Pharmacological NAD-boosting strategies improve mitochondrial homeostasis in human Complex I-mutant fibroblasts

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Running Title
NAD boosting strategies and mitochondrial defects

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Abbreviations
ATP5D, ATP synthase H+ transporting mitochondrial F1 complex delta subunit; NRF, nuclear respiratory factor; CI, complex I; COX, cytochrome c oxidase; mtDNA, mitochondrial DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nDNA, nuclear DNA; NDUFS1, NADH dehydrogenase (ubiquinone) Fe-S protein 1; NDUFV2, NADH dehydrogenase (ubiquinone) flavoprotein 2; ND2, NADH dehydrogenase 2; NR, nicotinamide riboside; NRF-1/2, nuclear respiratory factor 1/2; OXPHOS, oxidative phosphorylation; PARP, poly(ADP-ribose) polymerase; PGC1α/β, peroxisome proliferator-activated receptor gamma coactivator 1-alpha/beta; Phe, 6-(5H)-
phenanthridinone; PJ34, [N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide.HCl];
PPCR1, peroxisome proliferator-activated receptor gamma, coactivator-related 1; ROS, reactive
oxygen species; TFAM, mitochondrial transcription factor A; TFB1M, mitochondrial transcription
factor B1; TFB2M, mitochondrial transcription factor B2; TMRE, tetramethylrhodamine ethyl ester.
Abstract

Mitochondrial disorders are devastating genetic diseases for which efficacious therapies are still an unmet need. Recent studies report that increased availability of intracellular NAD obtained by inhibition of the NAD-consuming enzyme poly (ADP-ribose) polymerase (PARP)-1 or supplementation with the NAD-precursor nicotinamide riboside (NR) ameliorates energetic derangement and symptoms in mouse models of mitochondrial disorders. Whether these pharmacological approaches also improve bioenergetics of human cells harbouring mitochondrial defects is unknown. It is also unclear whether the same signaling cascade is prompted by PARP-1 inhibitors and NR supplementation to improve mitochondrial homeostasis. Here, we show that human fibroblasts mutant for NDUFS1 subunit of respiratory Complex I have similar ATP, NAD and mitochondrial content compared to control cells, but show reduced mitochondrial membrane potential. Interestingly, mutant cells also show increased transcript levels of mtDNA but not nDNA respiratory complex subunits, suggesting activation of a compensatory response. At variance with prior work in mice, however, NR supplementation but not PARP-1 inhibition increased intracellular NAD content in NDUFS1 mutant human fibroblasts. Conversely, PARP-1 inhibitors but not NR supplementation increased transcription of TFAM and mtDNA-encoded respiratory complexes constitutively induced in mutant cells. Still, both NR and PARP-1 inhibitors restored mitochondrial membrane potential and increased organelle content, as well as oxidative activity of NDUFS1-deficient fibroblasts. Overall, data provide the first evidence that in human cells harbouring a mitochondrial respiratory defect exposure to NR or PARP-1 inhibitors activate different signaling pathways not invariantly prompted by NAD increases, but equally able to improve energetic derangement.
Introduction

Mitochondrial diseases are devastating disorders due to various defects of mitochondrial functioning such as deficiencies in electron transport chain, protein importation, fusion/fission as well as regulation of programmed cell death. Mutations in nuclear or mitochondrial genes typically underpin the etiology of these disorders (Wallace, 1999; Dimauro and Schon, 2008). Despite the identification of the primary genetic defects, pathogenesis of mitochondrial disorders is not well understood. An intricate cascade of detrimental signals and metabolic impairment originates from the primary genetic mutations, and is responsible for cell demise and clinical symptoms (Kleist-Retzow et al., 1998; Skladal et al., 2003). Given the extreme difficulty in finding a cure for these genetic disorders, identification and targeting of death pathways prompted by the original gene defect represents a feasible strategy of relevance to symptomatic treatment of mitochondrial disease patients (Moslemi and Darin, 2007).

Respiratory Complex I (CI) deficiency is one of the most common cause of oxidative phosphorylation (OXPHOS) defects in childhood. Neurological symptoms occur at the age of ~4 months and the majority of patients die within the first 5 years of life (Dimauro and Schon, 2008; Spinazzola and Zeviani, 2009). A great deal of effort has been directed at identifying the molecular mechanisms responsible for neurodegeneration in children affected by OXPHOS defects. Evidence points to excessive production of reactive oxygen species (ROS) as the primary cause of cell demise in these patients and in particular in those affected by CI deficiency (Fato et al., 2008). Although pharmacological strategies able to counteract ROS-dependent oxidative stress in mitochondrial disease patients has a strong therapeutic rationale (Murphy and Smith, 2007; Dimauro and Rustin, 2009) the identification of compounds reducing tissue degeneration in children with respiratory chain disorders is still an unmet need (Dimauro and Mancuso, 2007).

During the last several years the biochemistry of pyridine nucleotide [NAD(H) and NADP(H)] biosynthetic pathways has been revisited in detail, and two main concepts on NAD homeostasis emerged. First, NAD-synthesizing enzymes and those responsible for its continuous consumption regulate an unexpected, large array of key cellular processes (Canto and Auwerx, 2012; Chiarugi et al., 2012; Koch-Nolte et al., 2009). Second, given that availability of pyridine nucleotides is
limiting several NAD-dependent processes, pharmacological approaches able to improve NAD availability holds remarkable therapeutic potential for different human disorders (Dolle et al., 2013; Houtkooper and Auwerx, 2012; Sauve, 2008).

As far as mitochondrial homeostasis is concerned, recent reports indicate that increased cellular NAD availability improves organelles’ functioning and cell metabolism (Bai et al., 2011b; Bai et al., 2011a; Pittelli et al., 2011; Canto et al., 2012; Cerutti et al., 2014; Khan et al., 2014). In this regard, both pharmacological and genetic suppression of the major NAD-consuming enzyme poly(ADP-ribose) polymerase (PARP)-1 increase NAD contents in different organs and overall oxidative metabolism in mice (Bai et al., 2011b). Similarly, diet supplementation with the NAD precursor nicotinamide riboside (NR) ameliorates metabolic defects of obese mice, boosts their energy expenditure and, overall, reverts metabolic impairment (Canto et al., 2012). Recent contributions report the ability of PARP-1 inhibitors or NR supplementation to improve mitochondrial defects and muscle fitness in both healthy mice and in those with mitochondrial myopathy (Khan et al., 2014; Cerutti et al., 2014; Pirinen et al., 2014). In keeping with this, diet supplementation with additional NAD precursors such as nicotinamide or nicotinamide mononucleotide partially normalizes mitochondrial defects in CI-deficient mouse cardiomyocytes (Karamanlidis et al., 2013).

As for the molecular mechanisms underpinning these therapeutic effects, it has been proposed that NAD precursors increase cellular NAD content and the activity of the NAD-dependent enzymes sirtuins (in particular Sirt1) which are master regulators of mitochondrial biogenesis, respiration and energy production/expenditure (Bai et al., 2011b; Bai et al., 2011a).

Of note, NR is a NAD precursor present in daily diet (Bieganowski and Brenner, 2004), whereas PARP-1 inhibitors reached the clinic for antineoplastic purposes, showing an acceptable safety profile (Yap et al., 2011). In the present study, we evaluated the effects of NR or PARP-1 inhibition on CI-deficient human fibroblasts obtained from a 6 month patient with progressive leukoencephalopathy (Bugiani et al., 2004), in order to confirm data obtained in mice and help defining the realistic translational potential of NAD-boosting strategies to mitochondrial disease patients.
Materials and Methods

Cell culturing

Control fibroblasts and patient’s fibroblasts (Iuso et al., 2006) were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 10% fetal bovine serum, and antibiotics. Cultures were brought to 50 to 70% confluence and used for the experiments. Cells were exposed to 6-(5H)-phenanthridinone (Phe), [N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide.HCl] (PJ34) or nicotinamide riboside (NR), directly dissolved in the culture media. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay and phase-contrast microscopy. An inverted Nikon TE-2000U microscope equipped with a charge-coupled device camera was used for cell visualization.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was evaluated by means of flow cytometry (Felici et al., 2014). NDUFS1 mutant fibroblasts were treated with vehicle, NR or with the PARP-1 inhibitors PJ34 (20 μM) or Phe (20 μM). Cells were then detached, incubated with tetramethylrhodamine ethyl ester (TMRE) 2.5 nM, and analyzed with a Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with the EXPO32 Flow Cytometry ADC software (Beckman Coulter).

NAD and ATP quantification

NAD contents were quantified by means of an enzymatic cycling procedure (Felici et al., 2013). Briefly, cells grown in a 48 well plate were killed with 50 ml HClO₄ 1N and then neutralized with an equal volume of KOH 1N. After the addition of 100 ml of bicine 100 mM pH 8, 50 ml of the cell extract was mixed with an equal volume of the bicine buffer containing 23 ml/ml ethanol, 0.17 mg/ml MTT, 0.57 mg/ml phenazine ethosulfate and 10 mg alcohol dehydrogenase. Mixture was kept at room temperature for 20 minutes and then absorbance a 550 nm was measured. A standard curve allowed quantification of NAD. The cellular ATP content were measured by means of an ATPlite kit from PerkinElmer Life and Analytical Sciences (Zaventem, Belgium) as described previously (Pittelli et al., 2011).
Evaluation of mtDNA content and respiratory subunits expression levels

Genomic DNA and total RNA were extracted from control and NDUFS1 mutant fibroblasts with the nucleoSpin TriPrep kit (Macherey-Nagel), and real-time polymerase chain reaction was performed as previously reported (Lapucci et al., 2011). Mitochondrial content was quantified by measuring the ratio between mitochondrial D-loop and nuclear 18S gene amplification products (Lapucci et al., 2011). The following primers were used: for COX1—forward 5'-TACCGGCTTCGGAATAATCTC-3' and reverse 5'-GATAGCGATGATTATGGTAGC-3'; for COX2—forward 5'-CTCCTTGACGTTGACAAT-3' and reverse 5'-CCACAGATTTAGAGCAGATTGA-3'; for mt-ND2—forward 5'-AGTGTAATCCTGCGATG-3' and reverse 5'-CGAGATACTAGTGGTCTGG-3'; for NDUFV2—forward 5'-GCTGTTTCTCAGTCTGGATTA-3' and reverse 5'-CAGAGTTTCGAAGCATGCAGGGAGT-3'; for COX15—forward 5'-CCCTGAGGGCCAGGCAAT-3' and reverse 5'-AATGCGCAATCTACCAGAGGAGCC-3'; for ATP5D—forward 5'-ATGTCTTATCCCTCGCTCTCC-3' and reverse 5'-GGAACCGCTGCTCACAAAGTATTGG-3'; for TFB1M—forward 5'-TGCAAGCAGGCAAGCAGCT-3' and reverse 5'-TTCAACCAGGAAGGTTCCGAG-3'; for TFB2M—forward 5'-GATCGGAGATTGGCTGAGAC-3' and reverse 5'-TCACTTTGAGCGCAACCAC-3'; for SIRT1—forward 5'-CCAGGCTGTGGAAGTCGACTGCG-3' and reverse 5'-GCAGGCTGTGGCAGATGCAGCTG-3'; for TFAM—forward 5'-CCGGCTGTGGAAGTCGACTGCG-3' and reverse 5'-TCCCTGCAAGCTGGGAATTCTCTC-3'; for PGC1α—forward 5'-CCAGGTCAAGATCTCCAGGAGG-3' and reverse 5'-ATATTGTGCTGGAAGCTTCTC-3'; for PGC1β—forward 5'-CGAAGGAATTCTTGAGAGACAGA-3' and reverse 5'-GTCAGCACCTCAGCTCACAT-3'; for NRF-1—forward 5'-GAAGATCAGCAAACACAGGCCA-3' and reverse 5'-GAATAATTCTGGGCAACGGGTACGAGG-3'; for NRF-2—forward 5'-GACCTCACACACTCAACATTTG-3' and reverse 5'-
Results

Bioenergetics and mitochondrial content in NDUFS1 mutant fibroblasts

Fibroblasts from a patient with a NDUFS1 gene mutation (C1564A) were used as a model of CI deficiency (Iuso et al., 2006). When compared with normal human fibroblasts, NDUFS1 mutant cells showed equal content of ATP and NAD (Fig. 1A and B). Conversely, we found a ~20% reduction of mitochondrial membrane potential in mutant cells (Fig. 1C). The latter result is in keeping with impairment of Complex I-dependent respiration in NDUFS1 mutant fibroblasts (Iuso et al., 2006). We also quantified mtDNA as an indicator of mitochondrial content (Lapucci et al., 2011; Felici et al., 2014), and found that it did not differ between mutant and control fibroblasts (Fig. 1D), suggesting therefore that mitochondrial dysfunction prompted by NDUFS1 mutation does not affect mitochondrial biogenesis. Finally, we asked whether respiratory defect due to NDUFS1 mutation alters expression levels of respiratory complex subunits as a sort of compensatory strategy. We therefore quantified in both cell types transcript levels for different respiratory complex subunits encoded by nuclear or mitochondrial DNA (nDNA and mtDNA, respectively). Interestingly, data shown in Fig. 1E indicate that mutant fibroblasts had higher transcript levels exclusively of those subunits encoded by mtDNA such as COX1, COX2 and ND2 (Fig. 1E). This prompted us to analyze expression levels of transcripts for different regulators of mitochondrial gene transcription and found that only TFB1M and PCCR1 were more abundant (about 3- and 4-fold, respectively) in mutant compared to control fibroblasts (Fig. 1F).
Effects of PARP-1 inhibitors on mitochondrial homeostasis of NDUFS1 mutant fibroblasts

Reportedly, genetic suppression of PARP-1 or chronic treatment with PARP-1 inhibitors increases intracellular NAD contents and mitochondrial biogenesis in mouse models of mitochondrial disorders (Cerutti et al., 2014). In order to reproduce these findings in human cells, we exposed NDUFS1-deficient fibroblasts for 7 days to chemical inhibitors of PARP-1 such as PHE and PJ34. We first checked for possible drug-induced cytotoxicity by means of intracellular ATP quantitation or LDH release assay, but found no evidence of changes in energy content or cell death (Fig. 2A and not shown). Remarkably, PARP-1 inhibition did not increase NAD contents of NDUFS1 mutant fibroblasts (Fig. 2B), whereas sufficed to augment their mitochondrial membrane potential (Fig. 2C and D). Furthermore, exposure to PHE and PJ34 increased mitochondrial content (Fig. 2E), consistent with the notion that suppression of PARP-1 activity promotes mitochondrial biogenesis in mice (Bai et al., 2011b). In light of our recent finding that both PHE and PJ34 increase expression of respiratory complex subunits in different tissues of \textit{Ndufs4} KO mice (Felici et al., 2014), we asked whether this also occurs in human cells harboring a mitochondrial defect. NDUFS1 deficient fibroblasts were therefore exposed to PARP-1 inhibitors for 7 days, and expression levels of respiratory complex subunits encoded by nDNA or mtDNA analyzed. Interestingly, we found that PHE and PJ34 increased transcript levels of different respiratory complex subunits encoded by mtDNA, having no effects on those encoded by nuclear genes (Fig. 2F and G). This finding prompted us to evaluate whether pharmacological inhibition of PARP-1 altered expression levels of mitochondrial transcription factors such as TFAM, TFB1M, TFB2M, NRF1, NRF2, and PPCR1 (Scarpulla et al., 2012). In light of the key role of Sirt-1 and PGC1α/β in mitochondrial biogenesis, their transcript levels were evaluated as well. As shown in Fig. 2H, both PHE and PJ34 had a selectively increased transcripts for TFAM, NRF1, NRF2 and PGC1β. mRNAs for PGC1α were highly increased by PJ43 only, suggesting a non-specific effect, unrelated to PARP-1 inhibition. Next, to understand whether the effects of PARP-1 inhibitors on NDUFS1 mutant fibroblasts translated into improved mitochondrial function, we evaluated fibroblast ability to reduce MTT which is a prototypical index of mitochondrial respiratory activity (Berridge and Tan, 1993). Of note, MTT reduction activity was increased by a 7 day incubation to PHE or PJ34 (Fig 2.
I and J). We also evaluated the effects of the two inhibitors on cultured fibroblasts from healthy subjects and found analogous results for ATP, NAD, mtDNA and TMRE. As for transcripts of respiratory subunits we found that in normal cells COX2 mRNA was not increased by the two compounds (Supplementary Fig. S1).

**Effects of NR on mitochondrial homeostasis of NDUFS1 mutant fibroblasts**

In addition to PARP-1 inhibition, exposure to NAD precursors is an alternative pharmacological strategy to increase intracellular NAD content. Among NAD precursors, NR has been intensively investigated as a NAD-boosting molecule because of its ability to cross the plasmamembrane and, at variance with nicotinamide, its inability to inhibit NAD-hydrolyzing enzymes such as PARP-1 and sirtuins (Chi and Sauve, 2013). We therefore exposed NDUFS1 mutant fibroblasts to NR for 2 or 7 days and evaluated whether it affected mitochondrial homeostasis in a way similar to PARP-1 inhibitors. As shown in Fig. 3A and B, exposure to NR did not affect ATP contents after 2 or 7 days of treatment, whereas led to a 24% increase in those of NAD after 7 days of incubation. Akin to PARP-1 inhibitors, a 7 day incubation with NR increased mitochondrial membrane potential (Fig. 3C, D) as well as mitochondrial content (Fig. 3E). Interestingly, however, NR had no effects on expression levels of nDNA- or mtDNA-encoded respiratory complex subunits (Fig. 3F). Notwithstanding the inability of NR to activate transcription of respiratory complex subunits, MTT reduction capacity of NDUFS1 mutant fibroblasts was augmented after a 7 day exposure to the NAD precursor (Fig. 3G and H). We also evaluated the effects of NR on cultured fibroblasts from healthy subjects and found analogous results for ATP, NAD, mtDNA, TMRE and transcripts of respiratory subunits (Supplementary Fig. S2). Finally, we evaluated whether NR also affects mRNA levels for mitochondrial transcription regulatory genes such as TFAM, TFB1M, TFB2M, NRF1, NRF2, PPCR1, SIRT-1 and PGC1α/β (Scarpulla et al., 2012). Interestingly, as shown in Fig. 3I, NR increased by about 3-fold selectively transcripts for PGC1α and PGC1β.
Discussion

In the present study we report that two pharmacological strategies previously shown to increase intracellular NAD content such as PARP-1 inhibition and NR supplementation improve mitochondrial homeostasis of human fibroblasts carrying a NDUFS1 mutation. These findings strengthen prior work in mice showing that suppression of PARP-1 activity or diet supplementation with NR ameliorates symptoms in experimental models of mitochondrial disorders (Cerutti et al., 2014). The present study, however, provides the first evidence that, at least in human cells, the two pharmacological approaches do not activate the same signalling pathways. Indeed, only NR supplementation was capable of increasing intracellular NAD content in NDUFS1 mutant fibroblasts, whereas only PARP-1 inhibitors increased transcription of TFAM and mtDNA-encoded respiratory complex subunits. Still, both NR and PARP-1 inhibitors increased mitochondrial membrane potential, organelle content, as well as MTT reduction capacity. The inability of PARP-1 inhibitors to increase cellular NAD content is in contrast with prior work (Bai et al., 2011b), but consistent with a recent contribution showing that PJ34 does not augment NAD content in different organs of Ndufs4 KO mice (Felici et al., 2014). At present we do not know the reason(s) of this apparent inconsistency. Evidence that inhibitors of PARP-1 do not increase NAD content in mouse tissues (Felici et al., 2014) and human cells rules out that the inconsistency might be merely due to specie differences. We speculate that different treatment paradigms and/or disease models may explain why treatment with chemical PARP-1 inhibitors is not invariantly related to NAD increases in cells or tissues. Even the higher apparent potency of PJ34 with respect to PHE for some parameters we have investigated here, may not be absolute, but related to the model or cell function analysed.

We also show that NR supplementation increases cellular NAD contents as well as mitochondrial membrane potential and content. However, the inability of NR to induce transcriptional activation of respiratory complex subunits indicates that an increased availability of NAD in human cells affects only specific parameters of mitochondrial homeostasis. Reportedly, improved mitochondrial functioning by increased NAD contents is due activation of the Sirt1/PGC1α axis (Canto et al., 2012; Bai et al., 2011b; Cerutti et al., 2014). In this light, given that NAD contents are not increased...
by PARP-1 inhibitors in NDUFS1 mutant cells, we reason that the chemicals affect TFAM and respiratory complex subunit transcription as well as or mitochondrial membrane potential in a Sirt1/PGC1α independent manner. Most likely, the transcriptional effects of PHE and PJ34 are due to their impact on PARP-1-dependent epigenetic regulation of gene expression. Indeed, pharmacological modulation of PARP-1 significantly alters gene expression profiles in different cell types in a positive or negative fashion according to the specific gene and its transcriptional context (Krishnakumar et al., 2008; Frizzell et al., 2009; Krishnakumar and Kraus, 2010). PARP-1 activity not only regulates formation of supramolecular complexes at the promoter levels, but also plays a key role at the level of nascent RNA (Soldatenkov et al., 2002). Further, excessive formation of poly(ADP-ribose) by PARP-1 at transcriptional active sites impairs basal transcriptional machinery (Soldatenkov et al., 2002). In this light, the finding that NDUFS1 mutant fibroblasts constitutively express higher transcript levels of respiratory complex subunits compared to control cells is of particular relevance. It is likely, indeed, that basal induction of these genes renders their transcriptional apparatus sensitive to PARP-1 inhibitors. Also, accumulation of reactive oxygen radicals [which are well known triggers of PARP-1 activation (Luo and Kraus, 2012)] in mutant NDUFS1 fibroblasts (Iuso et al., 2006) can further contribute to sensitization of these cells to PARP-1 inhibitors. Evidence that in PHE- and PJ34-exposed cells TFAM transcription was induced concomitant with that of those respiratory subunits which are constitutively induced corroborates the hypothesis that epigenetic modification triggered by PARP-1 inhibitors in NDUFS1 fibroblasts promotes a transcriptional program already primed by the respiratory deficit. The constitutive, selective induction of respiratory complex subunits encoded by mtDNA suggests that NDUFS1 mutation and ensuing derangement of mitochondrial homeostasis is sensed by the cells that activates transcription of mitochondrial respiratory genes as a sort of compensatory strategy. Nuclear respiratory factor (NRF)-1 might take part to this compensatory response. Indeed, NRF-1 promotes expression of numerous mitochondria regulating proteins including TFAM, and is negatively regulated by PARP-1 activity (Hossain et al., 2009). PARP-1 inhibitors might therefore unleash NRF-1 repression and indirectly promote expression of mtDNA-encoded respiratory complex subunits.
In conclusion, this study strengthens the therapeutic potential of NR and PARP-1 inhibitors in treatment of mitochondrial diseases. Even though the safety profile and pharmacokinetics of NR in humans is unknown, it can be speculated that, being this riboside a natural product present in daily consumed foods such as milk and yeast (Bieganski and Brenner, 2004), it is better tolerated than PARP-1 inhibitors which are xenobiotics with significant genotoxic potential. NR might have, therefore, a more rapid therapeutic application and clinical transferability.

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Authorship Contributions
Participated in research design: Felici, Chiarugi
Conducted experiments: Felici, Lapucci, Cavone, Berlinguer-Palmini
Contributed new reagents or analytic tools: Pratesi
Performed data analysis: Felici
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Reference List


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Legends to Figures

Figure 1. Bioenergetics and mitochondrial content in NDUFS1 mutant fibroblasts. Comparison of the ATP (A), NAD (B), mitochondrial membrane potential (C) and mtDNA content (D) between control and NDUFS1 mutant fibroblasts under resting conditions. The transcript levels of nDNA-encoded (COX15, NDUFV2, ATP5D) and mtDNA-encoded (COX1, COX2, ND2) respiratory complex subunits in NDUFS1 control or deficient cells is shown in (E). Columns represent the mean ± SEM of at least three experiments conducted in duplicate. **p<0.01, Student’s t-test.

Figure 2. Effects of PARP-1 inhibitors on mitochondrial homeostasis of NDUFS1 mutant fibroblasts. Effects of a 7 day incubation of NDUFS1 mutant fibroblasts to PHE (30 µM) or PJ34 (20 µM) on ATP (A) and NAD (B). The effects of an exposure to PHE (µM) and PJ34 (µM) on mitochondrial membrane potential (C, D), mtDNA content (E) and transcript levels of nDNA-encoded (COX15, NDUFV2, ATP5D) and mtDNA-encoded (COX1, COX2, ND2) respiratory complex subunits (F, G) is shown. (H) Effects of 7 day incubation to PHE (30 µM) and PJ34 (20 µM) on transcript levels of TFB1M, TFB2M, SIRT1, TFAM, PGC1α, PGC1β, NRF1, NRF2 and PPCR1. Phase contrast microscopy visualization (I) and quantitation (J) of MTT reduction activity of NDUFS1 fibroblasts under control conditions or after a 7 day incubation with PHE or PJ34. Columns represent the mean ± SEM of at least three experiments conducted in duplicate. *p<0.05, **p<0.01, ***p<0.001 ANOVA and Tukey’s post hoc test. In (I) an experiment representative of 4 is shown. Bar=10 µm.

Figure 3. Effects of NR on mitochondrial homeostasis of NDUFS1 mutant fibroblasts. Effects of a 2 and 7 day incubation of NDUFS1 mutant fibroblasts to NR (0.1-10 mM) on ATP (A), NAD (B), mitochondrial membrane potential (C, D) and mtDNA content (E). The effects of a 2 or 7 day exposure to NR on transcript levels of nDNA-encoded (COX15, NDUFV2, ATP5D) and mtDNA-encoded (COX1, COX2, ND2) respiratory complex subunits is shown in (F). Phase contrast microscopy visualization (G) and quantitation (H) of MTT reduction activity of NDUFS1 fibroblasts under control conditions or after a 2 or 7 day incubation with NR (0.1-10 mM). (I) Effects of 7 day
incubation to NR 1 mM on transcript levels of TFB1M, TFB2M, SIRT1, TFAM PGC1α, PGC1β, NRF-1, NRF-2 and PPCR1. Columns represent the mean ± SEM of at least three experiments conducted in duplicate. *p<0.05, **p<0.01, ***p<0.001 ANOVA and Tukey’s post hoc test. In (G) an experiment representative of 3 is shown. Bar=10 µm.
Figure 2
Figure 3