Development of a single-chain, quasi-dimeric zinc finger nuclease for the selective degradation of mutated human mitochondrial DNA

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SUPPLEMENTARY MATERIALS

Designing heteromeric ZFN in the standard configuration specific for the T8993G mitochondrial mutation

We designed highly specific ZFN constructs to target the site of the T8993G mutation (NARPa, NARPb and NARPd; sequences indicated in red in Supplementary Figure 1, Top) and tested them with ten different companion ZFNs (COMP) binding to the sites upstream of position 8993 (a sequence indicated in blue in Supplementary Figure 1). Each ZFN construct was designed to contain: (i) the mitochondrial targeting sequence of F1β subunit of mitochondrial ATP synthase (denoted F), (ii) zinc finger peptide (iii) the *Fok*I catalytic domain, (iv) long flexible linker (v) a nuclear export signal (NES) facilitating the mitochondrial targeting of chimeric ZFPs and (vi) epitope tags (Supplementary Figure 2A). We tested individual constructs and pairs of ZFNs (NARP and COMP) in an *in vitro* assay to assess their specificity toward the mutated sequence. For this purpose, proteins were synthesised *in vitro* in a reticulocyte lysate and incubated with a DNA probe containing either the T8993G mutation (denoted pCR4-NARP-G) or a wt probe (denoted pCR4-NARP-T). The DNA fragments were analysed by gel electrophoresis and the results are presented in Supplementary Figure 2B and 2C. Unexpectedly most of the COMP ZFNs (eight out of ten) cleaved both of the DNA substrates when supplied individually (i.e. without a partner nuclease) in our *in vitro*

assay. Therefore, these COMP ZFNs were excluded from further studies in cells, as their expression in mitochondria would potentially result in a depletion of the wt mtDNA. The best efficiency and specificity of the cleavage reaction *in vitro* by a ZFN pair was achieved by combining NARPd with COMPa or COMPb (Supplementary Figure 2C dashed line). Note that only approximately 30-50% of the substrate was digested by the above combinations of ZFN in our *in vitro* test and we were not able to improve this result by optimising the linkers between DBD and catalytic domain (data not shown).

Nevertheless, the above selected pairs were taken forward for further experiments in heteroplasmic cybrid cell lines, which harbour high levels (85-95%) of the T8993G mutation in their mtDNA. In order to facilitate mtDNA cleavage pairs of ZFN needed to be expressed simultaneously in the same cell (F-NARPd-Fok co expressed with either F-COMPa-Fok or F-COMPb-Fok). However, while expression of F-NARPd-Fok was readily detected in the mitochondria of the cybrid cells (see Figure 4 of the main text), we could not detect any expression of neither F-COMPa-Fok nor F-COMPb-Fok in analogous experiment. We have further tested alternative promoters (EF1alpha) and vectors, but none of these approaches succeeded in increasing levels of expression of F-COMPa-Fok and/or F-COMPb-Fok.

Supplementary Figure 1. Design of the constructs developed to target the mitochondrial T8993G mutation. The DNA sequence of both strands of human mtDNA (positions 8955-9011) is shown in the middle. The position of the T8993G mutation is underlined. The location of the binding sites for ZFPs specific to the T8993G mutation (named NARP) is indicted in red whereas the location of the binding sites for the companion ZFPs (named COMP) is in blue. The DNA target sequence and the primary structure of ZFPs are shown for each construct in the tables A (for the NARP constructs) and B (for the COMP constructs). Amino acid sequence of contacting helices within zinc fingers (F1-F6) is shown using a single letter code. All the presented ZFPs were designed and generated by Sangamo Bioscience Inc.

Supplementary Figure 2. *In vitro* assay of standard ZFNs designed to target the mitochondrial T8993G mutation. **(A)** Schematic structure of mitochondrially targeted nucleases with a long linker between ZFP and the *Fok*I domain. The mitochondrial targeting sequence of F1β subunit of mitochondrial ATP synthase (MTS F) was fused to the N-terminus of ZFP. The nuclear export signal (NES) facilitating mitochondrial targeting was added to the C-terminus of ZFP. All constructs use the HA epitope tag to facilitate a detection.

(B)-(E) *In vitro* assay testing the specificity of the F-ZFP-Fok constructs (for the protein sequences of the ZFP constructs please refer to Supplementary Figure 1). The DNA probes were constructed as described in Material and Methods by cloning a 200 bp fragment of wild type mtDNA or mtDNA fragment containing the T8993G mutation into the pCR4 vector (Invitrogen), the resulting plasmids were named pCR4-NARP-T and

pCR4-NARP-G, respectively. Linearised DNA probes were radioactively end-labeled. The ZFP constructs were synthesized in the *in vitro* transcription/translation reaction based on a reticulocyte lysate. Proteins as indicated were co-incubated either individually **(B)** or in pairs including NARP-specific and COMP ZFPs **(C-E)** with the 1 nM DNA targets for 1h at 37ºC. Next, reactions were loaded on 1% agarose, resolved and, after dying, subjected to autoradiography. Specific digestion at the T8993G mutation site results in the formation of 2.7 and 1.5 kb DNA fragments. The most specific pairs of ZFN are indicated by a dashed box.

Supplementary Figure 3. Designing a single-chain ZFN for targeting the mitochondrial non-coding region. **(A)** The results of *in vitro* cleavage assay testing the ability of the F-NCR-Fok or F-NCR-Fok-L35-Fok constructs to introduce dsDNA breaks in target DNA. The assay was performed as described in Material and Methods using a DNA probe containing a partial sequence of human mtDNA non-coding region (pCR-NCR). The results are an average from three experiments. **(B)** *In vitro* cleavage assay testing the specificity of the F-NCR-Fok or F-NCR-Fok-L35-Fok constructs. The assay was performed as described in Material and Methods. The following probes have been used: pCR-NCR (see point A.) and pCR4-NARP-G – containing a fragment of human mtDNA surrounding the T8993G substitution. f.p denotes free probe. Specific digestion near the binding site in the mtDNA non-coding region results in the formation of 2.8 and 1.7 kb DNA fragments. **(C)** Western blot illustrating that the constructs used in (B) are produced with the same efficiency in the *in vitro* transcription/translation system.

Supplementary Figure 4. Depletion of mtDNA upon expression of mitochondrially targeted ZFNs. **(A)** Western blot illustrating a time course of the inducible expression of the control F-NCR-Fok or F-NCR-Fok-L35-Fok constructs specific for the non-coding region in mtDNA. **(B)** Total mtDNA copy number in cells expressing F-NCR-Fok or F-NCR-Fok-L35-Fok in different time points presented in arbitrary units (a.u.). The results were normalized with respect to un-induced cells.

Supplementary Figure 5. Mitochondrial heteroplasmy in cells expressing ZFNs specific for the T8993G mutation after a long-term selection. Heteroplasmic cybrid cells containing 90% of mtDNA molecules with the NARP T8993G mutation were transfected with the pIRESpuro/EF1a vector encoding F-NARPd-Fok (see Supp. Figure 2), F-NARPd-Fok-L35-Fok or vector alone. After 10 days of puromycin selection individual clones from each transfection were collected and expanded for further 3-4 days. The heteroplasmy level was measured in each clone by PCR/RFLP. The difference in the percentage of the wt mtDNA molecules present in each clone is shown. The horizontal red bars represent an average of the wt mtDNA contents for each construct. The difference between mock and F-NARPd-Fok-L35-Fok transfected cells after 30 days is highly significant ($p=0.018$, 2-tailed T-test, unequal variance), whereas the difference between mock and the F-NARPd-Fok monomer is not significant ($p=0.74$, 2-tailed T-test, unequal variance),

Supplementary Table 1. Cell survival after transfection with the ZFNs constructs. The cybrid cells containing 85% of the T8993G mutation in mtDNA were transfected and

FACS analysed as described for Figure 5 of the main text. The percentage of eGFP positive cells after 48 h of expression of constructs specific for the T8993G mutation (A) and control construct specific for the mtDNA non-coding region (B) is shown in the Tables.