Gene Knockout Using Transcription Activator-like Effector Nucleases (TALENs) Reveals That Human NDUFA9 Protein Is Essential for Stabilizing the Junction between Membrane and Matrix Arms of Complex I*

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Background: Human mitochondrial complex I is composed of 44 subunits with an elaborate assembly mechanism.

Results: TALENs were used to knock out complex I subunit NDUFA9 in HEK293T cells, revealing a novel membrane arm subcomplex.

Conclusion: NDUFA9 is critical for stabilizing the junction between different arms of complex I.

Significance: TALEN-mediated knock-out represents an accessible approach to address protein function in human cells.

Transcription activator-like effector nucleases (TALENs) represent a promising approach for targeted knock-out of genes in cultured human cells. We used TALEN-technology to knock out the nuclear gene encoding NDUFA9, a subunit of mitochondrial respiratory chain complex I in HEK293T cells. Screening for the knock-out revealed a mixture of NDUFA9 cell clones that harbored partial deletions of the mitochondrial N-terminal targeting signal but were still capable of import. A cell line lacking functional copies of both NDUFA9 alleles resulted in a loss of NDUFA9 protein expression, impaired assembly of complex I, and cells incapable of growth in galactose medium. Cells lacking NDUFA9 contained a complex I subcomplex consisting of membrane arm subunits but not marker subunits of the matrix arm. Re-expression of NDUFA9 restored the defects in complex I assembly. We conclude that NDUFA9 is involved in stabilizing the junction between membrane and matrix arms of complex I, a late assembly step critical for complex I biogenesis and activity.

Defects in complex I activity and assembly are a major cause of mitochondrial disease (1, 2). In humans, complex I is composed of 44 subunits, seven of which are encoded by mitochondrial (mt) DNA and the remaining 37 of which are encoded by nuclear genes, synthesized in the cytosol, and imported into the organelle (1, 3). Complex I is an L-shaped structure with a matrix and membrane arm that undergoes conformational coupling to facilitate proton pumping (4). Its holoenzyme form is ∼1 MDa, but within mitochondria, it assembles into higher ordered supercomplexes with respiratory chain complexes III and IV (5). In recent years, the assembly pathway for complex I has been refined, and various assembly factors critical for complex I biogenesis have been identified (1). Briefly, mtDNA-encoded subunits are inserted into the inner membrane and associate in distinct modules with core subunits and specific assembly factors. Newly imported subunits of the matrix arm are incorporated at different times and can exchange with pre-existing ones, presumably to maintain the structural and functional integrity of the complex (6, 7). Still under debate is the role of so-called accessory (or supernumerary) subunits that are present in mitochondrial complex I but not in the bacterial counterpart (8, 9).

As the subunit composition of mitochondrial complex I is different between fungi and humans (8), studies into complex I assembly and disease most often use cultured mammalian cells such as patient cell lines. These primary cell lines, however, often grow poorly, whereas isogenic control cell lines are most often missing. Although RNA interference has been utilized to study the effect of down-regulating expression of assembly factors and complex I subunits, there are limitations with this technique due to the lack of complete knockdown and potential off-target effects.

In this study, we tested the feasibility of TALEN2-mediated gene disruption (10–15) for the study of complex I biogenesis in human cells. We chose to target NDUFA9, an accessory subunit found in all eukaryotes but having an unclear role in complex I function (8, 9). Homozygous disruption of the NDUFA9 gene resulted in loss of complex I activity and the appearance of a novel ∼600-kDa complex I subcomplex, which was rescued by re-expression of NDUFA9. Analysis of the subcomplex revealed it to contain subunits located in the membrane arm, but not marker subunits of the matrix arm. These results suggest that NDUFA9 is important in stabilizing the junction between matrix and membrane arms of complex I.

**EXPERIMENTAL PROCEDURES**

**TALEN Design and Construction**—The first exon and its immediate flanking regions of human NDUFA9 (NG_032124.1) were scanned for putative TALEN binding pairs using the TAL Effector Nuclease Targeter 2.0 (16) with the following constraints: (i) having a repeat array length of 20 repeat variable diresidue

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domains with the lowest number of Asn-Asn (NN) repeat variable dyeside domains, and (ii) having a spacer length of 14–20 nucleotides. The binding pairs NDUF9-A-L (NG NN HD HD NN HD NI NI NG HD HD HD NN NN NN NG NN) and NDUF9-A-R (NG HD HD HD NN NN NG NI HD NI HD NG NI HD NN NN NI NI) were respectively assembled into the pTALEN-v2-(NN) and pTALEN-v2-(NI) backbones, yielding pTALEN-NDUF9-A-L and pTALEN-NDUF9-A-R, according to Sanjana et al. (11).

Cell Culture and Screening—HEK293T cells grown in DMEM (Invitrogen) containing 10% (v/v) FBS at 37 °C under 5% CO₂. 95% air supplemented with 50 μg/ml uridine were transfected with a mixture of pTALEN-NDUF9-A-L, pTALEN-NDUF9-A-R, and pEGFP-N1 (Clontech) at a ratio of 9:9:1. Cells were trypsinized 2–6 days after transfection and resuspended in PBS containing 10% (v/v) FBS, and single cells were isolated using a BD Biosciences FACSAria III gated on GFP fluorescence. Clonal populations were expanded, and whole cell extracts were analyzed by SDS-PAGE and immunoblotting. To confirm NDUF9 disruption, the targeted exon was PCR-amplified from genomic DNA isolated from individual clones and analyzed by Sanger sequencing. For NDUF9 complementation, HEK293T or NDUF9−/− cells were transfected with pNDUF9. The following day, media were refreshed or exchanged with glucose-free DMEM (Invitrogen) containing 10% (v/v) FBS, 5 mm galactose, and 50 μg/ml uridine. Cells were grown an additional 24 h prior to harvesting.

Blue Native Gel Electrophoresis (BN-PAGE)—BN-PAGE was performed as described previously (17). Membrane protein complexes (solubilized in 1% (w/v) digitonin or 1% (w/v) Triton X-100 as indicated) were separated on a 4–13% acrylamide-bisacrylamide BN-PAGE gel. In-gel complex I activity staining was performed as described previously (18).

Radiolabeling of mtDNA-encoded Translation Products—Mitochondrial translation products were labeled with [35S]methionine/cysteine, and membrane protein complexes were solubilized in 1% Triton X-100 and separated on 4–13% acrylamide-bisacrylamide BN-PAGE gels. For two-dimensional PAGE, gel slices were excised and subsequently separated on 10–16% polyacrylamide Tris-Tricine gradient gels as described previously (17).

In Vitro Protein Import into Isolated Mitochondria—Precursor proteins labeled with [35S]methionine/cysteine were in vitro translated as described previously (6). Precursors were incubated with isolated mitochondria at 37 °C for various times as indicated. Proteinase K and dissipation of the mitochondrial membrane potential (ΔΨm) were performed as described previously (19).

Miscellaneous—Mitochondria were isolated from cells as described previously (6). Radiolabeled proteins were detected by PhosphorImager analysis (GE Healthcare). Standard techniques were used for immunoblotting and chemiluminescence detection. Polyclonal antibodies against NDUF9, NDUF6, NDUF5, and mtHSP70 were raised in rabbits. Monoclonal antibodies against NDUF52, SDHA, NDUF3, COX1, and CORE1 (Invitrogen) were used.

RESULTS

Targeted Gene Disruption of NDUF9—TALEN binding pairs for gene knock-out are designed to target directly downstream of the translation start codon, creating double-strand breaks that are repaired by nonhomologous end joining yielding out-of-frame deletions or insertions (10–15). Like the majority of nuclear encoded mitochondrial proteins, NDUF9 harbors an N-terminal cleavable presequence that targets the protein to mitochondria and is removed by specialized proteases in the mitochondrial matrix (6, 20). We targeted this region of NDUF9 for TALEN-mediated gene disruption, generating the TALEN binding pair shown in Fig. 1A (see “Experimental Procedures” for additional information) using the TALEN toolbox described by Sanjana et al. (11). We chose HEK293T cells due to their ease of culturing and high transfection efficiency. The cells were transfected with plasmids encoding the TALEN binding pair along with a limiting amount of marker plasmid expressing GFP for cell sorting. Clonal populations were screened by immunoblot analysis using NDUF9 antibodies. As can be seen (Fig. 1B), varying levels of NDUF9 were evident between clones. Sequencing of a clone with no detectable NDUF9 (Fig. 1B, lane 12, clone K; hereafter NDUF9−/−) revealed loss of 17 nucleotides in both alleles that disrupted NDUF9 expression (Fig. 1C).

Interestingly, analysis of another clone (Fig. 1C, Clone D) also revealed biallelic TALEN-mediated disruption of the NDUF9 gene; however, NDUF9 was still expressed (Fig. 1B, lane 5). In this case, a second in-frame translation start codon (corresponding to Met-15; Fig. 1A) might be used to initiate translation, thereby producing NDUF9 with a truncated presequence (NDUF9Δ1–14) leaving the mature protein unchanged. As shown by our in vitro import experiment, [35S]NDUF9Δ1–14 is equally capable of mitochondrial import as full-length NDUF9 (Fig. 1D, lanes 1–7), and when analyzed by BN-PAGE, it efficiently assembled into complex I (Fig. 1D, lanes 8–13). Taken together, these results demonstrate the importance of correct site selection in targets containing an N-terminal cleavable presequence and the verification of protein loss by immunoblotting.

NDUF9−/− Cells Harbor a Complex I Assembly Defect Reversible by Re-expression of NDUF9—We next assessed the effect of NDUF9 gene disruption on complex I activity. Mitochondria were isolated from HEK293T and NDUF9−/− cells and solubilized in digitonin, and complexes were resolved by BN-PAGE. Using an in-gel activity assay, we could detect complex I activity within a supercomplex that contains complexes III and IV (CI/CIII2/CIV) in HEK293T mitochondria but not in NDUF9−/− mitochondria (Fig. 1E, lanes 1 and 2). Immunoblot analysis using an antibody specific to NDUF86, a membrane arm subunit of complex I, showed the absence of the CI/CIII2/CIV supercomplex in NDUF9−/− mitochondria and the presence of faster migrating species (Fig. 1E, lanes 3 and 4). These species still contained complexes III and IV (Fig. 1E, lanes 5–8). The migration of complexes II and V, which do not form supercomplexes with complex I, also appeared normal in NDUF9−/− mitochondria (data not shown). We next used Triton X-100 to dissociate the majority of supercomplexes (17).
and analyzed the migration of the holocomplexes. Although NDUF6 was seen in the ~1-MDa mature complex I form in HEK293T mitochondria, it was found to migrate in a subcomplex of ~600 kDa in NDUFA9−/− mitochondria (Fig. 1E). Furthermore, the migration of complexes III and IV was unaffected in NDUFA9−/− mitochondria, pointing to an isolated defect in complex I assembly.

Cells with severe respiratory chain defects do not grow in the presence of galactose as the sole carbon source due to their dependence on oxidative phosphorylation for ATP generation (21). Consistent with a loss of complex I activity, we were unable to culture NDUFA9−/− cells in galactose-containing medium. However, when we transfected NDUFA9−/− cells with a plasmid expressing NDUFA9, growth in galactose medium was restored (data not shown). Analysis of mitochondria from these cells revealed that the levels of complex I were partially restored with a concomitant reduction in the amount of ~600-kDa assembly (Fig. 1F).

The ~600-kDa Subcomplex Contains All mtDNA-encoded Complex I Subunits—To understand how the ~600-kDa subcomplex is formed, we analyzed the assembly of newly synthesized mtDNA-encoded subunits into complex I using pulse-chase analysis (17). Mitochondria were isolated, solubilized in Triton X-100, and subjected to BN-PAGE analysis. In contrast to HEK293T mitochondria, in NDUFA9−/− mitochondria, the mtDNA-encoded subunits accumulated in a ~600-kDa complex instead of mature complex I (Fig. 2A). To identify the components within this complex, we used two-dimensional PAGE analysis of the 20-h chase. Following the labeling (0-h chase), the assembly profile of mtDNA-encoded complex I subunits between HEK293T and NDUFA9−/− mitochondria was indistinguishable with the presence of early assembly intermediates at ~400 and ~460 kDa and a later stage ~830-kDa intermediate (Fig. 2B). However, at a 20-h chase, the mtDNA-encoded complex I subunits accumulated in the ~600-kDa complex in NDUFA9−/− mitochondria instead of the mature complex I seen in HEK293T mitochondria. The ~600-kDa complex contained all mtDNA-encoded complex I subunits (ND1–6 and ND4L). The identity of the ~600-kDa complex and of mature complex I in control mitochondria was confirmed by immunoblotting for NDUF6 (Fig. 2B, lower panels). Taken together, we conclude that NDUFA9−/− cells lack mature complex I, instead harboring a ~600-kDa subcomplex containing at least all mtDNA-encoded subunits as well as other subunits including NDUF6.

*NDUFA9 Stabilizes the Matrix Arm of Complex I*—We analyzed the steady state levels of various nuclear encoded complex

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**FIGURE 1. Targeted gene disruption of NDUFA9.** A, schematic showing first coding exon of the NDUFA9 gene (translated sequence indicated by the dashed box). Highlighted areas indicate pTALEN-NDUFA9-L and pTALEN-NDUFA9-R binding sites and the predicted cut site. The translation initiator and second Met residues are indicated in bold. B, HEK293T cells and single cell clones derived from TALEN transfected cells were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. mHSP70, mitochondrial HSP70 as loading control. C, schematic showing nucleotide deletions in select clones. The second Met residue is indicated in bold. D, in vitro translated [35S]NDUFA9 or [35S]NDUFA9Δ1 were imported into HEK293T mitochondria for the indicated times in the presence or absence of the ΔV, m. Samples were split, and where indicated, treated with externally added proteinase K (PK), analyzed by SDS-PAGE (lanes 1–7), or solubilized in Triton X-100 and analyzed by BN-PAGE (lanes 8–13). Input lysate is also shown. p, precursor; m, mature. E, mitochondria isolated from HEK293T or NDUFA9−/− cells were solubilized in digitonin or Triton X-100, and protein complexes were separated by BN-PAGE. Gel strips were either stained for complex I in-gel activity (IGA; lanes 1 and 2) or analyzed by immunoblotting with the indicated antibodies. Complexes CI–CV are indicated. * indicates complex I subcomplexes detected in NDUFA9−/− mitochondria. F, mitochondria were isolated from mock− or pNDUFA9-transfected HEK293T or NDUFA9−/− cells grown in the indicated medium and solubilized in Triton X-100 as well as complexes analyzed by BN-PAGE and immunoblotting with the indicated antibodies.
I components in NDUFA9−/− mitochondria by SDS-PAGE and immunoblotting (Fig. 2C, lanes 1 and 2). In comparison with other subunits and control proteins, the level of matrix arm subunits NDUFS2 and NDUFS3 appeared reduced. Indeed both subunits were missing from the ~600-kDa subcomplex in NDUFA9−/− mitochondria (Fig. 2C, lanes 3–8). In contrast, NDUFS5, which has been proposed to associate with the membrane arm at a late stage in the assembly pathway (22–24), was detected in the ~600-kDa subcomplex. Due to the absence of antibodies against other subunits, we analyzed the import and assembly of matrix arm subunits NDUFS4 and NDUFA12 following their in vitro import. As can be seen (Fig. 2D), [35S]NDUFS4 and [35S]NDUFA12 could assemble into complex I in HEK293T mitochondria, but in NDUFA9−/− mitochondria, they failed to assemble into a complex, consistent with their absence in the ~600-kDa subcomplex. We conclude that NDUFA9 is a critical subunit for the function of complex I and is required for stability of a later stage assembly, involving the connection between the membrane and matrix arms.

**DISCUSSION**

Routine genome modification has the potential to revolutionize the use of cultured human cells as a model for the study of intracellular processes and for understanding disease. Although targeted methods such as zinc finger technology have existed for some years, widespread use has been prohibited by high costs associated with their often proprietary nature (11, 25, 26). TALEN-mediated gene disruption is a promising technique due to the open-source approach adopted by its establishing groups; reagents are freely available by request or deposited in nonprofit plasmid repositories (10, 11, 27). TALENs can be designed to generate out-of-frame deletions in the first coding exon of the targeted gene (10–15, 27); however, targets with multiple splice variants, especially those with internal splicing, pose significant TALEN design problems. As most nuclear encoded mitochondrial proteins are directed to mitochondria by a cleavable N-terminal presequence (28), we postulated that by disrupting the presequence and thus mitochondrial import, consideration of splice variants could be avoided.
REPORT: NDUF A9 in Complex I Assembly

We sought to apply TALEN-mediated gene disruption to the understanding of mitochondrial complex I assembly. NDUF A9 is an accessory subunit that is conserved in all euukaryotes that harbor mitochondrial complex I. The subunit was chosen based on (i) the availability of specific antibodies for this subunit to facilitate screening of clones; and (ii) the presence of an N-terminal, cleavable targeting signal for TALEN targeting. Although targeting of the NDUF A9 presequence resulted in efficient generation of out-of-frame deletions (we estimate >60% following sorting of transfected cells), we found that instead of a loss of detectable NDUF A9, many clones exhibited a reduced level of NDUF A9 expression. Our analysis suggested translation from a second in-frame start codon present within the NDUF A9 presequence to be the causative factor because the truncated presequence remained competent for mitochondrial import. Although our results show disruption of an N-terminal targeting signal to be sufficient to ablate protein expression, they also demonstrate that care must still be taken in target site selection and that appropriate experiments for expression studies are conducted.

Consistent with a recent patient study reporting loss of complex I activity in a patient harboring a point mutation in NDUF A9 (29), we found that NDUF A9 is critical for complex I activity. NDUF A9\(^{+/−}\) cells were also unable to utilize galactose as a carbon source, indicating that oxidative phosphorylation was disrupted. Although it was shown that complex I was deficient in NDUF A9 patient fibroblasts, it was not established how loss of NDUF A9 affected assembly (29). Furthermore, previous studies have reached different views into NDUF A9 biogenesis with suggestions that it is incorporated either at an early stage or at a late stage in complex I assembly (30–32). Pulse-chase analysis of membrane-integrated, mtDNA-encoded subunits revealed the presence of early complex I intermediates in NDUF A9\(^{−/−}\) cells, thus suggesting that NDUF A9 is required at a later stage of complex I assembly. A novel, steady state ~600-kDa subcomplex accumulated in NDUF A9\(^{−/−}\) cells that contained marker subunits of the complex I membrane arm, including all mtDNA-encoded subunits, but not marker subunits of the matrix arm. However, the earliest evidence for the incorporation of all mtDNA-encoded subunits appears to be when a late stage ~830-kDa intermediate is formed that is missing subunits of the matrix NADH dehydrogenase module (1). The pulse-chase studies indicated that in NDUF A9\(^{−/−}\) cells, the mtDNA-encoded subunits transiently assembled into a higher form before forming the stable ~600-kDa subcomplex. We suggest that the higher form represents the ~830-kDa intermediate, which, in the absence of NDUF A9, is unstable, leading to the dissociation of matrix subunits such as NDUF S2 and NDUF S3 (present in early intermediates (31, 32)) to produce the ~600-kDa membrane arm. These results would be consistent with the suggested location of NDUF A9 at the junction between the matrix and membrane arms of complex I. As the ~600-kDa subcomplex identified in this study could also be found in supercomplexes with complexes III and IV, our study also suggests that the membrane portion of complex I is involved in making major contacts with subunits of complex III and IV.

In summary, NDUF A9 is an essential subunit required for human complex I assembly. Although NDUF A9 is classified as an accessory subunit due to its absence in bacteria, it is nevertheless required for stabilizing the link between the membrane and matrix arms of mitochondrial complex I. We suggest that TALEN technology can also be used to determine the importance of other accessory subunits and assembly factors involved in complex I biogenesis and disease.

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