*) locus and regulated by *SREBF2* regulatory sequences [98]. Expression of miR-33-5p is influenced by dietary cholesterol in a highly sophisticated manner [99]: When exogenous cholesterol is low SREBFs are activated, transfer to the nucleus and activate genes responsible for increased cholesterol and FA synthesis. The simultaneous generation of miR-33a-5p is a convenient way of simultaneous inhibition of β-oxidation, contributing significantly to the increase in FA synthesis [97].

MiR-33a-5p is conserved across species [100], while in primates miR-33b-5p is embedded in the *SREBF1* locus [101]. Both miRs differ only in few nucleotides [97] and target central players in FA oxidation such as carnitine O-Octanoyltransferase (CROT), CPT1A and 3-ketoacyl-CoA thiolase (HADHB) as evidenced by a number of studies [95, 97, 100, 102]. In addition, miR-33a-3p, is also conserved, active and represses CROT, CPT1A as well as transcription factors in lipid metabolism [103]. In conclusion, the miR-33 precursors give rise to critical regulators in fatty acid metabolism.

The liver-specific and highly abundant miR-122-5p has major effects on lipid metabolism as thoroughly described in [99, 104–106]. Treatment of mice with anti-mir-122-5p reduces FA biosynthesis by 40 %, while increasing β-oxidation twofold [104]. By balancing aldolase A expression, mir-122-5p is additionally involved in control of glycolytic breakdown [107].

Indirectly, β-oxidation can be modulated by the mitomiRs miR-199a-5p [8] and miR-29a-3p [108] via the targeted δ-isoform of the peroxisome proliferator activated receptor (PPAR) family [109]. In heart tissue, expression of miR-199a-5p is driven by HIF leading to reduced PPARδ levels and reduced β-oxidation. This mechanism might also be relevant in liver tissue. MiR-199a-5p over-expression reduced the mitochondrial content and increased FA deposition [110]. However, mir-199a-5p was up-regulated

in mice fed a high fat diet [110], which could point to a negative feedback mechanism.

Other miRNAs with suggested influence on FA oxidation are miR-107 and mir-103 [93, 111, 112]. A feature of both miRs is their location within in intron of the *PANK1a* gene. The PANK product is involved in coenzyme A biosynthesis, a key cofactor of lipid metabolism. MiR-107 has also been detected in mitochondria [8, 11]. In adipocytes miR-224-5p impairs FA metabolism via its target acyl-CoA synthetase long chain familiy (ACSL4) [113]. The contribution of miRNAs to other paths of mitochondrial lipid metabolism (e.g., production of ketone bodies) is not well investigated. One report suggests miR-122-5p to target 3-oxoacid-CoA transferase 1 (OXCT1), an enzyme involved in the formation of ketone bodies. Upon mir-122-5p over-expression OXCT1 was repressed at mRNA and protein level [114].

Of note, although several mitomiRs have been shown to influence lipid metabolism, the miR-33 family and miR-122-5p seems to be the most important ones. Several studies provide evidence that a multitude of their target mRNAs are located within pathways of fat utilization [89, 103, 104, 115] and, thus, both miRNAs are strongly interwoven into lipid metabolism. Further information on the impact of miRNAs on lipid metabolism has also been excellently reviewed elsewhere (e.g., [98], [102], [100]).

One-carbon and nucleotide metabolism

The turnover of nucleotides and one-carbon metabolism are linked and mitochondria host parts of these metabolic pathways [116]. The mitochondrial enzyme 5,10-Methylenetetrahydrofolate reductase (MTHFR) converts homocysteine to methionine through the transfer of a methyl-group. MTHFR-mRNA is regulated by the mitomiR-149 [8, 117]. Additional mitomiRs (miR-125, miR-484 [8]) have been suggested to influence MTHFR expression [118] (Fig. 4).

Due to high DNA synthesis rates, nucleotide metabolism is important for cancer cells. The dihydroorotate dehydrogenase (DHODH), a mitochondrial enzyme [119], catalyzes the formation of orotate from dihydroorotate, and is targeted by miR-502 in HCT116 cells [120]. miR-502 over-expression resulted in tumor growth reduction partly mediated by DHODH's role in the cell cycle/G1 phase.

Transporter and carrier proteins

Sufficient transport of substrates and products is a major factor contributing to the metabolic activity of mitochondria. Most metabolites are not able to cross the mitochondrial membranes and depend on the import and export by specific transporter and carrier proteins. Table 3 summarizes the currently known miRNAs regulating mitochondrial transporters.

The adenine nucleotide transporter 1 (ANT1) has so far not been reported to be influenced by miRNAs. However, the expression of an interacting protein of ANT1, named ARL2, is reduced by miR-15b [146] (Table 3). Further, miR-141 targets the phosphate transporter SLC25A3, which provides phosphate for ATP production [144]. Carnitine-acyl carnitine translocase (CACT) in the outer membrane is a critical mitochondrial transporter in lipid metabolism. CACT directs activated fatty acids into mitochondria for β-oxidation. Upon CACT knock-down, carnitine-FA accumulate in the cytosol and FA oxidation is reduced [121]. This phenotype was mimicked by miR-132 and miR-212 over-expression, and both miRNAs directly interfere with CACT expression and are up-regulated in obese mice, providing a possible link to obesity-induced mitochondrial dysfunction.

In β-cells, the mitochondrial transit of glutamate is also subject to miRNA regulation. MiR-184 is enriched in islets and represses expression of SLC25A22, a mitochondrial glutamate carrier, in the MIN6 beta-cell line. In line with the ability of glutamate to potentiate insulin secretion [122], the study showed a reduced glucose-stimulated insulin secretion upon miR-184 over-expression [123]. MiR-184, together with its negative regulator glucose and its target AGO2, seems furthermore, to be part of a feedback loop involved in β-cell expansion during insulin resistance [124].

Thus, there is evidence that miRNAs control the regulation of metabolite transport in and out of the mitochondria, but this is an understudied area and should be examined more extensively in the future.

Mitochondrial fission and fusion

The mitochondrion is a highly dynamic organelle, forming a network-like structure with multiple contact points. The amount of mitochondria in cells is influenced by two opposing events: Fission and fusion (Fig. 5). The dynamics of this mitochondrial equilibrium impacts consequently the rate of the overall mitochondrial metabolism in a cell.

During fission, one mitochondrion is divided into two smaller organelles. The cytosolic GTPase dynamin related protein 1 (DRP1) is a central mediator of the events. DRP1 gets recruited to the outer mitochondrial membrane where it interacts with its adaptor proteins FIS1, mitochondrial fission factor (MFF), MID49 and MID51. The interactions facilitate the formation of a constricting ring structure around the mitochondria, which eventually causes scission in two separated organelles [125, 126]. There are miRNAs targeting DRP1 (miR-30a-5p [127], miR-499-5p [128]) or

Table 3 MiRNAs targeting mitochondrial transporters

MiRNA	Affected transporter	References
141	SLC25A3 (phosphate carrier)	[144]
155	SLC25A19 (thiamine transport; predicted target)	[145]
184	SLC25A22 (glutamate carrier)	[123]
132, 212	SLC25A20 (carnitine/acylcarnitine)	[121]
15b	ARL2 (interaction partner of ANT1)	[146]
15a, 133a	UCP2	[147, 148]
25	Mitochondrial calcium uniporter	[149]

ANT1 adenine nucleotide transporter 1, *ARL2* ADP-ribosylation factor-like protein 2, *UCP2* mitochondrial uncoupling protein 2

DRP1 adaptor proteins like MFF (miR-761 [129], miR-27 [130]) and FIS1 (miR-484 [131]) (Fig. 5).

In addition, miRNAs (miR-23 [132], miR-696 [133]) control the expression of PGC-1a, a key regulator of mitochondrial fission and biogenesis. However, upon physical activity this miRNA decreases and more mitochondria are formed [133]. This suggests that miRNAs could also function as mediators in the adaption to environmental factors such as exercise, e.g., by stimulating mitochondrial fission and biogenesis.

Mitochondrial biogenesis itself is depending on the mitochondrial transcription factor (mtTFA or TFAM). During differentiation of C2C12 muscle cells, both mtTFA (TFAM) expression and mtDNA content increase. Muscle differentiation in general is a complex event, which includes miR-1 as crucial stimulator of mitochondrial translation [134] and reduced miR-494 levels that give rise to mtTFA expression [135]. The down-regulation of miR-494 is somewhat surprising since mostly up-regulation of miRNAs have been reported in connection with differentiation processes. Of note, the presence of miR-494 in mitochondria has been reported several times [12, 16, 92].

The joining of two mitochondrial compartments is termed fusion and is considered a protective event since it promotes mitochondrial health, e.g., by content mixing [126]. Fusion depends on the membrane potential and starts with the merging of the MOM. The combination of the outer membranes is facilitated by mitofusin 1 (MFN1) and mitofusin 2 (MFN2) [126]. Because mitochondrial fusion can prevent apoptosis, the down-regulation of mitofusins by miR-106b and the mitomiR miR-140 [12] has been implicated with mitochondrial dysfunction and apoptosis [136, 137]. The MOM fusion is followed by the fusion of the inner membranes. For this process, the protein optic atrophy 1 (OPA1) is a central player. OPA1 binds to cardiolipin, which is an essential component of the inner mitochondrial membrane. So far no miRNAs have been experimentally shown to target OPA1, but fusion events

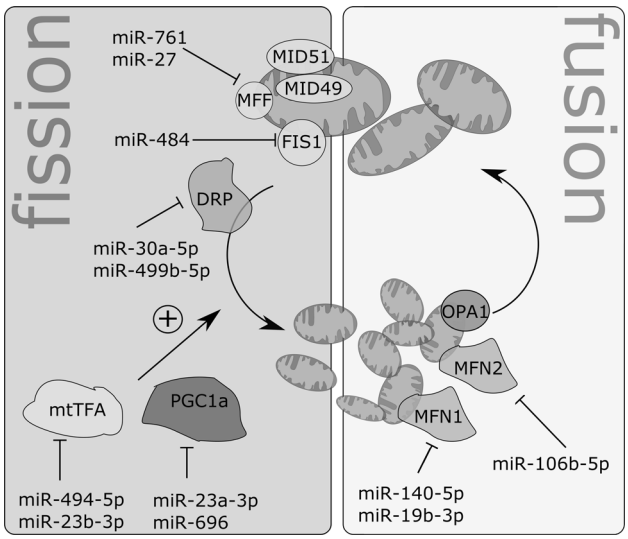

Fig. 5 Fission and fusion of mitochondria is regulated by multiple proteins. The figure summarizes involved proteins that are subject to miRNA regulation, such as MFF, MIDs, FIS1 or mitofusins. Moreover, miRNAs regulate fission via mtTFA and PGC1A, which are general enhancers of mitochondrial biogenesis. *DRP1* dymanin-related protein 1, *FIS1* mitochondrial fission protein 1, *MFF* mitochondrial fission factor, *MFN* mitofusin, *MID* mitochondrial dynamics protein, *mtTFA* mitochondrial transcription factor A, *OPA1* optic atrophy protein 1, *PGC1a* peroxisome proliferator-activated receptor gamma coactivator 1-alpha

could also be altered by changes in cardiolipin biosynthesis, because cardiolipin is an important lipid constituent of mitochondrial inner membranes. The CDP-DAG-synthase 2 is an enzyme in this pathway and a validated target of miR-16 [138]. However, the impact of this connection on mitochondrial dynamics needs to be further investigated. In summary, miRNAs can regulate proteins key in mitochondrial fission and fusion events and may control the equilibrium point of mitochondrial dynamics.

Conclusions and further perspectives

It is a common view that miRNAs may serve as fine tuning tools controlling the cellular environment. Most mitochondrial proteins are made in the cytosol. The regulation of mitochondrial proteins by miRNAs can, therefore, act via the canonical miRNA pathways of translational inhibition or transcript destabilization, but recent evidence supports an even closer interplay of miRNAs and mitochondria: A growing number of reports show the presence of miRNAs inside mitochondria of different cell types and tissues. Since these findings were obtained by the use of different detection techniques and biological specimens, they provide strong evidence that mitomiRs are more than a mere lab artifact. Moreover, a small subset of 7 mitomiRs co-occurred in more than three independent studies,

thereby further increasing the validity of mitochondrial miRNAs.

Although the miRNA-field is growing, most reports only investigate total quantities of miRNA-transcripts, thus neglecting their cellular localization. This limits the number of available publications on mitomiRs and provides further challenges for important matters such as finding a consensus in the quantification of mitomiRs. The suitability of frequently used reference genes, like the small nuclear RNA U6, is doubtful due to their often low mitochondrial abundance. Still, mitomiRs are an unattended topic in the current debate around miRNA-normalization and quantification.

In this context, it is also important to consider the strengths and limitations of different model systems. Cell culture models allow the unwrapping of mechanistic effects of miRNAs in a homogenous cell population. Still, they provide limited information compared to animal models, which yield close insight into systemic events. On the other hand, the complex interaction of numerous miRNA effects across tissues makes it often difficult to interpret data from in vivo systems. Last but not least, caution is required when transferring findings between different models and across species. Although conservation, e.g., of targets or miRNAs, might provide an indication, the notion is that a portion of mitomiRs are specific to primates or even humans [16, 37]. Translating results obtained using non-human specimens requires, therefore, further validation in human model systems.

In general, mitochondrial miRNA populations vary from different tissues and in different pathological states, suggesting that mitochondrial miRNA populations are subject to yet unidentified mechanisms of regulation. Unraveling ways of mitomiR trafficking and how to block them, would provide tremendously useful tools to study their biological functions. Since miRNAs can interact with a multitude of target mRNAs, it is a major drawback of current studies that they do not allow to study exclusively cytosolic and mitochondrial effects, respectively. Thus, more sophisticated experimental systems must be developed to resolve the role and functions of miRNAs in mitochondria.

So far, a limited number of studies point to mitomiRs as potential contributors to pathological conditions. It is a well-known feature that miRNA expression is altered in many forms of cancer [106, 139, 140]. Similarly, it is known that mitochondrial dysfunction is occurring in cancer [141, 142]. The coincidence of these two features will be interesting to explore further. Interestingly, ca. 20 % of known mitomiRs have also been described in a (non-cancerous) disease-linked context as up-regulated biomarkers. Given the up-regulation of these miRNAs in serum or blood, it seems possible that also intracellular concentrations are increased, thus also affecting mitochondrial targets. It remains to be closer investigated, how relevant the mitochondrial localization is in context of up-regulated biomarkers. But all in all, mitochondrial effects caused by miRNAs are also potential targets for therapy and should be closer investigated in the future.

Credits

The software Inkscape 0.91 was used to assemble all figures.

Acknowledgments J. Geiger and L.T. Dalgaard are supported by a grant from the Danish Independent Research Council | Health Sciences (DFF-FSS) and Roskilde University. The funders had no role in decision to publish or preparation of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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