Sequence-specific modification of mitochondrial DNA using a chimeric zinc finger methylase

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We used engineered zinc finger peptides (ZFPs) to bind selectively to predetermined sequences in human mtDNA. Surprisingly, we found that engineered ZFPs cannot be reliably routed to mitochondria by using only conventional mitochondrial targeting sequences. We here show that addition of a nuclear export signal allows zinc finger chimeric enzymes to be imported into human mitochondria. The selective binding of mitochondria-specific ZFPs to mtDNA was exemplified by targeting the T8993G mutation, which causes two mitochondrial diseases, neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) and also maternally inherited Leigh's syndrome. To develop a system that allows the monitoring of site-specific alteration of mtDNA we combined a ZFP with the easily assayed DNA-modifying activity of hDNMT3a methylase. Expression of the mutation-specific chimeric methylase resulted in the selective methylation of cytosines adjacent to the mutation site. This is a proof of principle that it is possible to target and alter mtDNA in a sequence-specific manner by using zinc finger technology.

gene therapy | mitochondria | mitochondrial diseases | synthetic zinc finger peptides | nuclear export signal

Mitochondria are cellular organelles that play a central role in energy metabolism, apoptosis, and aging (1). Human mitochondria contain their own DNA (mtDNA) of 16,569 bp, which encodes essential components of the oxidative phosphorylation machinery. There are still considerable uncertainties about how mtDNA is replicated, maintained, and expressed. Point mutations, deletions, or rearrangements in human mtDNA disrupt oxidative phosphorylation, leading to a range of genetic diseases for which there are no treatments (1).

The ability to manipulate or modify particular mtDNA sequences in mitochondria within cells would facilitate investigations of normal mtDNA processes and also enable development of therapies for mtDNA diseases. Unfortunately, the inaccessible location of the mtDNA and its association with mitochondrial proteins and membranes make this task difficult. Standard gene therapy approaches, such as delivering WT copies of DNA into mitochondria in a heritable manner, have not been successful despite many attempts (2, 3). Furthermore, although alternative approaches for the modification of mtDNA are being sought, none of them has so far proven to be widely applicable (4, 5).

The Cys_2His_2 class of zinc fingers has proven to be particularly effective for engineering customized DNA binding proteins with high specificity and affinity for a given DNA sequence. These have been used as powerful tools for intervening in nuclear gene expression and modifying DNA in a sequence-specific manner (6–11). In all of the previous approaches the engineered zinc finger peptides (ZFPs) have been expressed from exogenous DNA templates, synthesized in the cytoplasm, and imported to the nucleus. However, the engineered-zinc-finger technology has not yet been tested outside the nucleus.

We set out to determine whether we could adapt this technology for targeting the DNA in mitochondria. This raised some immediate problems that had to be solved: (*i*) zinc finger proteins needed to be imported to mitochondria; (*ii*) they had to be correctly folded in the mitochondrial matrix to facilitate binding to target DNA; and (*iii*) the binding site had to be accessible.

Thus, our first step was to develop a general method of delivery of active ZFPs to mitochondria. Second, a simple assay system was used to monitor simultaneously the correct folding of zinc finger proteins inside mitochondria as well as their ability to bind and react with mtDNA. To this end we have used a chimeric enzyme comprising a zinc finger DNA binding domain fused to the catalytic domain of DNMT3a methylase. Here we report the successful mitochondrial import of this chimeric zinc finger methylase and provide evidence for its selective binding to mtDNA and for site-specific methylation of the target DNA.

Results and Discussion

Strategies for Delivering ZFPs to Mitochondria. Zinc fingers are predominantly DNA binding proteins adapted to operate in the nucleus. Even in the absence of nuclear localization signals they often localize in the nucleus (10). To use designer ZFPs to manipulate mtDNA, they have to be both effectively targeted to mitochondria and at the same time excluded from the nucleus to avoid binding to nuclear DNA, which could be toxic (12). The majority of mitochondrial proteins are encoded by nuclear genes, and many of these are imported from the cytoplasm by means of a cleavable N-terminal mitochondrial targeting sequence (MTS). The MTSs vary greatly in length and composition and appear to be individually tailored to different proteins (13). Fusing an MTS to N termini can deliver exogenous proteins of various kinds to mitochondria.

To develop and optimize ZFP delivery to mitochondria we tested a library of four-finger ZFPs, engineered by fusing pairs of two-finger units (14), for their ability to enter mitochondria with the aid of MTSs from various natural mitochondrial proteins (Fig. 1*A*). The ZFPs tested were closely related and differed predominantly in the amino acid residues contained within DNA-contacting helices (Fig. 1*A*) [for full sequences see supporting information (SI) Fig. 5]. The intracellular localization studies of zinc finger fusions with different MTSs, in the presence or absence of an additional C-terminal GFP, revealed three possible intracellular destinations for the ZFPs (Fig. 1*A*), namely exclusively nuclear, mitochondrial and nuclear in various proportions, or exclusively mitochondrial. The same diverse localization patterns were also observed for a family of three-finger proteins (data not shown). Other experiments suggested a

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Abbreviations: MTS, mitochondrial targeting sequence; NARP, neurogenic muscle weakness, ataxia, and retinitis pigmentosa; NES, nuclear export signal; ZFP, zinc finger peptide.

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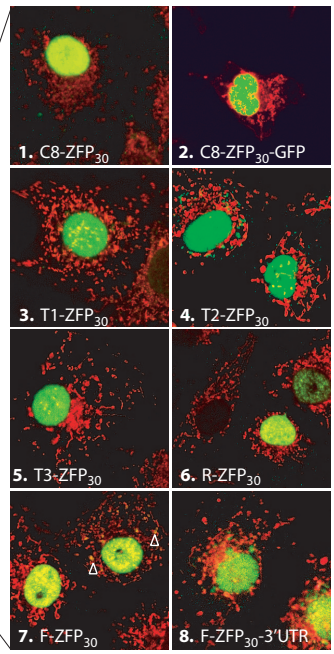
A

	Clone	ZFP sequence F1 −1123456	F2 −1123456	linker	F3 −1123456	F4 −1123456	Localisation C8-ZFP-GFP	C8-ZFP	F-ZFP
L1	5	RSDVLSA	RNDHRIN	–	RSDTLSR	HNHHRKT	-	-	M
	9	RSDHLST	HSNTRKN	GGG	RSDSLST	AGANRTT	-	-	M/N
	10	RSDVLSV	TNQHRTK	–	RSDHLSE	NNSSRTR	N	N	M/N
	11	RSDHLSQ	TSSNRKT	–	RSDHLSN	RSDDRKK	N	N	M/N
	12	RSDHLSE	RKDARIT	GGG	RSDHLSN	RSDDRKK	N	N/M	M
	15	RSDHLSN	RNDDRKK	–	RSDHLSN	QSATRIT	-	-	M/N
	18	RSDHLSQ	TSSNRKT	–	RSDNLST	RSDNRTK	-	-	M/N
	19	RSDHLSE	DSSHRTR	GGG	RSDSLSV	QNQHRIN	-	-	M/N
	21	RSDHLSE	RNDNRKR	–	RSDHLSQ	TSANRTT	N	N	M/N
	22	RSDSLSQ	NSSNRKN	GGG	RSDSLSQ	TSSNRKT	-	-	M/N
	26	RSDHLSN	TRDTRKK	GGG	RSDALSV	DSSHRTR	-	-	M/N
	29	RSDTLSE	RNDHRTT	GGG	RSDHLSN	RNDDRKK	N	N/M	M/N
	30	RSDHLSE	RNDNRKR	–	RSDHLSN	RNDDRKK	N	N	N/M
	31	RSDHLSE	RNDHRTT		RSDHLSE	RRDSRTN	-	-	N/M
	34	RSDHLST	HSNTRKN	GGG	RSDHLSE	RNDHRTT	-	-	M/N
	35	RSDVLSV	QNNHRIT	–	RSDHLST	ASSARKT	-	-	N/M
	37	RSDALSV	HSDTRTK	–	RSDVLSV	QNNHRIT		-	M
L2	20	RSDHLTK	NSDHLSR	–	RSDSLSV	QNQHRIN	N	M/N	M
	27	RSDHLTK	NSDHLSR	–	RSDHLSN	TRDTRKK	-	-	M/N
	32	TNDDLNT	TSSHLSR	GGG	RSDHLSE	RNDNRKR	-	-	N/M
	33	TNDDLNT	TSSHLSR	–	RSDHLSE	RNDHRTT	-	-	N

B



C

Clone	ZFP sequence F1 −1123456	F2 −1123456	linker	F3 −1123456	F4 −1123456	Localisation F-ZFP-NES	F-GFP-ZFP-NES	F-ZFP-meth-NES	F-GFP-ZFP-meth-NES
11	RSDHLSQ	TSSNRKT	–	RSDHLSN	RSDDRKK	M	M	M	M
12	RSDHLSE	RKDARIT	GGG	RSDHLSN	RSDDRKK	M	-	M	-
18	RSDHLSQ	TSSNRKT	–	RSDNLST	RSDNRTK	M	M	M/N	M
21	RSDHLSE	RNDNRKR	–	RSDHLSQ	TSANRTT	M	M	M	M
30	RSDHLSE	RNDNRKR	–	RSDHLSN	RNDDRKK	M	M	M	M

D

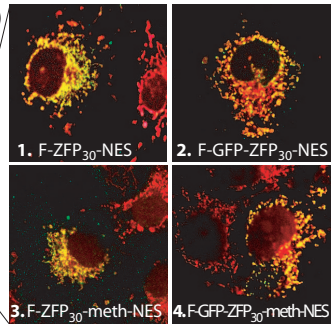


Fig. 1. Efficient mitochondrial targeting of multidomain ZFP proteins requires a combination of a specific MTS and NES. (*A*) ZFPs containing four fingers (F1–F4) were selected from two libraries (L1 and L2) to bind targets in mtDNA (SI Fig. 5). All ZFPs (with or without a GGG linker) were closely related. Sequences of recognition helices (positions −1 to 6) are shown with DNA-contacting amino acids in boldface. ZFPs were fused to N-terminal MTSs of the F1β subunit of the human mitochondrial ATP synthase (F-ZFP) or subunit VIII of human cytochrome *c* oxidase (C8-ZFP) in the presence or absence of C-terminal GFP (C8-ZFP-GFP), and their localization was assessed as exclusively nuclear (N), exclusively mitochondrial (M), or mixed with either predominantly mitochondrial (M/N) or predominantly nuclear (N/M). (*B*) The localization of a particular ZFP, ZFP_{30}, was analyzed further in the context of different MTSs with or without additional sequences such as GFP (image 2) or 3′ UTR of the mRNA for F1β subunit of human ATP synthase (32) (image 8). In addition to C8 (images 1 and 2) and F (images 7 and 8), we tested the MTS from the subunit 6 of ATP synthase from *Chlamydomonas reinhardtii* (R) (image 6) and MTSs from the following zinc finger proteins: MP42 from *Trypanosoma brucei* (T1) (image 3), MP63 from *T. brucei* (T2) (image 4), and 7b from *Leishmania tarentolae* (T3) (image 5). In the merged immunofluorescence images mitochondria are stained in red and ZFPs are labeled in green, and partially mitochondrial localization of $F\text{-}ZFP_{30}$ is marked by arrows (image 7). (*C* and *D*) A number of F-ZFPs were fused with the C-terminal NES (F-ZFP–NES) and tested for their ability to enter mitochondria when attached to additional domains including GFP (F-GFP–ZFP–NES, 48 kDa), a catalytic domain of the hDNMT3a methylase (F-ZFP–meth–NES, 61 kDa), or both (F-GFP-ZFP–meth–NES, 86 kDa). Intracellular localization of individual proteins was assessed and presented (*C*) and additionally, for clone ZFP_{30}, illustrated by images in *D* (abbreviations as in *A* and *B*).

possible size restriction to mitochondrial import of ZFPs. Addition of a C-terminal GFP to MTS–ZFP fusion impaired mitochondrial import, and a six-finger ZFP conjugated to MTS was not imported at all (data not shown). The variability of localization pattern between very closely related ZFPs and size restriction on import were initial setbacks to our aim of directing ZFPs and their derivatives to mitochondria. Therefore, our first challenge was to develop a universal system for routing ZFPs to mitochondria.

To investigate this we chose ZFP clone 30 (ZFP_{30}) as a case study because it was the most difficult to import into mitochondria (see Fig. 1*A*). We tested several MTSs in fusion with ZFP_{30}, including the MTSs from endogenous mitochondrial proteins containing zinc finger motifs, as well as the 3′ UTR from human mRNA for the F1β subunit of ATP synthase, which is known to aid mitochondrial import (15) (Fig. 1*B*). All of these fusion proteins localized exclusively in the nuclei (Fig. 1*B*), with the exception of ZFP_{30} fused to the MTS from the F1β subunit of the human mitochondrial ATP synthase (denoted $F\text{-}ZFP_{30}$), which occasionally also localized in mitochondria (Fig. 1*B*, image 7, arrow).

Predominantly nuclear localization and the absence of all of the MTS–ZFP_{30} fusions from the cytoplasm (Fig. 1*B*) indicated very efficient nuclear targeting. To counteract the nuclear import of ZFPs we hypothesized that a nuclear export signal (NES) might facilitate mitochondrial import by either preventing sequestration of the nascent polypeptide in the nucleus or rerouting it out again, thus giving it more opportunity to be taken up by mitochondria. To test this we fused the $F\text{-}ZFP_{30}$ and the NES from the nonstructural protein 2 of minute virus of mice

(16) to generate the F-ZFP$_{30}$–NES protein. Immunofluorescence studies of F-ZFP$_{30}$–NES fusion showed that it was efficiently targeted to mitochondria and was absent from the nucleus (Fig. 1*D*). In a control experiment a fusion protein comprising ZFP$_{30}$ and NES but lacking a MTS was still found in the nucleus, which indicates that NES alone cannot function as a mitochondrial import signal (data not shown). Even increasing the size of the F-ZFP–NES protein by fusing additional domains still led to efficient mitochondrial uptake (Fig. 1*D*). Using NES in conjunction with the N-terminal F MTS facilitated the efficient mitochondrial uptake of a range of ZFP fusion proteins (Fig. 1*C*) including three- and six-finger ZFPs (data not shown). This ability to deliver into mitochondria proteins composed of large exogenous domains fused to a ZFP opened up the possibility of constructing chimeric enzymes targeted to specific mtDNA sequences.

Construction of a Mitochondria-Targeted ZFP That Binds a Mutated mtDNA Sequence. We next generated ZFPs that bound selectively to particular mtDNA sequences. The three-finger protein F-ZFP$_{NARP}$ was designed to bind to a 9-bp sequence, GCCC**G**GGCC, in mtDNA (Fig. 2*A*); the bold G at position 8993 in mtDNA indicates a T→G mutation responsible for the mitochondrial diseases called neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) and maternally inherited Leigh's syndrome. *In vitro* F-ZFP$_{NARP}$ specifically bound an oligonucleotide containing GCCC**G**GGCC, as assessed by a gel retardation assay (Fig. 2*B*). In contrast, oligonucleotides containing either GCCC**T**GGCC (WT) or GCCC**C**GGCC were not bound by F-ZFP$_{NARP}$ (Fig. 2*B*). Furthermore, *in vitro* binding studies of ZFPs showed that the addition of NES did not affect DNA binding (data not shown). A control, F-ZFP$_{cont}$, of the same size as F-ZFP$_{NARP}$ did not bind to any of the three DNA sequences tested (Fig. 2*B*) and hence could be further used as a control for any nonsequence-specific effects of targeting a ZFP to mitochondria. Therefore, F-ZFP$_{NARP}$ binds highly specifically to a sequence containing the T8993G mutation, but not to WT mtDNA that differs by a single base pair.

It was unclear whether an exogenous ZFP would still incorporate zinc and fold correctly within mitochondria. This question was addressed by using a mitochondrial extract from the cells expressing F-ZFP$_{NARP}$ for binding studies *in vitro* (Fig. 2*C*). These experiments showed that mitochondria from these cells contained a DNA binding activity that bound GCCC**G**GGCC in the same way as F-ZFP$_{NARP}$ expressed *in vitro*. This was not due to nonspecific DNA binding, because the mitochondrial extracts from the cells expressing F-ZFP$_{cont}$ did not retard these DNA oligomers (Fig. 2*C*). Therefore, ZFPs delivered into mitochondria by our combined MTS–NES system fold in their active form and are capable of the selective binding to target DNA sequences.

Construction and Intramitochondrial Localization of a Chimeric ZFP-Methylase. The next step was to investigate whether F-ZFP$_{NARP}$ could direct a DNA-modifying activity selectively to the mutant GCCC**G**GGCC mtDNA sequence. As a DNA-modifying activity we chose the catalytic domain of the human DNMT3a DNA methyltransferase. This enzyme predominately methylates cytosines in CpG sites to form 5-methylcytosine but can also modify general CpN sites less efficiently. Methylation was chosen deliberately as a marker activity because it is practically absent from mtDNA (17), it is easy to assay (18), and it had been previously used in chimeric ZFP enzymes (19–21).

The F-ZFP$_{NARP}$ construct was fused to the methylase domain of the human DNMT3a (meth) by using a flexible linker and C-terminal NES to give F-ZFP$_{NARP}$–meth–NES (Fig. 3*A*). When F-ZFP$_{NARP}$–meth–NES was expressed in human cells the MTS

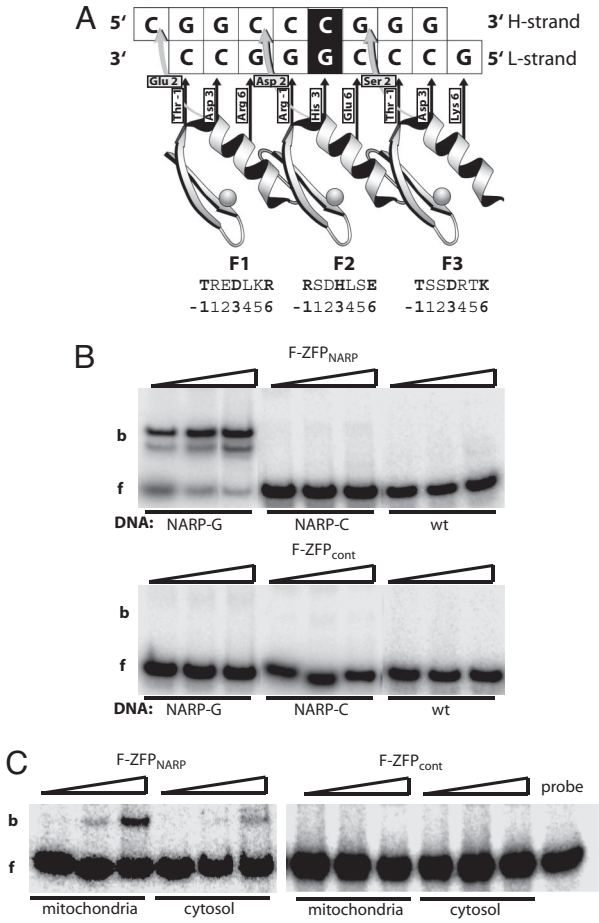


Fig. 2. Design and DNA binding of the NARP-specific mitochondrial F-ZFP$_{NARP}$. (*A*) DNA recognition by a three-finger protein, F-ZFP$_{NARP}$. A Zif268-based F-ZFP$_{NARP}$ has been selected to bind a sequence containing the T8993G mutation in the L-strand of mtDNA (the 8993G is marked as a black box in a target site). The amino acid sequences of the α-helices of zinc fingers F1, F2, and F3 are listed below with a single-letter code. The fingers F1, F2, and F3 are represented by α-helix and two β-strands stabilized by a zinc ion depicted as a gray sphere. Predicted contacts by residues in positions −1, 3, and 6 with the L-strand of mtDNA are shown as solid black arrows. The curved gray arrows indicate possible cross-strand interactions (33) between the amino acid in position 2 and the complementary H-strand at the interface between adjacent 3-bp binding sites for each finger. (*B*) F-ZFP$_{NARP}$ discriminates between closely related sequences. *In vitro*-synthesized F-ZFP$_{NARP}$ and a control ZFP F-ZFP$_{cont}$ were tested in the gel retardation assays for their binding to the target DNA, which contained the mutant T8993G (NARP-G) or T8993C (NARP-C) or the WT sequence 8993T (WT). All of the peptides were used in successive 5-fold dilutions (marked as gradient symbols), and DNA probes were used at a concentration of 0.3 nM. The letter ''f'' denotes free DNA, and ''b'' denotes protein-bound complexes. Two mobility forms of protein bound complexes ''b'' can be attributed to two different degrees of compaction of F-ZFP$_{NARP}$-DNA, occurring in the presence or absence of a ''cross-strand interaction.'' Note that F-ZFP$_{NARP}$ has not been optimized for these interactions. (*C*) F-ZFP$_{NARP}$ retains its binding ability upon import to mitochondria. Gel retardation assay on the DNA target containing the T8993G mutation (NARP-G) was performed on the mitochondrial extract from the cells transiently expressing mitochondrially targeted F-ZFP$_{NARP}$ or F-ZFP$_{cont}$. The cytosolic fraction was used as a control. Sequential dilutions of the proteins and concentration of the probe were as in *B*.

F was cleaved off from the mature protein, which is consistent with uptake through the conventional mitochondrial import pathway. The mature form of F-ZFP$_{NARP}$–meth–NES was protected from proteolysis to the same extent as the mitochondrial matrix protein TFAM (22) when the isolated mitochondria were

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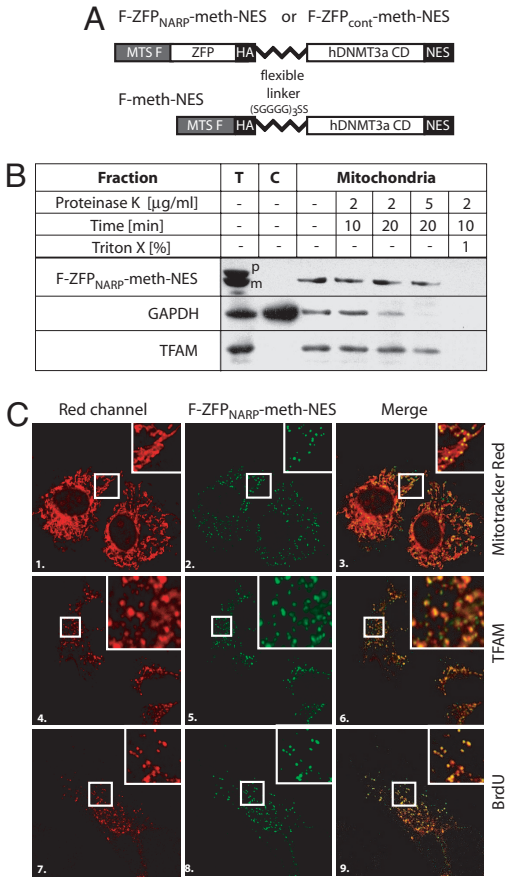

Fig. 3. Chimeric zinc finger methylase F-ZFP$_{NARP}$–meth–NES is targeted to the mitochondrial matrix and colocalizes with mtDNA. (*A*) Schematic structure of mitochondrially targeted methylases. To construct NARP-specific (F-ZFP$_{NARP}$–meth–NES) or control (F-ZFP$_{cont}$–meth–NES) chimeric methylases F-ZFP$_{NARP}$ or F-ZFP$_{cont}$ was linked by using a 17-aa flexible linker of (SGGGG)$_3$SS to a catalytic domain (residues 592–909) of the human DNMT3a DNA methylase (hDNMT3a CD). The NES was added to the C terminus. As an additional control the mitochondrially targeted methylase lacking the DNA binding domain was constructed (F–meth–NES) by deleting ZFP from the F-ZFP$_{NARP}$–meth–NES construct. Both constructs use the HA epitope tag to facilitate further detection. (*B*) F-ZFP$_{NARP}$–meth–NES zinc finger methylase localizes inside mitochondria. The NARP cells transiently overexpressing F-ZFP$_{NARP}$–meth–NES were fractionated, and the protein fractions were analyzed by Western blotting using anti-HA mAb. The localization of the F-ZFP$_{NARP}$–meth–NES precursor (p) and its mature (m) form in total cell lysate (T), cytosolic (C), and a mitochondrial fraction treated with proteinase K under various conditions, as indicated, was compared with the localization of marker proteins. The precursor of F-ZFP$_{NARP}$–meth–NES was found in the mitochondrial fraction but was clearly located outside the mitochondria, because it was accessible to protease digestion. In contrast, the mature form of the chimeric methylase was protected and became accessible to proteolysis only after the mitochondria were lysed with Triton X-100. The following endogenous proteins were used as fractionation markers: (*i*) GAPDH, previously reported as electrostatically associated with mitochondrial outer membrane (23, 24); and (*ii*) TFAM, the transcription factor that is localized in the mitochondrial matrix (25). (*C*) F-ZFP$_{NARP}$–meth–NES zinc finger methylase colocalizes with mitochondrial nucleoid. The intracellular localization of F-ZFP$_{NARP}$–meth–NES was analyzed by immunofluorescence in transiently transfected NARP cells. Mitochondria were stained with MitoTracker CMX Red (red), and F-ZFP$_{NARP}$–meth–NES was detected with antibodies against the HA epitope tag followed by secondary antibodies conjugated to FITC (green). The F-ZFP$_{NARP}$–meth–NES exhibits a punctate intramitochondrial staining pattern (images 1–3). Moreover, the majority of transiently expressed F-ZFP$_{NARP}$–meth–NES colocalized with TFAM, a well known protein of the human mitochondrial nucleoid, stained here with polyclonal antibodies and visualized with Texas red (images 4–6). Intramitochondrial foci that were positive for F-ZFP$_{NARP}$–meth–NES colocalized with mtDNA labeled with BrdU (images 7–9).

incubated with proteinase K. In contrast, GAPDH [a protein associated with the mitochondrial outer membrane (23, 24)] was degraded, indicating that the protease K was active outside the mitochondria (Fig. 3*B*).

Immunofluorescence experiments revealed that F-ZFP$_{NARP}$–meth–NES was distributed in a punctate pattern within mitochondria (Fig. 3*C*, images 1–3), typical of proteins found in the mitochondrial nucleoid (25). Its localization in the nucleoid was confirmed by comparing its distribution with that of the mitochondrial transcription factor TFAM (Fig. 3*C*, images 4–6) and also the mitochondrial single-strand DNA binding protein mtSSB (data not shown), which are known to be a part of the nucleoid (25). The final confirmation came from showing that F-ZFP$_{NARP}$–meth–NES colocalized with mtDNA itself labeled with BrdU (Fig. 3*C*, images 7–9). Therefore, the F-ZFP$_{NARP}$–meth–NES is taken up by mitochondria within cells and localizes to the mtDNA in the matrix.

Sequence-Specific *in Vivo* Methylation of mtDNA by a Chimeric ZFP-Methylase. The final goal was to determine whether F-ZFP$_{NARP}$–meth–NES selectively increased the ^{5}C methylation of cytosines in CpG sites adjacent to the targeted GCCC**G**GGCC sequence. The F-ZFP$_{NARP}$–meth–NES construct was expressed in mutant NARP cells, which contain a G at position 8993 in their mtDNA, and also in WT cells, which have T at this position. To assess the sequence specificity of mtDNA methylation by F-ZFP$_{NARP}$–meth–NES we used the bisulfite method (18). Methylation of CpG dinucleotides surrounding the GCCC**G**GGCC sequence was observed in 23% of clones derived from the NARP cells expressing F-ZFP$_{NARP}$–meth–NES (Fig. 4*B*). In contrast, in WT cells expressing F-ZFP$_{NARP}$–meth–NES the number of CpG methylation events in the analogous region was ≈6-fold lower and was indistinguishable from background levels of CpG methylation. Similarly, when the control F-ZFP$_{cont}$–meth–NES construct was expressed in the NARP cells there was an ≈6-fold-lower level of CpG methylation in the analyzed region. As an additional control, a mitochondrially targeted methylase domain without a ZFP, F–meth–NES (see Fig. 3*A*), was expressed in the NARP cells. It caused far lower CpG methylation levels (≈2.6-fold) than F-ZFP$_{NARP}$–meth–NES, and this methylation was spread throughout the entire analyzed region (Fig. 4*A*). The lower methylation levels observed for F-ZFP$_{cont}$–meth–NES or F-ZFP$_{NARP}$–meth–NES expressed in the WT cells as compared with F–meth–NES might be due to the attenuation of the methyltransferase DNA affinity by fusion to the ZFPs as reported (19). These controls indicate that the increased CpG methylation around the GCCC**G**GGCC sequence upon expression of F-ZFP$_{NARP}$–meth–NES was not a simple consequence of the presence of a methylase in mitochondria. Furthermore, the increased CpG methylation is a result of the sequence-specific binding of F-ZFP$_{NARP}$–meth–NES to GCCC**G**GGCC because there was no increase in CpG methylation in other regions of mtDNA well away from this site (regions 380–570 and 13,500–13,650 in SI Fig. 6*A*) or in a region containing the closest related site, CCCCTGGCC (SI Fig. 6*B*).

Furthermore, in the NARP cells expressing F-ZFP$_{NARP}$–meth–NES we observed a preference for methylation of particular CpG sites as well as enhanced methylation of CpA, CpT, and CpC dinucleotides, in ≈4%, 2.5%, and 1% of clones, respectively, as compared with the controls (Fig. 4*A*). Both of these effects are consistent with the known preferences of the DNMT3a methylase for certain flanking sequences in CpGs (see SI Fig. 7) (26) and its ability to methylate cytosines in non-CpG dinucleotides, albeit much less effectively (27–29). Additionally, the overall relative proportions of methylation found in the dinucleotides CpG, CpA, CpT, and CpC correlate well with the known propensities of the DNMT3a methylase for these sites. Elevated non-CpG methylation of the region surrounding the targeted GCCC**G**GGCC sequence could therefore be a result of

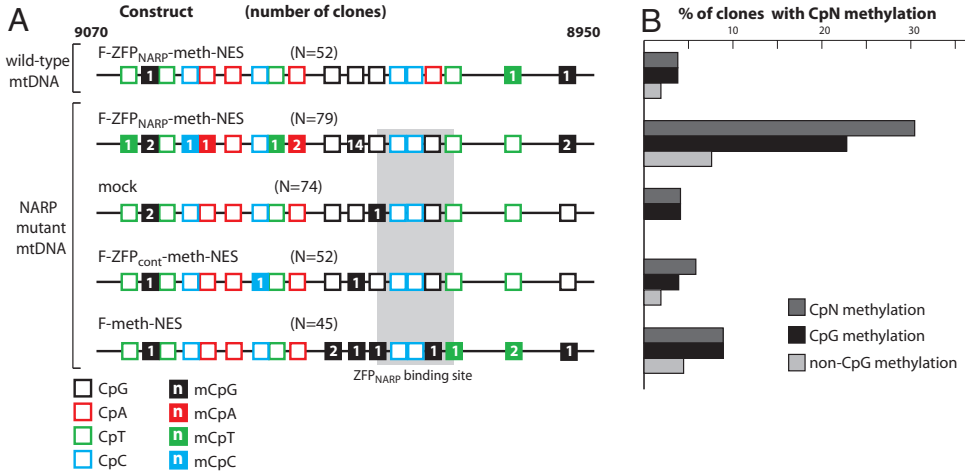

Fig. 4. F-ZFP$_{NARP}$–meth–NES selectively methylates mtDNA *in vivo* in the vicinity of its predetermined binding site. (*A*) We determined the methylation status of cytosine residues in the H-strand of mtDNA surrounding the NARP mutation site (positions 8950–9070 of mtDNA as indicated at the top) upon expression of mitochondrial ZFP methylases. For each indicated construct total cellular DNA was subjected to bisulfite conversion (to convert all cytosines to uracils while leaving 5-methylcytosine unchanged). Then the region of interest was amplified by PCR and cloned into *Escherichia coli*. For each construct a number of clones from two independent experiments (the total number of clones from the two experiments is indicated by *N*) was randomly chosen, sequenced, and analyzed to identify which cytosine had been methylated. The diagrams represent the mtDNA fragments originated from either the NARP cells or control WT cells, where unmethylated CpN dinucleotides are represented by open squares and the methylated CpN sites (mCpN) are depicted by filled squares and are colored according to the key. The numbers inside the filled squares represent the frequency of mCpN detected for each construct. The ZFP$_{NARP}$ binding site is shaded. (*B*) For each construct the percentage of clones containing at least one mCpN, mCpG, or methylated non-CpG is presented on the graph. The data presented are combined from two independent experiments that gave very similar results.

a high concentration of the enzyme directed there by the ZFP. In summary, we have shown sequence-specific methylation of sites in mtDNA by the targeted methylase.

The efficient nuclear export and mitochondrial uptake of the chimeric zinc finger methylase F-ZFP$_{NARP}$–meth–NES suggested that its interaction with the nuclear DNA would be negligible. Nevertheless, it was important to determine whether there was any increased methylation of potential target sites in the nuclear DNA within the cells expressing the chimeric methylase. Although the target sequence GCCCGGGCC is predicted to have $\approx 1.1 \times 10^4$ copies in the nuclear genome, most are likely to be inaccessible. Therefore, instead of analyzing genomic DNA, we introduced into the cells a nuclear reporter plasmid carrying a fragment of mtDNA containing the NARP mutation. These cells were cotransfected with an empty vector or plasmids encoding either F-ZFP$_{NARP}$–meth–NES or a version of ZFP$_{NARP}$–meth, which lacks MTS and NES and localizes exclusively in cell nuclei (SI Fig. 8*A*). The level of nuclear reporter methylation was not enhanced by expression of F-ZFP$_{NARP}$–meth–NES, relative to the background (mock, SI Fig. 8*B*). In contrast, the enzyme without the MTS F and NES domain (ZFP$_{NARP}$–meth) efficiently methylated the reporter DNA (SI Fig. 8*B*). These results indicate that combination of F and NES is sufficient to reroute F-ZFP$_{NARP}$–meth–NES from the nucleus and prevent accidental methylation of any potential targets in the nuclear DNA.

Concluding Remarks and Perspectives. We have shown that it is possible to target an enzymatically active chimeric ZFP to mitochondria and to bind it selectively to a mutant mtDNA sequence. Overcoming the tendency of ZFPs to localize in the nucleus was a major challenge because conventional MTSs were not sufficient to ensure reliable mitochondrial localization. Therefore, we developed a system that involved incorporation of a NES into the fusion protein in addition to the MTS, which proved effective at directing ZFPs exclusively to mitochondria. As well as being essential for the use of mitochondria-targeted ZFPs, this strategy of incorporating NES may be of general use in targeting proteins to mitochondria and to other subcellular compartments where nuclear localization is problematic.

Within mitochondria, the ZFP folded correctly and bound the appropriate DNA sequence with the same sequence discrimination as in *in vitro* studies. Moreover, the experiments demonstrate that ZFPs expressed in mitochondria can discriminate between mtDNA differing by one of nine base pairs, as was reported for nuclear ZFPs (30). This degree of discrimination allows for selective recognition of point mutations in mtDNA.

The ZFP targeted a DNA-modifying enzyme activity to a particular location on the mtDNA. The activity chosen here, as a proof of principle, was a methylase domain because assaying sequence-specific methylation is straightforward. Our studies demonstrated that conjugation of the methylase to the ZFP led to the sequence-specific modification of mtDNA in the vicinity of the target site. Because it is possible to design a wide range of ZFPs with various sequence specificities, the approach reported here could be used for selective targeting of a large range of DNA sequences within mitochondria. This would entail attaching particular effector domains to ZFPs and directing them to predetermined DNA sequences in mitochondria. For example, a mitochondrially targeted ZFP conjugated to a nuclease domain could selectively disrupt mutant mtDNA while sparing the WT DNA and thus could be a potential therapy for heteroplasmic mitochondrial diseases (1). By lowering the copy number of the mutant mtDNA the cell could become repopulated by WT mtDNA, thus restoring mitochondrial function and eliminating the disease phenotype (4, 5). More immediately the zinc finger technology could be used to investigate regulatory regions for mtDNA replication and transcription.

Methods

Engineering and Design of Zinc Finger Proteins for Binding mtDNA Targets. The assembly of the ZFPs as well as the detailed description of all of the constructs used in these studies can be found in *SI Text*, and the exact nucleotide and amino acid sequences of the important construct are additionally presented in SI Fig. 9.

Gel Retardation Assays. The three-finger peptides ZFP_{NARP} and ZFP_{cont} and their derivatives (containing additional domains such as N-terminal MTS F, and/or C-terminal methylase domain, and/or NES) were synthesized *in vitro* and subjected to gel retardation assay as described in *SI Text*.

The gel retardation assays were also performed on mitochondrial extracts from cells transiently expressing mitochondrially targeted ZFP_{NARP}. In this case, 24 h after transfection the cells were harvested and intact mitochondria were isolated as described below (see *Cell Fractionation and Limited Proteolysis*). Afterward, mitochondrial proteins were solubilized by sonication, and the mitochondrial extracts ($\approx$2–2.5 μg of proteins) were used in the band-shift assays as described in *SI Text*.

Cell Lines, Transfections, Selection, and Immunodetection Methods. Maintenance, transfection, selection, and immunodetection methods for the COS-7, 143B (TK−) WT cell lines and 143B (TK−) NARP cybrid cell line (referred to as NARP cells), which contained the T8993G mutation in 100% of its mtDNA, used in these studies are described in *SI Text*.

Labeling of mtDNA. Metabolic labeling of mtDNA in 143B (TK−) WT or NARP cells using BrdU was performed according to Garrido *et al.* (25) with the modifications described in *SI Text.*

Cell Fractionation and Limited Proteolysis. Mitochondria from the 143B WT or NARP cells were isolated as described by Minczuk *et al.* (31). The mitochondrial fractions were then incubated in 1× IB buffer (40 mM Tris·HCl, pH 7.4/25 mM NaCl/5 mM $MgCl_2$) supplemented with proteinase K at the concentrations indicated in Fig. 3*B*. The subcellular fractions normalized for protein content were analyzed with anti-HA mAb to detect ZFP protein constructs. Blotting using antibodies against marker proteins (anti-TFAM serum and anti-GAPDH mAb; Abcam, Cambridge, U.K.) was also performed to verify the fractionation.

Detection of Cytosine Methylation. Overall methylation of mtDNA was analyzed by the McrBC nuclease digestion followed by Southern blot, and the specific methylation of mtDNA was assessed by bisulfite method as described in *SI Text.*

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