Can Mitochondrial DNA be CRISPRized: Pro and Contra

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Research Communication

Can Mitochondrial DNA be CRISPRized: *Pro* and *Contra*

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Abstract

Mitochondria represent a chimera of macromolecules encoded either in the organellar genome, mtDNA, or in the nuclear one. If the pathway of protein targeting to different sub-compartments of mitochondria was relatively well studied, import of small noncoding RNAs into mammalian mitochondria still awaits mechanistic explanations and its functional issues are often not understood thus raising polemics. At the same time, RNA mitochondrial import pathway has an obvious attractiveness as it appears as a unique natural mechanism permitting to address nucleic acids into the organelles. Deciphering the function(s) of imported RNAs inside the mitochondria is extremely complicated due to their relatively low abundance, which suggests their regulatory role. We previously demonstrated that mitochondrial targeting of small noncoding RNAs able to specifically anneal with the mutant mitochondrial DNA led to a decrease of the mtDNA heteroplasmy level by inhibiting mutant mtDNA replication. We then demonstrated that increasing level of expression of such antireplicative recombinant RNAs increases significantly the antireplicative effect. In this report, we present a new data investigating the possibility to establish a CRISPR-Cas9 system targeting mtDNA exploiting of the pathway of RNA import into mitochondria. Mitochondrially addressed Cas9 versions and a set of mitochondrially targeted guide RNAs were tested *in vitro* and *in vivo* and their effect on mtDNA copy number was demonstrated. So far, the system appeared as more complicated for use than previously found for nuclear DNA, because only application of a pair of guide RNAs produced the effect of mtDNA depletion. We discuss, in a critical way, these results and put them in a broader context of polemics concerning the possibilities of manipulation of mtDNA in mammalians. The findings described here prove the potential of the RNA import pathway as a tool for studying mtDNA and for future therapy of mitochondrial disorders. © 2018 The Authors. IUBMB Life published by Wiley Periodicals, Inc. on behalf of International Union of Biochemistry and Molecular Biology, 00(00):1–7, 2018

Keywords: *mitochondria; mitochondrila DNA; CRISPR; Cas9; heteroplasmy; human*

INTRODUCTION

Mitochondria are intracellular organelles involved in a multitude of mechanisms ranging from respiration to apoptosis (1, 2). Their implication in metabolic, bioenergetics, and homeostatic processes make them indispensable for virtually all eukaryotic cells with only few contrary examples of replacement of essential pathways by alternative bacterial counterparts (3). Mitochondria possess their own genome, mtDNA, which can be organized in different ways among species, encoding between one and several dozens of proteins, different number of tRNAs and rRNAs. The most of proteins and some small RNAs are targeted from the nucleoplasmic compartment in different sub-compartments of mitochondria (4, 5). These sub-compartments are defined by a complex bimembrane envelope with several multiprotein complexes-receptors. Loss of function(s) of mitochondria has drastic effect on the overall cellular functioning, in humans

Abbreviations: *mtDNA, mitochondrial DNA; gRNA, guide RNA; KSS, Kearns Sayre Syndrome; PCR, polymerase chain reaction; RNP, ribonucleoprotein*

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provoking severe and mostly incurable diseases (6). Both to study mitochondrial functions and to try to cure mtDNA-caused dysfunctions, it is essential to be able to address directly mtDNA, but it still remains an important challenge. Last years, several alternative approaches to manipulate mtDNA were developed. One successful strategy was based on the TALEN technology, adapted for mitochondria (mito-TALENs) (7–10), the second – on sequence-specific designed Zn-fingers nucleases targeted in the organelles (11–13). As one of the largely used approach to manipulate DNA *in vivo* is currently the CRISPR system (in all its versions), the legitimate idea was to apply it to mitochondria (discussed in Reference (14)). Some attempts seemed to be promising if even provoking debate (15). In the opinion of other researchers, mtDNA could be even the single known target impossible for "CRISPRization" (16). So far, to investigate mitochondrial functions, dysfunction and evolutionary aspects such a system would be of great help.

For many years, our team invested in studying of the pathway of RNA targeting into mitochondria, in yeast S*accharomyces cerevisiae* and in human cells. We discovered several important features of this mechanism (17–25) and exploited it to address into mitochondria active RNA molecules able to either functional replace of the mutant ones (strategy called as "allotopic") (26, 27) or to affect in a specific way the propagation of mutant mtDNA ("anti-replication" strategy) (28–30). We now tried to adapt our know-how to develop the CRISPR-Cas9 system for human mitochondria using cultured human cells. The results described herein are in favor of the possibility of such a system, but it is clear that additional adaptation and optimization will be required to make it as efficient and specific as in nucleus.

MATERIAL AND METHODS

Strains, Lines, Antibodies

Escherichia coli XL1-Blue (Stratagen) were used for all cloning procedures and Rosetta™ (DE3) – for overexpression of hCas9.

Human KSS cells were a stable cybrid line based on 143B osteocarcinoma rho0 cells and contained 65% of the deletion in mtDNA (m.8363-15438del) (30). Human HepG2 cells, issued from hepatocyte carcinoma and available commercially, were used as wild type line. Flp-In™ T-rex™ cells permitting controlled insertion of the MTS^{COX8A}-hCas9 gene in the unique FRT site were purchased in Invitrogen (Carlsbad, CA, USA).

For Western analysis, we used primary monoclonal antibodies against Cas9 (Diagenode), polyclonal antibodies against enolase (C-19, Santa Cruz Biotechnolgy, Dallas, TX, USA) and PNPase (Abcam, Cambridge, UK), TOM20 (Abcam), and corresponding secondary antibodies from either GE Healthcare (Pittsburgh, PA, USA) or Molecular Probes (Life Technologies, Waltham, MA, USA).

In Vitro MtDNA Cleavage Assay

Mature hCas9 (devoid of NLS sequence) was purified after overexpression from the pMJ806 plasmid, as described in Reference (31). The cleavage substrates represented PCR-amplified fragments of mtDNA of various sizes (1–4 kb) containing (or not containing) the target sequences. gRNAs were produced by T7 transcription from DNA fragments generated by annealing of two oligonucleotides followed by PCR. The cleavage assay contained 500 nM of gRNA, 500 nM of recombinant hCas9, and 300 ng of the template DNA, as described elsewhere (31). For the assay of native mtDNA cleavage, after incubation with hCas9 the reaction mix was deproteinized by phenol-chloroform extraction and treated with endonuclease BlpI (FastDigest) to facilitate the Southern blot identification of Cas9 cleavage sites.

RNA Mitochondrial Import Assay

Mitochondria and mitochondrial RNA were isolated as described previously (29, 30). Total and mitochondrial RNA were separated by urea-PAGE and analyzed by Northern hybridization using ^{32}P-5′-labeled oligonucleotide probes. As a control of cytosolic contamination, we tested 5.8S rRNA or $tRNA^{Met}$, as a control of the integrity of mitochondrial RNA, mt $tRNA^{Lys}$.

Heteroplasmy and MtDNA Content Measurements

The heteroplasmy level of the KSS deletion was measured by qPCR with the Bio-Rad qPCR iCycler CFX96™ as described (30) and always included two calibration series corresponding to either only wild type or all (wild type and deleted) mtDNA. The level of heteroplasmy was then calculated as the % = (1−mtDNAwt/total) × 100. To measure the mtDNA copy number by qPCR, the mitochondrial gene *MT-RNR1* (12S rRNA) and nuclear gene *TST* (thiosulfate sulfurtransferase) were quantified; each assay included two calibration series for absolute quantification. Data obtained on independently cultivated cells (n = 3) by 3–4 independent qPCR measurements were statistically processed using the two-tailed Student's *t*-test; values of $P \leq 0.05$ were considered to be statistically significant.

Microscopy

Colocalisation analyses were performed by either confocal microscopy of fixed permeabilized samples with the Zeiss LSM 700 system with antibodies against Cas9 or TOM20 (presented in the result section) or super-resolution PALM/STORM with Cas9 antibodies and the co-expressed fusion TWINKLE-mEos2 (not presented here but providing similar results). The images were analyzed with ImageJ software.

Informatics

Secondary structures of mito-gRNAs were predicted using the *Mfold* software. Analysis of the annealing between mito-gRNAs and the mtDNA was performed by *RNAcofold* web server, University of Vienna. To estimate melting temperatures for RNA–DNA duplexes, we used IDT Sci-Tools *OligoAnalyzer 3.1* software.

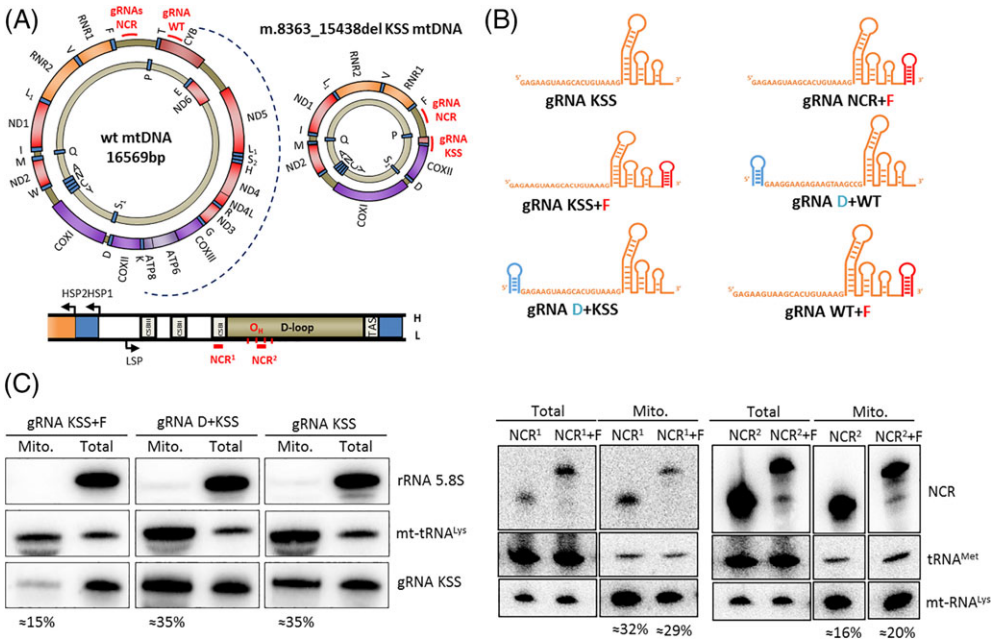

FIG 1

Design of mito-gRNAs. (A) Genetic map of human wild type mtDNA (at the left) and of the deleted "KSS" mtDNA (at the right). Pointed line indicated the KSS deletion. At the bottom, the NCR of mtDNA is enlarged: CSB, OriH, promoters, and D-loop region are indicated. Locations of the sequences that are targeted by mito-gRNAs are indicated in red; (B) Predicted 2D structures of mito-gRNAs. Linear orange part corresponds to the sequence targeting mtDNA. Orange structured part is the conserved sequence recognized by the Cas9 enzyme. Red or blue hairpins represent mitochondrial import determinants "F" or "D"; (C) Mitochondrial import of mito-gRNAs. Results of northern-hybridization are presented. Probes are indicated at the right of the panels, the mito-gRNAs used for cell transfection are indicated earlier, as well as the origin of RNA (mitochondrial or total). Mitochondrial import efficiencies (% of the cellular pool present in mitochondrial fraction) are indicated below the panels.

RESULTS

Recombinant Guide RNAs can be Targeted into Human Mitochondria

We designed guide RNAs targeting different regions of either wild type mtDNA or specifically recognizing the border of the KSS deletion, m.8363-15438del (Fig. 1A). To make gRNAs targetable in mitochondria, we introduced import determinants described in our previous studies. We used hairpin structures F and D (Fig. 1B), initially described in Selex experiments and further successfully tested for anti-replication strategy in a variety of cell lines (28–30, 32, 33). When designing gRNAs, we first analyzed *in sillico* predicted secondary structures, to ensure the correct folding of the import determinants and of the hairpins recognized by the Cas9 protein.

We then tested if these gRNAs are mitochondrially addressed. To this end, we transiently transfected cultured cells with corresponding T7 transcripts and analyzed total RNA and RNA isolated from purified mitochondria by Northern hybridization. Control hybridizations show that isolated mitochondria were devoid of nuclear or cytosolic contamination (5.8S rRNA) and contained intact mtDNA derived transcripts. For gRNAs tested, we observed the mitochondrial targeting with slightly different but comparable efficiencies (Fig. 1C). Surprisingly, gRNA molecules lacking the import determinants were also detected in purified mitochondria, suggesting that gRNA hairpins can substitute them.

Cas9 Protein can be Targeted into Human Mitochondria

The enzymatic part of the system we developed was based on the *Streptococcus pyogenes* Cas9 gene adapted for expression in human cells (31). The coding sequence of the gene was modified to eliminate the C-terminal NLS motif, and to add the mitochondrial targeting signal of the mitochondrially imported Cox8A protein at the N-terminus (Fig. 2A). The resulting construct, MTS^{COX8A}-hCas9 was tested for expression and mitochondrial targeting in cultured human cells. We first demonstrated mitochondrial import of MTS^{COX8A}-hCas9 in model HepG2 cells by Western blot on purified mitochondria (Fig. 2C). In parallel, we developed a cell line, further referred for as HEK293-T-Rex-MTS^{COX8A}-hCas9, expressing the MTS^{COX8A}-hCas9 in an inducible way, by using the Flp-In™ T-Rex™ system (Invitrogen), and tested the mitochondrial localization of MTS^{COX8A}-hCas9 by immunofluorescent microscopy (Fig. 2B).

Mt-gRNA can Specifically Cleave Mitochondrial DNA *In Vitro*

The two components of the system (mito-gRNAs) and the mature form of MTS^{COX8A}-hCas9 were then tested for cleavage activity in *in vitro* assays. First, we demonstrated that two purified components (T7 polymerase generated mito-gRNA and recombinant hCas9) are able to cleave the PCR fragment

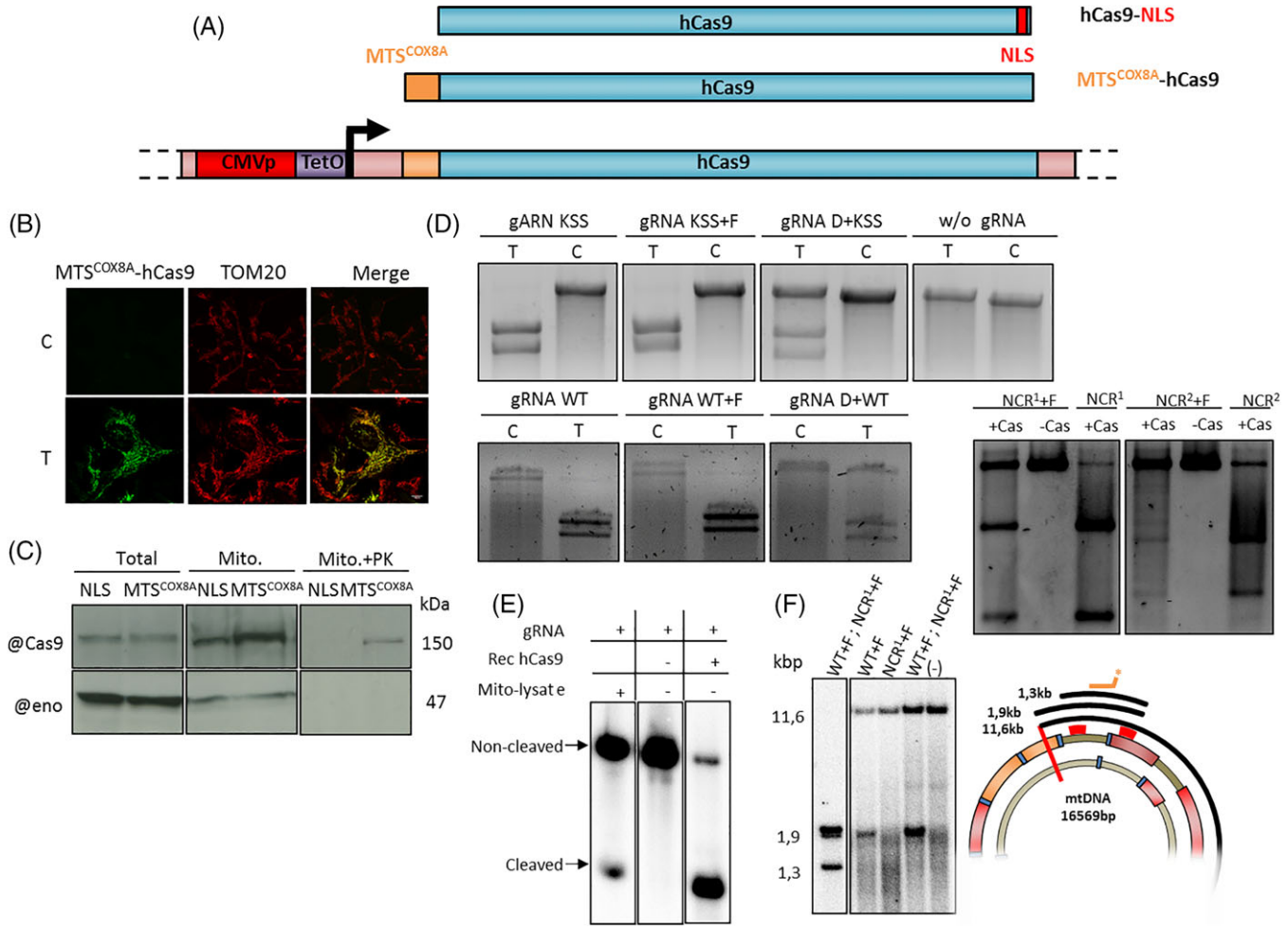

FIG 2

Design of MTS^{COX8A}-hCas9. (A) Constructs containing the Cas9 gene. The NLS signal is in red, the COX8A MTS sequence is in orange. (B) Confocal immunofluorescent microscopy of cells expressing MTS^{-COX8A}-hCas9. C – control non-transfected cells, T – transiently transfected cells. (C) Detection of hCas9 in cells and mitochondria by western immunoblotting. PK, mitochondria were treated with proteinase K. @eno – antibodies against enolase. (D) In vitro *cleavage activity of the hCas9 in the presence of mito-gRNAs on fragments of mtDNA. "C" corresponds to the control mtDNA fragment, which does not contain the target sequence; "T" – to that containing the target sequence. (E) Cleavage activity of the hCas9 extracted from isolated mitochondria (mito-lysate) compared to recombinant protein (Rec hCas9). (F) Nucleoid mtDNA cleavage by recombinant hCas9 detected by southern-hybridization. Possible predicted cleavage products are shown at the right. The orange line indicates the hybridization primer; the red bar – BlpI cleavage site. First panel at the left represents the cleavage products of purified mtDNA; mito-gRNAs used in the test are shown above the panels.*

containing the target sequence (Fig. 2D). These assays gave an obvious positive result: only the target, but not a control mtDNA sequence was recognized and cleaved. The gRNA versions bearing the hairpin D at the 5′-end (Fig. 1B), as well as noncoding region (NCR)[2] gRNA, could not induce a complete cleavage of the DNA substrate and thus have been excluded from further analysis. Next, we used mitochondrial lysates of human cells expressing MTS^{COX8A}-hCas9 as a source of protein. The efficiency of this system was clearly lower than with purified recombinant protein, but still provided a specific cleavage (Fig. 2E). Taking into account that *in vivo* mitochondrial DNA is organized in a complex way, forming the nucleoid nucleoprotein complexes, we also assayed the cleavage of native mtDNA, isolated from purified mitochondria without deproteinization. The data obtained also demonstrated that the target sequence can be partially cleaved (Fig. 2F). Noteworthy, we detected only cleavage induced by gRNA WT + F targeting a region of Cyt b gene (producing a fragment 1.9 kb detected by Southern blot). Another gRNA NCR + F could induce only a partial cleavage (producing 1.3 kb fragment) of purified mtDNA.

The Effect of Mitochondrial Targeting of CRISPR-Cas9 System on MtDNA *In Vivo*

We next analyzed the effect of the mitochondrial CRISPR-Cas9 system *in vivo*. The assays were performed either by transient transfection of cultured cells with vectors expressing the MTS^{COX8A}-hCas9 protein together with mito-gRNAs, or by induction of the protein expression in the cell line HEK293-T-

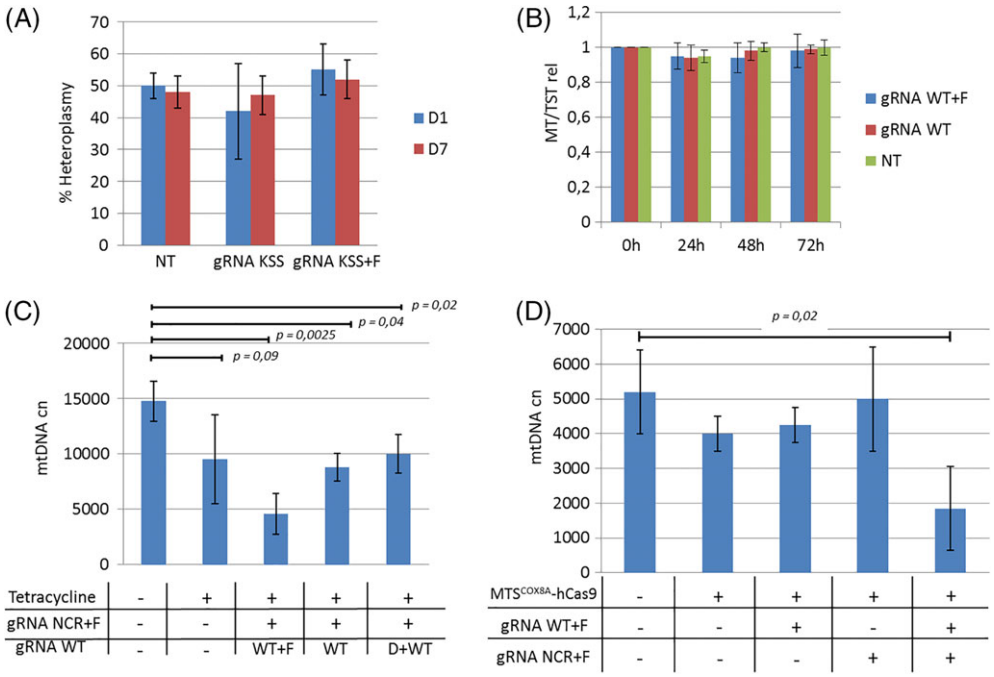

FIG 3

In vivo *effect of mitochondrial crispr-hcas9 system. Levels of heteroplasmy in KSS cybrid cells (A) and mtDNA copy number in cells transfected with vector expressing the MTS^{COX8A}-hCas9 protein together with mito-gRNAs (A, D) or in cells expressing MTS^{COX8A}-hCas9 in inducible way (B, C). (**A**) KSS heteroplasmy levels (% of mutant mtDNA), gRNAs indicated below the graphs; NT, nontransfected KSS cybrid cells; D1 and D7, days after transfection. (B) relative mtDNA copy number HEK293-T-Rex-MTS^{COX8A}-hCas9 cells, gRNAs are indicated at the right, time after protein induction and transfection with a gRNA – below the graphs. (**C**) mtDNA copy number decrease in HEK293-T-Rex-MTS^{COX8A}-hCas9 cells in 48 h after the protein induction and transfection with two gRNAs, as indicated. (D) mtDNA copy number in HepG2 cells transfected with vector expressing the MTS^{COX8A}-hCas9 protein and with one or two gRNAs, as indicated below the graphs. Error bars correspond to two (for B) or three independent experiments. Statistical differences were determined with a two-tailed Student's* t*-test,* P*-values are indicated.*

Rex-MTS^{COX8A}-hCas9 followed by transient transfection with mito-gRNAs. When KSS cybrid cells were transfected with one mito-gRNA targeting the border of the KSS deletion, we did not observe any significant shift of the mutant mtDNA proportion (Fig. 3A). No significant effect was observed on mtDNA content when the Cyt-b gene region of wild type mtDNA was addressed either (Fig. 3B). In contrast, when two mito-gRNAs, one targeting the NCR of mtDNA and the second – a sequence in the Cyt-b gene, were applied simultaneously, we observed a reproducible depletion of mtDNA by 2–3 times compared to the control (Fig. 3C,D). The more important effect was demonstrated in HEK293-T-Rex-MTS^{COX8A}-hCas9 cells transfected with gRNAs bearing the 3' F hairpin (Figs.1B and 3C). The similar mtDNA depletion induced by the combination of two gRNAs was obtained in transient co-transfection assays on another cell type, HepG2 (Fig. 3D). We demonstrated a significant decrease of mtDNA copy number in the cells transfected simultaneously with two gRNAs, but not in the cells transfected with each of these gRNAs separately.

DISCUSSION

The main goal of this study was to demonstrate the feasibility to establish a CRISPR-Cas9 system addressing human mtDNA and based on the RNA mitochondrial import pathway. We obtained clear evidence that the Cas9 enzyme can be adapted to mitochondria and that it is able to cleave specifically a variety of mtDNA targets *in vitro*. We also created several guide RNAs able to be targeted in human mitochondria due to short additional hairpin structures that we previously characterized as import determinants (29, 30, 33). So far, neither of the single target mt-gRNAs was able to influence the mtDNA content and there was no expected shift when one mtDNA species was specifically targeted in the heteroplasmic context. It was recently demonstrated that cleaved mtDNA molecules are rapidly degraded *in vivo* (34, 35). According to this finding, all our attempts to detect either point mutations or insertions–deletions at the cleavage sites or to visualize the mtDNA fragments corresponding to the targeted regions were unsuccessful (data not shown). Nevertheless, we reproducibly were able to induce significant mtDNA depletion when two mtDNA regions were targeted simultaneously.

Of note, one of these regions was the NCR of the mtDNA, bearing the classical origin of the H-strand replication and promoter regions for both strands' transcription. NCR was shown to have a very particular structure; its large part often incorporates a third linear DNA strand, which forms a stable displacement (D-) loop. Other mtDNA molecules contain RNA as a third

strand, which is complementary to D-loop DNA (36). Conserved sequence blocks of the human NCR were characterized by the presence of stable G-quadruplex structures ((37, 38)). The function of the NCR is still poorly understood. This region can be involved in mtDNA topology and segregation and its association with the inner mitochondrial membrane (39). Taken together our *in vitro* and *in vivo* data, one can suggest that the docking of the hCas9/gRNA complex in the NCR might disturb the D-loop formation, mtDNA replication or/and topology, which can be a prerequisite for the successful mtDNA cleavage by a second hCas9/gRNA complex located in the Cyt b gene region. Thus, if even the hCas9/NCR gRNA complex cannot cleave mtDNA *in vitro*, its action on the triple-strand D-loop (or alternatively R-loop) structure can make another mtDNA region accessible for the cleavage, thus inducing further rapid degradation and mtDNA depletion.

Another interesting hypothesis is generated by recent report claiming that the temperature in functional mitochondria might be much higher than in the rest of the cell (40), which can also explain why the system working so well in nucleus is only partially active in the organelle. As a matter of fact, the overall functionality of the RNP complex leading to the cleavage of the DNA molecule may be somehow affected at 50°. Moreover, due to high AT-content of mtDNA, the most of the possible target sequences (N20GG) are characterized by predicted Tm < 50°, which can disturb the gRNA annealing with mtDNA target. It was indeed demonstrated that at different temperatures the CRISPR-Cas9 system may be affected – low temperature decrease its efficiency, whereas higher – enhance the off-target effect (41). To resolve these obstacles, one might imagine to create new enzymes with better abilities than Cas9 at elevated temperature, and to test them in a similar way we did in this study, for example modified thermo-stable Cas9 versions may be used (42). As the temperature issue can affect the extent of the off-target cleavages, and taking into account that natural small-, medium- or large-size noncoding RNA may be present in mitochondria (4, 43–45), one could expect that the presence of active Cas9 enzyme can induce additional cleavages of mtDNA in the absence of gRNAs. Without ruling out such a possibility, our results are rather against this hypothesis. Indeed, as it is shown on Fig. 3C,D, both transient and induced expression of MTS^{COX8A}-hCas9 do not significantly influence mtDNA copy number. Finally, it is worth to mention that our previous assays in the frame of the anti-replicative strategy to address heteroplasmic mtDNA mutations proved to be more effective when imported recombinant RNAs with higher melting temperatures were applied (29, 46, 47), which can indirectly indicate that the temperature issue is to be seriously taken into account also for CRIPRization.

The last but not least important point is that the RNA import is a widely spread process (4), so far it is not efficient, meaning that only a minor part of the cellular pool of a given imported RNA reaches the matrix compartment. This intrinsically low efficiency of the pathway and often still not elucidated functions of the imported RNA species generated recent doubts about the usefulness of this mechanism for correction of mtDNA deficiencies. So far, many of our data indicate on a clear possibility to exploit it for both allotropic and anti-replication strategies (25–30). To counter the problem of low efficiency, new RNA molecules can be created with greater mitochondrial import capacities, and our previous reports indicate that it is possible. As a matter of fact, our recent studies with 5S rRNA based RNA vectors clearly are supporting this strategy, as increasing expression and import of recombinant RNA also produce a more pronounced anti-replication effect on mtDNA (47). All in all, we believe that the current study provides matter to be moderately optimistic about the possibility of CRISPRization of human mitochondrial DNA. If even this system cannot cleave mutant mtDNA in specific way, our findings indicate that it might be developed for the future mtDNA editing strategies.

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