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Targeting of CRISPR-Cas12a crRNAs into human mitochondria

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ABSTRACT

Mitochondrial gene editing holds great promise as a therapeutic approach for mitochondrial diseases caused by mutations in the mitochondrial DNA (mtDNA). Current strategies focus on reducing mutant mtDNA heteroplasmy levels through targeted cleavage or base editing. However, the delivery of editing components into mitochondria remains a challenge. Here we investigate the import of CRISPR-Cas12a system guide RNAs (crRNAs) into human mitochondria and study the structural requirements for this process by northern blot analysis of RNA isolated from nucleases-treated mitoplasts. To investigate whether the fusion of crRNA with known RNA import determinants (MLS) improve its mitochondrial targeting, we added MLS hairpin structures at 3′-end of crRNA and demonstrated that this did not impact crRNA ability to program specific cleavage of DNA in lysate of human cells expressing AsCas12a nuclease. Surprisingly, mitochondrial localization of the fused crRNA molecules was not improved compared to non-modified version, indicating that structured scaffold domain of crRNA can probably function as MLS, assuring crRNA mitochondrial import. Then, we designed a series of crRNAs targeting different regions of mtDNA and demonstrated their ability to program specific cleavage of mtDNA fragments in cell lysate and their partial localization in mitochondrial matrix in human cells transfected with these RNA molecules. We hypothesize that mitochondrial import of crRNAs may depend on their secondary structure/sequence. We presume that imported crRNA allow reconstituting the active crRNA/Cas12a system in human mitochondria, which can contribute to the development of effective strategies for mitochondrial gene editing and potential future treatment of mitochondrial diseases.

1. Introduction

Mitochondria play a vital role in eukaryotic cells, serving various functions, but their primary significance lies in the production of energy in the form of ATP through oxidative phosphorylation in the mitochondrial respiratory chain [1]. Although most of the organelle's macromolecules are encoded by the nuclear genome, mitochondria possess their own 16.5 kb DNA (mtDNA) which encodes 13 subunits of four out of the five OXPHOS complexes, 22 tRNAs and 2 ribosomal RNAs [2–4] (Fig. 1A). Mutations in these proteins, tRNAs or ribosomal RNAs can affect mitochondrial ATP production, resulting in a wide range of mitochondrial diseases, often associated with neuromuscular disorders [5,6]. The severity of mitochondrial diseases is highly associated with heteroplasmy level – a state when mutant and wild-type (WT) mtDNA molecules coexist in the same cell [7]. Taken together, mtDNA pathogenic mutations affect 1 in 5000 individuals worldwide [8]. To date, these pathologies have not found adequate treatment [9].

Mitochondrial gene editing offers a promising therapeutic approach to restore healthy mitochondrial function by reducing mutant mtDNA heteroplasmy level [10]. Currently, two main strategies have been proposed. The first strategy involves decreasing of mtDNA heteroplasmy level by inducing targeted cleavage of mutant mtDNA molecules. Due to the lack of repair mechanisms for double-strand breaks in human mitochondria, linearized mtDNA will be rapidly degraded, enabling the replication of WT mtDNA molecules to reconstitute the mtDNA pool [11]. Various mitochondria-targeted nucleases have been developed to degrade mutant mtDNA and adjust heteroplasmy ratios. Mitochondria-

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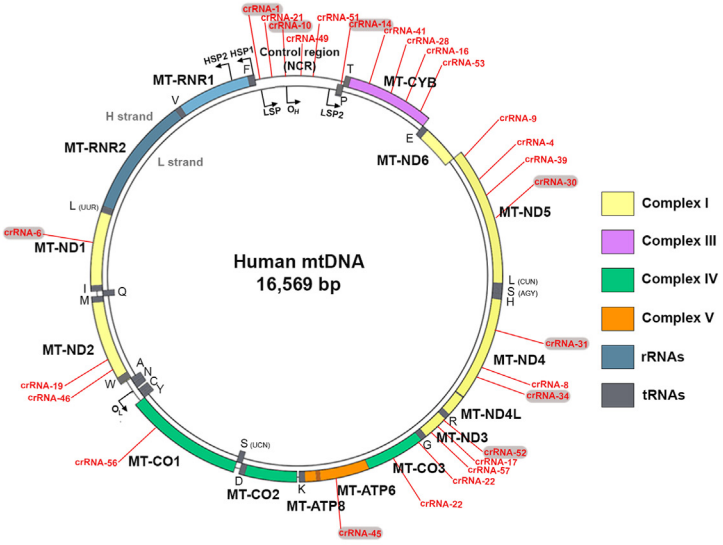
A



B

Name	Target	Sequence: 5'- 3'
crRNA-1	D-loop	UAAUUUCUACUCUUGUAGAUCCCUCCCACUCCCAUACUAC
crRNA-6	D-loop	UAAUUUCUACUCUUGUAGAUACCUCUCCACCCUUAUCACA
crRNA-10	MT-ND1	UAAUUUCUACUCUUGUAGAUCACACAGACAUCAUAACAAA
crRNA-14	mt tRNA^{Pro}	UAAUUUCUACUCUUGUAGAUACUCCACCAUUAGCACCCAA
crRNA-30	MT-ND5	UAAUUUCUACUCUUGUAGAUGAUGAUGGUCUUUGGAGU
crRNA-31	MT-ND4	UAAUUUCUACUCUUGUAGAUCACUCACAACACCCUAGGCU
crRNA-34	MT-ND4	UAAUUUCUACUCUUGUAGAUGCUGUUCCCCAACCUUUUCC
crRNA-45	MT-ATP6	UAAUUUCUACUCUUGUAGAUCCCCUCUAUUGAUCCCCACC
crRNA-52	MT-ND3	UAAUUUCUACUCUUGUAGAUUAGAUCAAAUAAUAAGAAGG

Fig. 1. Human mtDNA map with the representation of binding sites for 27 AsCas12a crRNAs bearing TTTV protospacer adjacent motif, designed for targeting different regions of mtDNA. (A) HSP, HSP2, LSP and LSP2 are heavy and light chain promoters; O_H and O_L are the origins of heavy and light chain replication; and the following genes are presented: NADH dehydrogenase subunits (*MT-ND1-ND6* and *ND4L*), cytochrome *b* (*MT-CYB*), cytochrome *c* oxidase subunits (*MT-COI-III*), ATP synthase 8 and 6 subunits (*MT-ATP6, MT-ATP8*), rRNA 12S (*MT-RNR1*) and 16S (*MT-RNR2*) and tRNAs (denoted by the one-letter code of the corresponding amino acids). The 9 crRNAs for which the possibility of mitochondrial import was evaluated are highlighted in grey. **(B)** Sequences of the 9 crRNAs used for mitochondrial import assays. The scaffold and spacer sequences in crRNA molecules are colored in black and blue, respectively. The targeted gene in human mitochondrial DNA is specified for each crRNA.

targeted restriction endonucleases (mitoREs), both native and artificial ones (mitoARCUS), have been successfully used to reduce mutated mtDNA levels in human cultured cells and mouse models [12–15]. However, such systems are highly limited by the occurrence of unique recognition sites, allowing targeting of specific pathogenic mutations in mtDNA. Additionally, the development of artificial nucleases demands significant time and resources. The mitoREs limitations were addressed by creating nucleases that are able to target specific sites in mtDNA by utilizing specific DNA recognition modules - zinc-finger proteins for mitoZFNs and TAL effector proteins for mitoTALENs. These approaches have demonstrated efficacy in modifying heteroplasmy ratios in cybrid cells and mouse models [16–24].

The second strategy, which can also be applicable for homoplasmic mutations, consists of the use of base editors in order to reverse mutations without introducing a break in mtDNA. Two types of base editors are currently employed for mtDNA editing: mitochondria-targeted TALE-linked or ZFN-linked double-stranded DNA deaminase (DddA)-derived cytosine base editors (DdCBEs) and mitochondria-targeted TALE-linked adenine deaminases (TALEDs) [25–29]. Although strategies based on mitoZFNs and mitoTALENs show promise for mtDNA targeting, they still have limitations. Designing and engineering monomers for specific mtDNA recognition is a challenging and resource consuming task. Moreover, the large size of protein coding sequences exceeds the capacity of current virus-based delivery systems, such as adeno-associated virus (AAV) [30,31].

In this regard, CRISPR-Cas systems, while having shorter coding sequences, offer greater flexibility for DNA recognition experiments compared to protein-only systems due to their reliance on DNA–RNA interactions for specific mtDNA site recognition [32]. Thus, they enable faster change of the DNA recognition site by use of different guide RNAs keeping the protein component constant. However, the two-component system necessitates the assembly of a functional machine within the mitochondria, which requires direct delivery of both the protein and RNA components into the mitochondrial matrix [33]. The import of proteins into mitochondria can be effectively achieved by fusing a mitochondrial targeting sequence (MTS) to the N-terminus of the protein of interest [34]. The MTS consists of an amphipathic α-helical peptide sequence that is recognized by receptors of the translocases of the outer membranes (TOM) that direct the protein to the mitochondrial matrix. Recent studies from our and other laboratories demonstrated mitochondrial targeting of Cas nucleases bearing various N-terminal MTS (listed in Table 1).

In contrast, the import of gRNA component into mitochondria

Table 1
Recently developed mitochondrial CRISPR/Cas systems.

Cas nuclease	sgRNA/crRNA	RNA delivery	Detection of sgRNA/crRNA localization	Effect on mtDNA	Reference
SpCas9	Unmodified sgRNA; sgRNA bearing yeast tRK1 F- and D-hairpins	RNA transfection	Northern blot hybridization of total and mitoplast RNA	decrease of mtDNA copy number; no shift in heteroplasmy level	[33]
SpCas9	Unmodified sgRNA	Transcribed from plasmid under control of U6 promoter	Not verified	decrease of mtDNA copy number;	[47]
LbCas12a	Unmodified crRNA; crRNAs with the stem-loop motifs from RNase P RNA and from yeast tRK1	Transcribed from plasmid under control of U6 promoter	Northern blot hybridization of total and mitochondral RNA	Increase of mtDNA copy number	[48]
SpCas9	Unmodified sgRNA; sgRNA with the stem-loop motif from RNase P RNA	Transcribed from plasmid under control of U6 promoter	Laser scanning microscopy of Alexa fluor 488-labeled RNA; RT-qPCR on total and mitochondrial RNA	Small reduction of mutant mtDNA	[49]
SpCas9	Unmodified sgRNA; sgRNA with the stem-loop motif from RNase P RNA	Transcribed from plasmid under control of U6 promoter	Not verified	Generation of InDels (<0.05%)	[50]
SpCas9 SpCas9-BE3	Unmodified sgRNA; sgRNA with the stem-loop motif from RNase P RNA	Transcribed from plasmid under control of U6 promoter	Not verified	No change in mtDNA copy number; Unspecific C to T substitutions	[51]
SpCas9	Unmodified sgRNA	Transcribed from plasmid under control of U6 promoter	Laser scanning microscopy of FAM-labeled sgRNA	decrease of mtDNA copy number;	[52]

poses a significant challenge for applying CRISPR-Cas systems. In most of recent publications (Table 1), CRISPR/SpCas9 system have been applied, but the mitochondrial import of gRNA has never been convincingly demonstrated, thus raising uncertainty on the possible use of this system for mtDNA manipulations [35]. We suggest two main reasons for this. First, the use of plasmids expressing gRNAs in the nucleus, being well established for the nuclear DNA applications, seems to be not convenient for gRNA mitochondrial targeting due to the need of efficient gRNA export from the nucleus (Fig. S1A), which cannot be assured in the absence of the signals for RNA export [36–38]. Second, sgRNAs of Cas9 system are rather long (about 100 bp) (Fig. S1B) [39], which can strongly decrease their mitochondrial targeting [40].

To address these problems, in this study, we deliver gRNAs into cells by direct Lipofectamine transfection (Fig. S1A), which had been used in our lab previously to provide mitochondrial import of various types of small non-coding RNAs [41,42], including SpCas9 sgRNA [33]. Then, we use an alternative CRISPR/Cas system, the Type V CRISPR/Cas12a, characterized by a shorter crRNA of 40–44 nucleotides (Fig. S1B) [43], which may represent a better substrate for mitochondrial import compared to sgRNA. Finally, we apply our long-term expertise and recent findings [44] to avoid possible false-positive data and provide evidence of AsCas12a crRNA targeting into mitochondria in transfected human cells.

Studies by our and other laboratories had shown that the motif required for the transport of RNA molecules into mitochondria is not a sequence, but a hairpin structure (Fig. S1C) [45,46]. These structures, referred to as MLS (mitochondrial localization signal), were initially discovered in our Selex experiments and further successfully tested for anti-replication strategy in a variety of human cell lines [33,41,42].

We aim to critically investigate whether various AsCas12 crRNAs can be successfully imported into human mitochondria and to study the structural requirements for this process. We show that fusion of crRNAs with MLS is not required for their targeting into mitochondria.

2. Materials and methods

2.1. Design of AsCas12a crRNAs

RNAfold [53] and Mfold web servers [54] were used to predict secondary structures of AsCas12a crRNAs. To predict secondary structures of crRNAs upon dimer formation, RNAcofold web server was used [53]. In order to search for the potential off-target sites of AsCas12a RNA-guided endonuclease, we used Cas-OFFinder algorithm [55].

In the reference human mtDNA sequence (GenBank NC_012920 gi:251831106), 530 sequences representing the canonical AsCas12a "TTTV" PAM (Fig. S1B) were found [56]. Following the PAM sites, subsequent 5' – 3′ 20 bp sequences of spacers were supplemented at the 5' -end by the AsCas12a scaffold sequence – "UAAUUUCUACUCUUGUAGAU" [43]. crRNAs were analyzed for predicted probability of introducing unwanted changes (off-targets) into the nuclear genome by the AsCas12a-crRNA complex, as well as for predicted secondary structures. crRNAs were rated according to these parameters and 27 of them were chosen for mitochondrial targeting assays (Table S3).

2.2. In vitro transcription of AsCas12a crRNAs bearing import determinants

crRNAs were *in vitro* transcribed using HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs) according to the manufacturer's protocol. As a DNA template, we used PCR amplicons obtained from the plasmids encoding non-modified (NM) crRNAs and modified versions bearing RNA import determinants (MRP, HD, HF) using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific) (Fig. S2). T7 promoter was added to the sequence of forward primer (Table S1). *In vitro* transcribed crRNAs were purified using TRIzol™ Reagent (Invitrogen) according to the manufacturer's protocol. RNA concentrations were measured using NanoDrop 1000 Spectrophotometer V3.5 (Thermo Scientific). The quality of

synthetic crRNAs was verified by electrophoresis in 8 M urea denaturing 10% polyacrylamide gel. Following 1 h migration at 20 mA, EtBr-stained gel was visualized by Hero Lab Transilluminator UVT-28 M (Herolab GmbH).

2.3. Chemical synthesis of AsCas12a crRNAs

The oligoribonucleotides were synthesized using an automatic DNA/RNA synthesizer ASM 2000 (Biosset Ltd.) at 500 nmol scale on 1000 Å Universal support CPG (Primetech ALC, 41 μmol/g) as described before [57]. Synthetic crRNAs were protected with terminal 2′-O-Methyl (2′-O-Me) and phosphorothioate (PS) modifications [58] (Fig. S3).

2.4. Cell culture conditions and RNA transfections

HEK 293 cells and the hAsCas12a HEK 293 T-REx stable cell line [57] were maintained at 37 °C, 5% CO_2 in essential modified Eagle's medium (EMEM) (Sigma-Aldrich) containing 1 g/L D-glucose and supplemented with 1.5 g/L sodium bicarbonate (Sigma-Aldrich) and 0.11 g/L sodium pyruvate (Sigma-Aldrich), 10% fetal bovine serum (Gibco, Fisher Scientific), 100 mg/L Penicillin-Streptomycin (Sigma-Aldrich) and 2.5 mg/L Amphotericin B (Sigma-Aldrich). The human osteosarcoma cell line 143B was cultivated under the same conditions, with the exception of the medium, which was based on Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) containing 4.5 g/L of D-glucose.

Before each experiment, cell cultures were tested for mycoplasma contamination by PCR with DreamTaq Green PCR Master Mix (Thermo Scientific), as described [59] (see primers in Table S1). Total DNA isolated with NucleoSpin® Tissue Mini Kit (Macherey-Nagel) according to the manufacturer's protocol was used as a PCR template.

For AsCas12a crRNAs mitochondrial import analysis, cells were transfected at 80% confluency in 75 cm^2 cell culture flask with 6 μg of chemically-synthesized or T7-transcribed crRNA using 20 μL of Lipofectamine™ 2000 transfection reagent (Invitrogen) in 9 mL Opti-MEM™ reduced serum medium (Gibco, Fisher Scientific). 6 h after transfection, the medium was changed to 12 mL of EMEM, and cells were left to propagate for another 48 h.

2.5. Test of AsCas12a/crRNAs activity in cell lysate

As DNA substrates for *in vitro* cleavage tests, we used mtDNA fragments amplified with Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific) from genomic DNA isolated from T-REx cell line (T-REx™-293 Cell Line, Invitrogen, kat. #R71007) by NucleoSpin® Tissue Mini Kit (Macherey-Nagel) according to the manufacturer's protocol. DNA oligonucleotides used for the template DNA amplification are listed in Table S1.

For the *in vitro* cleavage assay, the hAsCas12a HEK 293 T-REx stable cell line was used [57]. Cells with inducible expression of the AsCas12a nuclease were cultured in 6-well plate. The expression of the nuclease was activated at 80% confluency by the addition of tetracycline to a final concentration of 100 ng/mL 24 h after induction, cells were washed with 1 × phosphate buffered saline (PBS) and lysed by 50 μL/cm^2 lysis buffer (20 mM HEPES-KOH pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 0.1% Triton X-100 and Protease Inhibitor cocktail (Roche Diagnostics)) and then rocked for 10 min at 4 °C. To remove cell debris, lysates were centrifuged for 10 min at 1000 g, 4 °C. The resulting supernatant was divided into aliquots and stored at −80 °C.

Cleavage reactions were carried out in a total volume of 20 μL and contained 18 μL of cell lysate, 150 ng of PCR-obtained DNA substrate and 50 ng of crRNA. Reactions were incubated for 30 min at 37 °C. After incubation, reactions were purified with NucleoSpin® PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer's protocol. Analysis of cleavage products was performed by electrophoresis in 1% agarose gel (w/v) in 1 × TAE buffer. EtBr-stained gels were visualized by Hero Lab Transilluminator UVT-28 M (Herolab GmbH).

Alternatively, to verify AsCas12a/crRNA functionality in cells, the hAsCas12a HEK 293 T-REx stable cell line was cultured in a 12-well plate and transfected at 80% confluency with increasing amounts of AsCas12a crRNA (175, 250, 375 ng/cm^2) using Lipofectamine™ 2000 transfection reagent (Invitrogen) in 500 μL of Opti-MEM™ reduced serum medium (Gibco, Fisher Scientific). After 6 h of transfection, the medium was changed to 2 mL of EMEM supplemented with 100 ng/mL of tetracycline to activate the nuclease expression. After 24 h, the cell lysates were prepared as described above. The *in vitro* cleavage reactions contained the appropriate DNA template, but no additional crRNA was added (except for the control reaction).

2.6. Mitochondria isolation and fractionation

Mitochondria were mainly isolated as described [42] with modifications. All procedures were carried out at 4 °C, unless specified differently. Briefly, transfected cells were washed once with 1 × phosphate buffered saline (PBS) and then detached and resuspended in 1 mL of prechilled Mito buffer (0.6 M sorbitol, 10 mM HEPES-KOH, pH 7.5, 1 mM EDTA). 50 μL of resuspended cells were taken for total RNA isolation. The rest of the cells were disrupted by 30 passages through 2 mL syringe with needle No26G (0,45 × 12 mm). Nuclei, cell debris and unbroken cells were pelleted by centrifugation for 10 min at 1000 g at 4 °C. The procedure was repeated 3 times with transferring the resulting supernatant each time to a new ice-cold tube. Mitochondria-rich pellet was obtained after high-speed centrifugation at 15,000 g at 4 °C for 30 min and then carefully resuspended in 150 μL of Mito buffer. 30 μL of resuspended mitochondria were taken for lysate preparation (see below). To decrease possible cytosolic RNA contamination, 120 μL of 2 × RNase A solution (20 μg/mL ribonuclease A from bovine pancreas, 4 mM $MgCl_2$, 200 mM NaCl) was added to the purified mitochondria. The reaction was incubated at room temperature for 7 min and then stopped by adding 500 μL of Mito buffer containing 4 mM EDTA. RNase A was washed out by centrifugation at 15,000 g at 4 °C for 15 min 3 times with 500 μL of Mito buffer, each time discarding the resulting supernatant. After the last centrifugation, mitochondria were resuspended in 300 μL of Mito buffer. In order to obtain mitoplasts, mitochondria were treated with 20 μg of digitonin (Sigma-Aldrich) for 7 min at room temperature. The resulting mitoplasts were centrifuged for 15 min at 15,000 g at 4 °C and washed ones with 500 μL of Mito buffer. To proceed with RNA isolation, the mitoplast pellet was resuspended in 500 μL of TRIzol™ Reagent (Invitrogen) and was either stored at −80 °C or treated immediately.

To prepare mitochondrial lysate, mitochondria were treated with n-dodecyl-β-D-maltoside (Sigma-Aldrich) (0.5% final concentration (w/v)), 20 μg of digitonin (Sigma-Aldrich) and with an equal volume of 2 × RNase A solution to degrade mitochondrial RNA and to demonstrate possible presence of RNase-resistant contaminant. After a brief pipetting, the mixture was left for incubation at room temperature for 10 min. To proceed with RNA isolation, the mitochondrial lysate was resuspended in 500 μL of TRIzol™ Reagent (Invitrogen).

Submitochondrial fractionation was performed as previously described [44,60] with modifications. In brief, the mitochondria-rich fraction corresponding to the pellet was obtained by differential centrifugation as described above. For the RNase A/T_1

accessibility test, the mitochondrial pellet was carefully resuspended in 300 μL of Mito buffer and split into three aliquots. The mitochondrial pellet was sedimented again with an excess of Mito buffer. After that, mitochondria were carefully resuspended in 100 μL of Mito buffer (for intact mitochondria), swelling buffer (10 mM HEPES-KOH, pH 7.5, 1 mM EDTA) (for mitoplasts) and lysis buffer (10 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 0.5% (w/v) n-dodecyl-β-maltoside) (to lyse mitochondria). Each tube was split into 2 aliquots, in which one was treated with 1.5 μL of RNase A/T_1 mix (2 mg/mL, 5.000 U/mL; Thermo Fisher Scientific) for 10 min at room temperature. For RNA extraction, 500 μL of TRIzol™ Reagent (Invitrogen) were added to the reaction.

2.7. Northern blot analysis

RNA was purified from all the samples using TRIzol™ Reagent (Invitrogen) according to the manufacturer's protocol, followed by precipitation with isopropanol. RNA pellets were diluted in 15 μL of nuclease-free water. Northern blot analysis was performed as described [42]. Purified RNA samples were separated on a 10% (w/v) denaturing polyacrylamide gel. After electrophoresis at 20 mA in 1 × TBE until the bromophenol blue dye reached the middle of the gel, samples were electroblotted onto a nylon Amersham HybondN + membrane (GE Healthcare) in a wet transfer camera in 0.5 × TBE at 4 °C, 10 V, overnight. RNAs were fixed on the membrane by irradiation in a cross-linking UV chamber (Amersham Life Science), for 3 min at each side of the membrane at constant energy 1.500 × 100 μJ/cm^2. After that, membranes were pre-hybridized by rotating in a hybridization oven in 6 × SSC, 0.1% SDS, 10 × Denhardt solution for 1 h at 65 °C. The pre-hybridized membranes were incubated overnight with continuous rotation in the prehybridization buffer supplemented with 5′-^{32}P-labeled antisense oligonucleotide probe at 56 °C. DNA oligonucleotide probes (Table S2) were radiolabeled with γ-^{32}P-ATP using T4 polynucleotide Kinase (Thermo Scientific) and then purified by size exclusion chromatography with Micro Bio-Spin™ Chromatography Columns (Bio-Rad) according to the manufacturers' protocols. After hybridization, membranes were washed 3 times for 10 min in 5 × SSC, 0.1% SDS buffer, and sealed between two polyethylene sheets and exposed on the phosphoimager screen overnight. The hybridization signal was visualized on Typhoon Trio (GE Healthcare) and analyzed with ImageQuant TL (v. 7.0, GE Healthcare). Before proceeding with hybridization with other probes, membranes were stripped 3 times for 10 min in 0.02 × SSC, 0.1% SDS buffer at 80 °C.

2.8. Quantitative analysis of RNA import

After quantification using the Typhoon Trio scanner, the relative efficiency of RNA import into mitochondria was calculated as a ratio between the signal obtained with a probe specific for AsCas12a crRNA used for cell transfection and that obtained with the probe against the host mitochondrial tRNA (mt *tRNAVal*) [42]. To detect possible AsCas12a crRNAs' protection against treatment with RNases not linked to the mitochondrial import, a control experiment consisting of the lysis of the portion of mitochondria was considered.

3. Results

3.1. Design of AsCas12a crRNAs targeting various regions in human mitochondrial DNA

The selected 27 crRNAs are shown in Fig. 1A, Table S3. Capacity to be imported into mitochondria has been evaluated for nine crRNA from this list (Fig. 1A and B), characterised by different predicted secondary structures, probability of off-target cleavage in the nuclear genome and homodimer formation (Tables S4, S5 and S6).

3.2. AsCas12a crRNA can be imported into human mitochondria without additional import determinants

To ensure function of AsCas12a nuclease within human mitochondria, the presence of crRNA in the mitochondrial matrix is essential. To investigate whether the fusion of crRNA with known RNA import determinants [40] improve its mitochondrial targeting, we modified one of the designed crRNAs by adding stem-loop motifs of human RNase MRP RNA [45] and *S. cerevisiae* $tRNA^{Lys}_{CUU}$ (tRK1) D-hairpin and F-hairpin [46] (Fig. S1C).

Previously published crystal structure of the AsCas12a-RNA complex revealed that the crRNA 5′ handle adopts a pseudoknot structure, which is crucial for recognition and interaction between AsCas12a and crRNA [43]. It is worth noting that several nucleotides of the 5′ scaffold, including the terminal ones, are conserved among the CRISPR-Cas12a systems [61]. However, in the crystal structure, the absence of electron density for the terminal nucleotides of the 3′ guide segment and target DNA strand suggests that these regions are flexible and disordered [43]. Based on these published data, we hypothesized that this part of the crRNA could be modified without affecting the overall function of the complex. Therefore, we fused the MLS to the 3′-end of crRNA (Fig. 2A and B). Subsequently, we evaluated AsCas12a nuclease activity *in vitro*, using modified or unmodified crRNA, and observed complete cleavage of targeted DNA in all reactions (Fig. 2C and D).

We then tested whether these crRNAs could be delivered to mitochondria *in vivo*. For this, we transiently transfected HEK 293 cells with corresponding T7 transcripts and then analyzed total RNA and RNA isolated from mitoplasts using Northern blot hybridization (Fig. 3A). Control hybridization was carried out to verify that isolated mitoplasts were depleted of nuclear or cytosolic contamination (5.8S rRNA). A mitochondrial RNA ($tRNA^{Val}$) was used as a loading control. We observed strong enrichment of the crRNA hybridization signal in the mitoplast fraction only for non-modified and F-hairpin-modified crRNAs which indicates on the targeting of these molecules into mitochondria (Fig. 3B). In contrast, other MLS structures (MRP and HD) reduced the mitochondrial localization of the crRNA. Noteworthy, these data show that crRNA can be imported into mitochondria without additional hairpin structures, in agreement with our former observation for Cas9 gRNA [33] and with that of Antón et al., 2020 [48] for LbCas12a crRNA.

3.3. Mitochondrial import for AsCas12a crRNAs targeting various regions in human mtDNA

Once confirmed that AsCas12a crRNA does not require an additional MLS, the next question we addressed was: if crRNAs, designed to target various regions in human mitochondrial DNA and thus possessing different sequences, can be imported into mitochondria with comparable efficiency. For this, we selected 9 crRNAs (Fig. 1B), chosen based on variations in predicted secondary structures, with 2 of them possessing potential off-target sites in the nuclear genome (Tables S4 and S5). Prior to the evaluation of their mitochondrial import, we first confirmed the activity of all 9 crRNAs by *in vitro* cleavage assay in lysate of human cells expressing AsCas12a nuclease (Fig. 4).

To evaluate the import efficiency of the selected crRNAs, we included, as a control, an aliquot of purified mitochondria lysed with detergent and treated with RNase, which aimed to detect potential protection of RNA of interest unrelated to mitochondrial

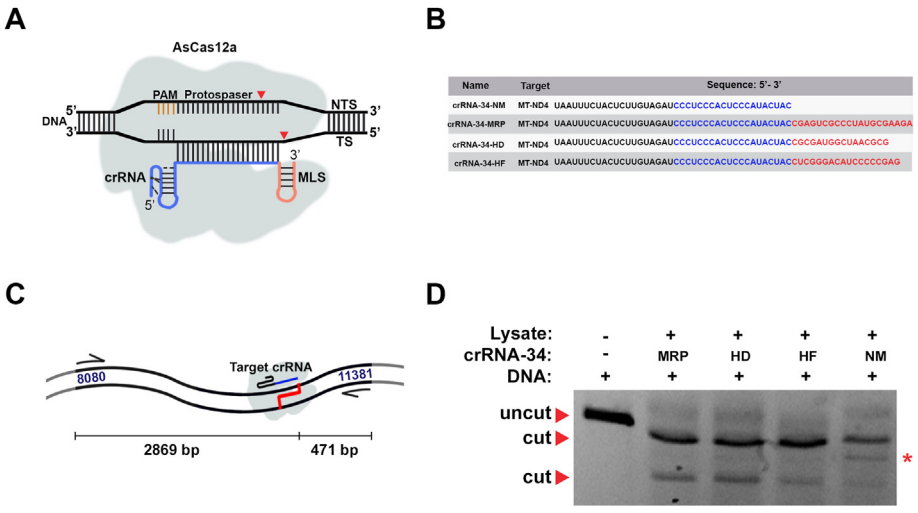


Fig. 2. Fusion of crRNA with MLS did not affect AsCas12a cleavage ability *in vitro*.
(A) Schematic representation of AsCas12a-crRNA-DNA complex. Mitochondrial localization signals (MLS) (see Fig. S1C) were added to the 3′-end of AsCas12a crRNA (shown in coral). **(B)** Sequences of the 4 crRNAs used in the import assays. The scaffold and spacer sequences in crRNA molecules are colored in black and blue, respectively. The targeted region in human mitochondrial DNA for each crRNA is specified. Additional 3′-end RNA import motifs of tRK1 (HD and HF), RNase MRP RNA (MRP) are shown in red. **(C)** Schematic representation of target DNA (PCR fragment of human mtDNA, nucleotide numbers are indicated) containing AsCas12a PAM sequence. The spacer moiety of target crRNA-34 is shown in blue, the scaffold part is shown in black, the cleavage site is marked with a red line. The sizes of the fragments corresponding to cleaved DNA template are given below scheme. **(D)** *In vitro* cleavage of target DNA by AsCas12a in complex with non-modified (NM) crRNA-34 or modified with the RNA import motifs (MLS) from tRK1 (HD and HF) or RNase MRP RNA (MRP). The addition of cell lysate containing AsCas12a nuclease, crRNA and DNA template is indicated above the gel. *In vitro* cleaved products were resolved by 1% (w/v) agarose gel electrophoresis and revealed with ethidium bromide staining. Additional DNA band marked with red asterisc originates from the cell lysate.

import (Fig. 5A). Surprisingly, the data demonstrated that crRNA-1 and 14 appeared to be protected from the nuclease treatment (Fig. 5B). This protection may be due to interaction with unknown proteins and/or to a stable secondary structure which was not predicted by the software used. In any case, we therefore cannot conclude about mitochondrial import of crRNA-1 and 14 in the used experimental conditions.

In contrast, we observed that crRNAs 31, 45, and 52 (Fig. 5B) as well as crRNAs 6, 10, and 34 (Fig. 5C) were efficiently degraded in mitochondrial lysate and still present in mitoplasts after RNase treatment indicating on their import into human mitochondria. The weak hybridization signals for crRNAs 45 and 34 in total RNA fraction may be due to the low transfection efficiency of the cells. We do not provide here the quantification of RNA import efficiency (as described in our previous papers [33,42], since the standard deviation obtained in 2–3 biological replicates exceeded 20%. The variability between biological replicates may be explained by the poor reproducibility of numerous experimental manipulations that can potentially affect the final outcome, such as cell transfection with RNA molecules, mitochondria isolation, and mitoplast preparation, but this does not affect the statement concerning the mitochondrial targeting.

Noteworthy, crRNAs 31 and 52 showed a higher enrichment in RNase-treated mitoplasts compared to the other tested crRNAs. To explain this observation, we analyzed the predicted alternative 2D structures for all crRNAs tested, as well as predicted secondary structures upon dimer formation (Tables S4 and S6). For crRNA-52 another possible secondary structure characterized by longer hairpin stem and a stable homodimer were predicted. Homodimer formation was also observed for crRNA-31. Based on our analysis, we can hypothesize that the import efficiency may be enhanced by the formation of stable helix structures that could act as import determinants [45].

To further confirm the data on the mitochondrial targeting of AsCas12a crRNAs' alternative predicted secondary structures and dimers, we compared the import of crRNA-45 and crRNA-30 in the hAsCas12a HEK 293 T-REx stable cell line and 143B osteosarcoma cells, respectively, using our recently developed CoLoc method [44]. We treated isolated mitochondria, mitoplasts, and mitochondrial lysate with a mix of RNase A and T_1 and compared the results to the untreated control samples (Fig. 6A). After RNase A/T_1 treatment of intact or swollen mitochondria, crRNA-45 remained resistant to digestion and only disappeared when the mitochondria were fully lysed, similar to the matrix-localized mitochondrial *tRNAVal* (Fig. 6B). Extensive nuclease treatment caused partial degradation of mitochondrial *tRNAVal*, however, the signal corresponding to crRNA persisted, thus providing further confirmation that AsCas12a crRNAs are indeed localized in the mitochondrial matrix.

We obtained comparable results for crRNA-30 import into mitochondria of cell line 143B (Fig. 6C), indicating that the crRNA import pathway is not cell line-specific. Interestingly, crRNA-30 characterised by a very compact predicted secondary structure and a stable homodimer, was not protected from the nucleases in the mitochondrial lysate, but is clearly enriched in the mitoplasts, supporting our hypothesis on the positive impact of the RNA secondary structure on its mitochondrial targeting.

We also confirmed the functionality of the AsCas12a/crRNA complex in the hAsCas12a HEK 293 T-REx stable cell line by performing an *in vitro* cleavage assay on the cell lysate obtained 48 h after transfecting the cells with increasing concentrations of crRNA-45 and activating the nuclease expression (Fig. 6D). The reactions contained the appropriate DNA template, but no additional crRNA (except for the control reaction). Specific crRNA-dependent DNA template cleavage was detected, demonstrating that crRNA designed to target mitochondrial DNA, introduced into human cells, can induce specific AsCas12a nuclease activity.

Recently, we demonstrated that AsCas12a crRNA can be split into two 20 bp parts, namely scaffold and spacer, without affecting its cleavage activity (Fig. S4A) [57]. We compared the mitochondrial import of crRNA-45 and its spacer part and found that the spacer part of crRNA can be targeted into human mitochondria of the hAsCas12a HEK 293 T-REx stable cell line (Fig. S4B). Thus, we

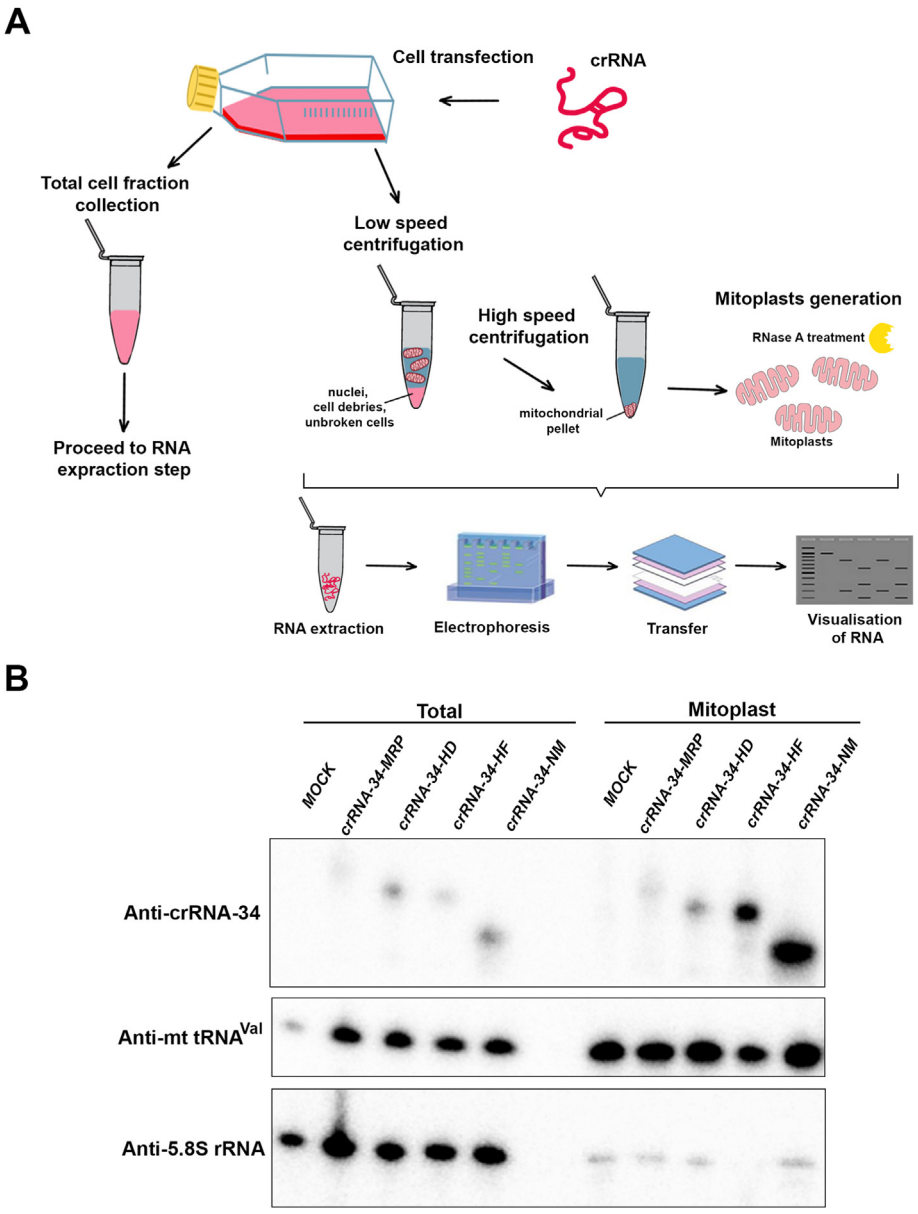


Fig. 3. Mitochondrial import of AsCas12a crRNAs bearing RNA import determinants.
(A) Schematic representation of the experiment pipeline. To evaluate the mitochondrial import of various AsCas12a crRNAs, we use Northern blot hybridization of total and mitochondrial RNA isolated from cultured human cells transfected with crRNAs. To improve the purity of isolated mitochondria and degrade RNA adhered to mitochondria from the cytosol, we treat them with RNase A with subsequent generation of mitoplasts. **(B)** Northern blot hybridization of the total or purified mitoplasts' RNAs isolated from the control HEK 293 cells (MOCK) or HEK 293 cells transfected with either non-modified crRNA-34 (NM) or crRNA-34 modified with 3′-MRP, 3′-HD, 3′-HF import determinants (Fig. 2B). The hybridization probes are shown on the left: crRNA-34 (top panel), mitochondrial *tRNAVal* (middle panel) and cytoplasmic 5.8S rRNA (lower panel).

suggest that the split AsCas12a crRNA variant could be used in further *in vivo* experiments for mtDNA targeting.

Therefore, we demonstrated that crRNA for AsCas12a system designed to target mitochondrial DNA, being introduced into human cells, can be imported into mitochondria and can induce specific DNA cleavage by AsCas12a nuclease. This finding opens the possibility to use CRISPR/Cas12 system for specific cleavage of mtDNA.

4. Discussion

Multiple alterations of human mitochondrial DNA were shown to be associated with severe impact on cellular respiration therefore leading to the onset of many diseases, mostly muscular or neurodegenerative, but also common diseases as cancer and diabetes (reviewed in Refs. [6,62]). Mitochondria are surrounded by a double membrane bearing a negative charge; therefore, there is no possibility to direct large DNA or mRNA molecules into the organelles with the aim to substitute the defective genes. Only some non-coding RNAs can be translocated into mitochondria from the cytosol (reviewed in Refs. [40,63–65]). Can this pathway be adapted for mitochondrial delivery of crRNAs, thus developing a mito-CRISPR/Cas system? This is currently a matter of controversy, since some speculative arguments (e.g., artefactual nature of the RNA import due to inability of imported RNA to perform their canonical functions) have been forwarded [35]. Nevertheless, several

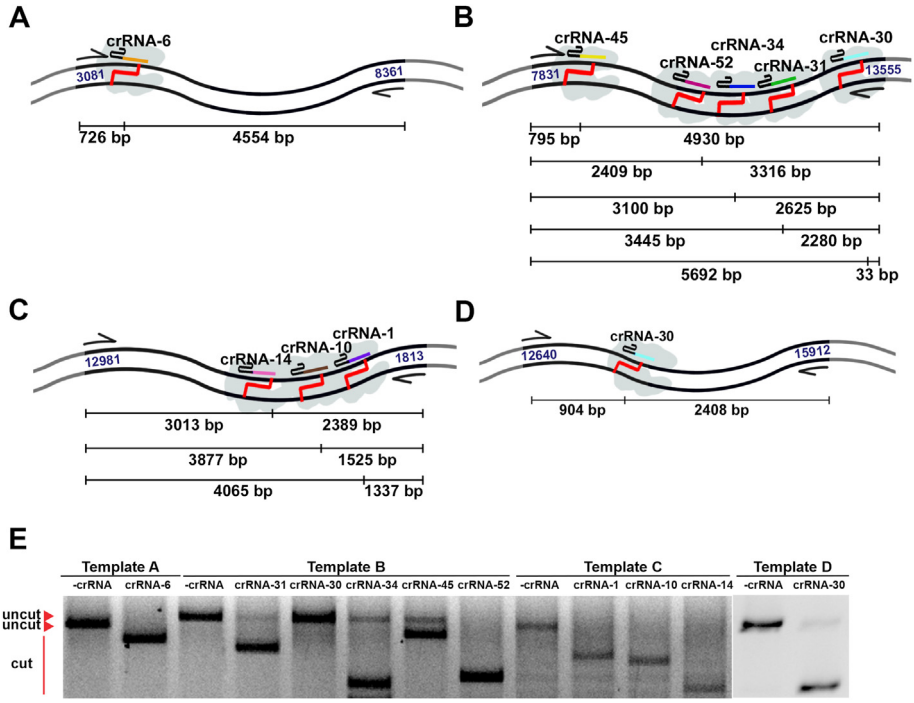


Fig. 4. AsCas12a can be programmed with crRNAs crRNAs targeting various regions in human mitochondrial DNA.
(A)–**(D)** Schematic representation of target DNA (PCR fragment of human mtDNA with corresponding numbers of nucleotides). The spacer moieties of target crRNAs are shown in color, the scaffold part is shown in black, and the cleavage site is marked with a red arrow. The size of the fragments corresponding to cleaved DNA template are given below the scheme. DNA "Template A" **(A)** contains protospacer sequence for crRNA-6; "Template B" **(B)** - for crRNAs 45, 52, 34, 31 and 30; "Template C" **(C)** - for crRNAs 14, 10, 1; and "Template D" **(D)** for crRNA-30. **(E)** *In vitro* cleavage of target DNA templates induced by crRNAs. DNA templates and crRNAs used in the reaction are indicated above the gel. "-crRNA" corresponds to a control reaction that does not contain any crRNAs. Since crRNA-30 generates short cleavage fragments **(B),** which cannot be distinguished from a non-cleaved DNA template, the cleavage was made with the use of DNA template **(D)**. *In vitro* cleaved products were resolved by 1% (w/v) agarose gel electrophoresis and revealed with ethidium bromide staining.

laboratories still work in this direction [33,47–52]. We have previously demonstrated the possibility to address SpCas9 nuclease and a set of sgRNA molecules into mitochondria of cultured human cells and obtained a consequent decrease of mtDNA copy number [33], but could not detect specific mtDNA cleavage. We hypothesized that the main challenge consists in sub-efficient delivery of a rather long sgRNA (~100 bases) for CRISPR/Cas9 system into the mitochondria.

Several studies demonstrated that while long non-coding RNAs (75–300 nucleotides) can be imported into mitochondria only with the help of specific protein factors, which limits the amount of translocated molecules [66,67], the shorter RNA molecules such as miRNA and siRNA are targeted in a much more efficient and probably non-specific way and can be not only detectable, but also functional [68–70].

Here we demonstrate that 40 nucleotide-long crRNA of Type V CRISPR/Cas12 system, designed to target human mtDNA and introduced into human cells by lipophilic transfection, can be translocated into mitochondrial matrix. We show that crRNA can be imported into the mitochondria of HEK293 (Figs. 3B) and 143B osteosarcoma cells (Fig. 6C); therefore, this pathway does not depend on the expression of the hAsCas12a nuclease and is non cell line-specific. Given the functional activity of crRNA in the lysate of human cells expressing AsCas12a nuclease (see Fig. 6D), we hypothesize that these imported crRNA can make it possible to reconstitute an active Cas12a-crRNA system *in vivo*.

Molecular mechanisms of RNA translocation across mitochondrial membranes in humans remain poorly understood [40]. The TOM complex and/or VDAC channel may facilitate translocation through the OMM (reviewed in Ref. [71]), while polynucleotide phosphorylase (PNPase) involved in RNA import from the intermembrane space into the matrix [45,72,73]. The mechanism by which PNPase participates in RNA import is still unclear but could involve its conformational changes upon recognition of the stem-loop RNA structures (reviewed [40]). This model supports previous data of our and other labs on identified RNA hairpins (here referred for as MLS) which strongly improved the mitochondrial targeting of various RNA molecules [74–76] (Fig. S1C). Here we show that hairpin structures fused to the 3′-end of crRNA do not impact its ability to program specific cleavage of DNA *in vitro* (Fig. 2). Surprisingly, mitochondrial localization of the fused crRNA molecules was not improved by HF stem-loop and even decreased by hairpins MRP and HD compared to non-modified version (Fig. 3B). The finding that crRNAs are imported without addition of MLS can be explained by the secondary structure of crRNA for CRISPR-Cas12a system, which consists of two nearly equally sized moieties, a 5′-handle (scaffold) and a guide segment complementary to the target DNA sequences (referred to as the spacer) (Fig. S4A). Crystal structures of AsCas12a-crRNA complexes revealed that the crRNA scaffold adopts a pseudoknot structure [43]. One can hypothesize that this structure functions as MLS, assuring crRNA mitochondrial import by specific interaction with PNPase, while addition of supplementary MLS could make a longer RNA less efficient in mitochondrial translocation.

Analysis of mitochondrial import of crRNAs targeting different regions in mtDNA revealed that two crRNAs (31 and 52) exhibited a higher enrichment in RNase-treated mitoplasts compared to other tested crRNAs (Fig. 5). Further analysis revealed unique

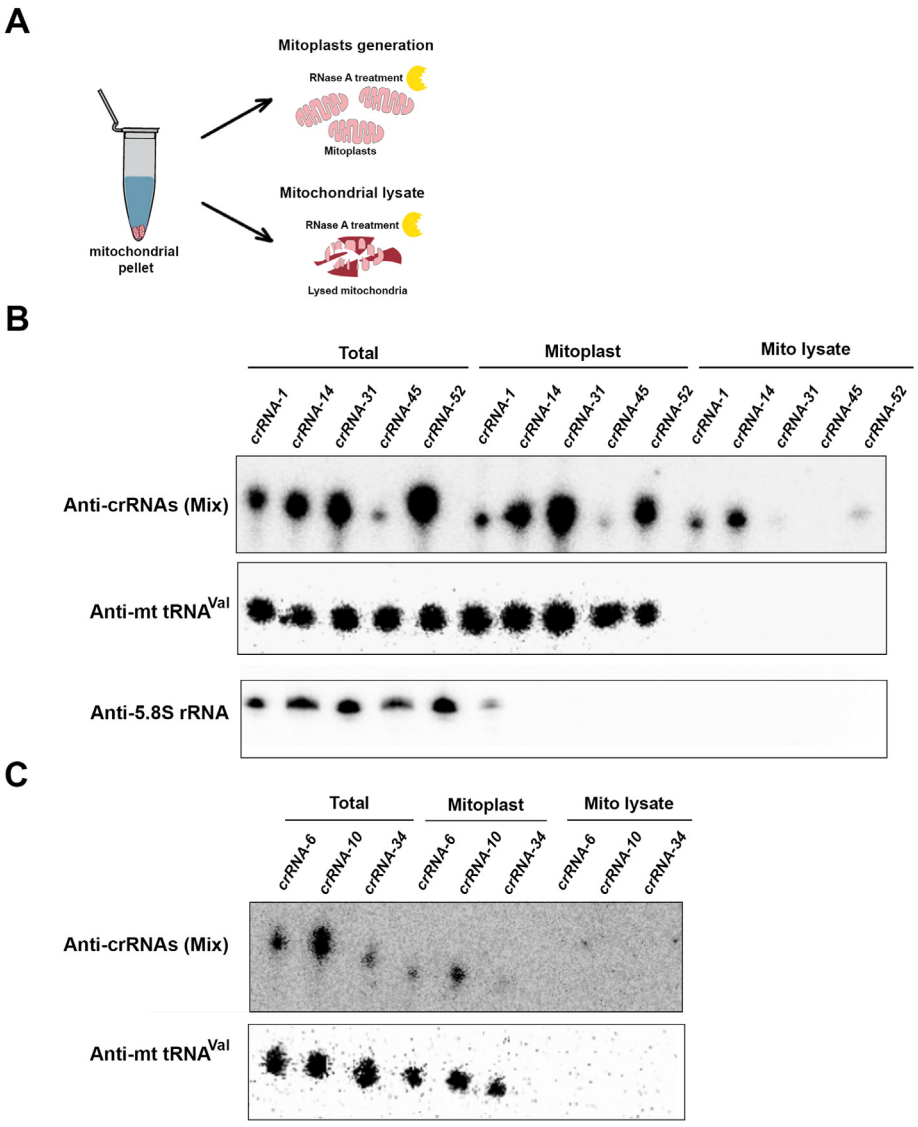


Fig. 5. Evaluation of mitochondrial import for AsCas12a crRNAs targeting various regions in human mtDNA.
(A) Schematic representation of a control experiment. (**B, C**) Northern blot hybridization of RNA isolated from the hAsCas12a HEK 293 T-REx stable cell line transfected with crRNAs 1, 14, 31, 45, 52 **(B)** and crRNAs 6, 10, and 34 **(C)**. RNA was isolated from cells ("Total"), RNase treated mitoplasts ("Mitoplast") and RNase treated mitochondrial lysates ("Mito lysate"). Hybridization probes used are indicated on the left: crRNAs (top panel), mitochondrial *tRNAVal* (middle panel) and cytoplasmic 5.8S rRNA (lower panel). Anti-crRNAs (Mix) probe represents the combination of probes to corresponding crRNAs (Table S2).

characteristics of these crRNAs, such as longer hairpin stems and stable homodimer formation (Tables S4 and S6), suggesting that the presence of stable helix structures may facilitate their import into mitochondria.

Recently, we demonstrated that AsCas12a crRNA can be split into two 20 bp parts, namely scaffold and spacer, without affecting its cleavage activity [57]. We, therefore, compared mitochondrial import of crRNA-45 and its spacer part and found that the spacer part of crRNA can be targeted into human mitochondria *per se* (Fig. S4B). This is in line with the reports on the import of small non-coding RNAs, miRNAs and siRNAs [70]. We are fully aware that the experimental estimation of RNA mitochondrial import can be biased and can give overestimated data [44]. For this reason, to remove possible RNA contaminants from the cytosol, we treated isolated mitochondria and mitoplasts with nucleases. To ensure accurate crRNA detection and mitigate false-positive results, we performed control experiments by lysing a portion of mitochondria before nuclease treatment. Detection of crRNA in both intact mitochondria and mitoplasts but not in the lysates, confirmed their localization in the mitochondrial matrix (Fig. 5A and 6A). Nevertheless, the final proof of concept should be obtained by a direct demonstration of specific mtDNA cleavage *in vivo*. We believe that our data provided here will be useful for further *in vivo* mtDNA targeting experiments.

Development of mitochondrial CRISPR/Cas12a system is challenging but very promising since it can be used as a therapeutic approach for specific cleavage of mutant mtDNA molecules, as well as a research tool to introduce InDel modifications in different parts of mtDNA for a better understanding of the molecular mechanisms of its replication, processing of the primary transcripts and the role of non-coding regulatory regions.

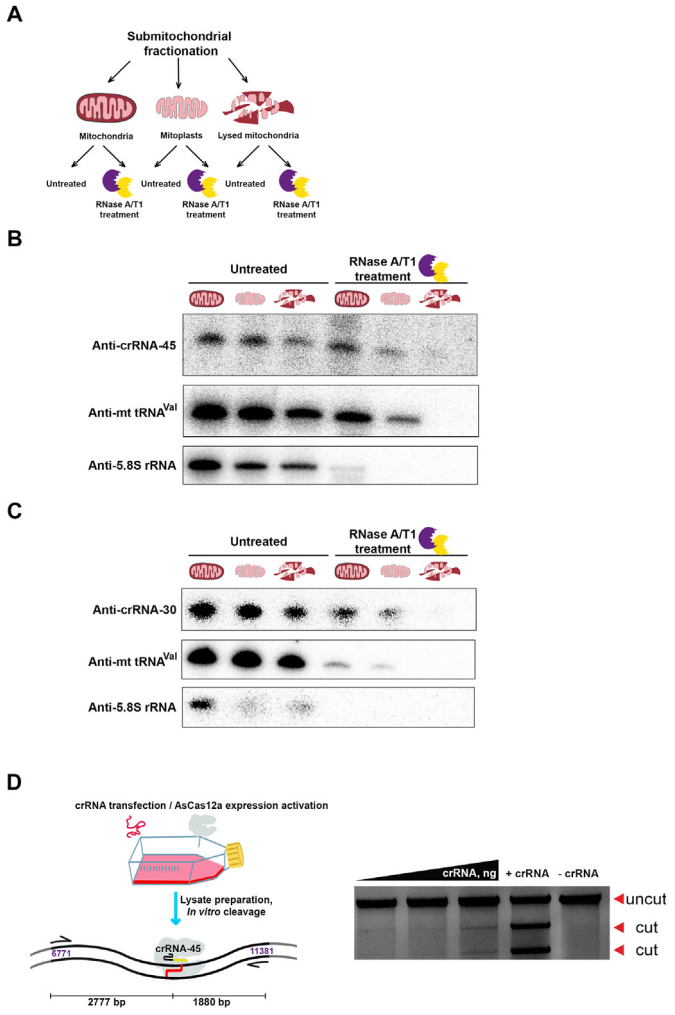


Fig. 6. Submitochondrial localization of AsCas12a crRNAs.
(A) Schematic representation of submitochondrial fractionation. (**B, C**) Intact mitochondria, mitoplasts and lysed mitochondria from the hAsCas12a HEK 293 T-REx stable cell line transfected with crRNA-45 (B) and from 143B cells transfected with crRNA-30 (C) were treated or not with the mix of RNase A/T_1, and the residual RNA molecules are analyzed by Northern blot hybridization. The hybridization probes are shown on the left: crRNA-45/30 (top panel), mitochondrial *tRNAVal* (middle panel) and cytoplasmic 5.8S rRNA (lower panel). Mitochondria, mitoplasts and lysed mitochondria are schematically indicated above the panels, as in **(A)**. **(D)** On the right, schematic representation of the *in vitro* cleavage of target DNA (PCR fragment of human mtDNA with corresponding numbers of nucleotides) in a cell lysate generated from the hAsCas12a HEK 293 T-REx stable cell line, transfected with crRNA-45 and induced with 100 ng/mL of tetracycline. The spacer moiety of crRNA-45 is shown in yellow, the scaffold part is shown in black, and the cleavage site is marked with a red arrow. The size of the fragments corresponding to cleaved DNA template are given below the scheme. On the left, *in vitro* cleavage of target DNA template induced by the increasing amounts of crRNA-45 used for the cells transfection (175–375 ng/cm^2). "-crRNA" corresponds to a control reaction that does not contain any crRNAs. "+crRNA" represents a positive control reaction for which 40 ng of chemically synthesized crRNA were added. *In vitro* cleaved products were resolved by 1% (w/v) agarose gel electrophoresis and revealed with ethidium bromide staining.

Data availability

All data presented in the manuscript is available upon request.

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Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2023.09.006.

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