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Complementation of mitochondrial electron transport chain by manipulation of the NAD⁺/NADH ratio

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Abstract

A decline in electron transport chain (ETC) activity is associated with many human diseases. Although diminished mitochondrial ATP production is recognized as a source of pathology, the contribution of the associated reduction in the ratio of the amount of oxidized nicotinamide adenine dinucleotide (NAD⁺) to that of its reduced form (NADH) is less clear. We used a water-forming NADH oxidase from *L. brevis* (*LbNOX*) as a genetic tool for inducing a compartment-specific increase of the NAD⁺/NADH ratio in human cells. We used *LbNOX* to demonstrate the dependence of key metabolic fluxes, gluconeogenesis, and signaling on the cytosolic or mitochondrial NAD⁺/NADH ratios. Expression of *LbNOX* in the cytosol or mitochondria ameliorated proliferative and metabolic defects caused by an impaired ETC. The results underscore the role of reductive stress in mitochondrial pathogenesis and demonstrate the utility of targeted *LbNOX* for direct, compartment-specific manipulation of redox state.

One Sentence Summary

We developed a genetically encoded tool for raising NAD⁺/NADH ratios and showed it can complement an impaired electron transport chain in human cells.

A decline in electron transport chain (ETC) activity has been linked to numerous human disorders, ranging from rare genetic syndromes to common diseases such as neurodegeneration, cancer, and diabetes, as well as the aging process itself (1, 2). How a decline in ETC activity gives rise to the spectrum of observed pathology cannot be readily explained by a simple deficiency in adenosine triphosphate (ATP) production (1). A key

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challenge in deciphering mitochondrial pathogenesis stems from the fact that the ETC performs at least two coupled functions: redox transfer of electrons from NADH [the reduced form of nicotinamide adenine dinucleotide (NAD⁺)] to oxygen and a simultaneous conversion of the free energy of the electromotive force into a proton gradient across the mitochondrial inner membrane. In principle, pathology could stem from an excess of reducing equivalents (termed reductive stress or pseudohypoxia, which includes stalling of NAD⁺-coupled reactions) or a reduced proton gradient (impairing pH and voltage-coupled processes, including ATP synthesis by the F₁F₀-ATPase). Currently there are no methods for dissecting the redox function of the ETC from its proton pumping function.

Here, we report the application of a genetically encoded tool for compartment-specific manipulation of the NAD⁺/NADH ratio. Our tool is based on the flavin adenine dinucleotide (FAD)-dependent H₂O-forming NADH oxidases, which catalyze the four-electron reduction of O₂ to two molecules of H₂O (Fig. 1A). We focused on bacterial oxidases with specificity for NADH over NADPH (3–7), whose natural function is protection of redox balance and defense against oxygen toxicity (8). Such oxidases have been successfully expressed in bacteria and yeast for biotechnological applications (9–11). We screened several H₂O-forming NADH oxidases by expressing their human codon-optimized, epitope-tagged versions in cultured human cancer-derived epithelial (HeLa) cells. The enzyme from *L. brevis* (*LbNOX*) was most highly expressed and had the highest oxidase activity when targeted to mitochondria (fig. S1).

We evaluated the biochemical properties of *LbNOX* modified to contain a C-terminal FLAG tag and a cleavable N-terminal hexahistidine tag, and overexpressed in *E. coli*. Purified *LbNOX*-FLAG has a yellow color in solution and a characteristic UV-visible absorption spectrum ($\lambda_{\max} = 371$ and 444 nm) consistent with the presence of FAD, which can be reduced upon the addition of sodium dithionite (Fig. 1B). Our recombinant enzyme consumes oxygen and is strictly specific for NADH rather than NADPH with K_M for NADH of $69 \pm 3 \mu\text{M}$, V_{\max} of $758 \pm 33 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and k_{cat} of $648 \pm 28 \text{s}^{-1}$ which is more active than previously reported (3, 12) (Fig. 1C and D). The molecular size of *LbNOX*-FLAG was determined to be $197 \pm 4 \text{kD}$, which indicates that the protein is a tetramer in solution. Although enzymes in this family often produce H₂O₂, *LbNOX*-FLAG produces amounts of H₂O₂ that constitute only 1 to 2 % of the amount of H₂O produced during its catalytic cycle (fig. S2A) (4, 6, 7). The apparent K_M for O₂ of *LbNOX*-FLAG was below 2 μM ($\sim 0.17 \%$ O₂), as estimated from enzyme-monitored turnover experiments (fig. S2B), which is less than one-tenth of the concentration of oxygen in human venous blood (13). Thus, we expect *LbNOX* to be active in most animal tissues. The enzymatic properties of *LbNOX*-FLAG in solution were well founded in the 2.4 Å resolution X-ray structure of this protein that we determined (Fig. 1E, Table S1). Our structure is generally similar to the reported structures of H₂O-forming NAD(P)H oxidases from *L. sanfranciscensis* (PDB ID 2CDU) and *S. pyogenes* (PDB ID 2BC0) (14, 15). However, our structure captures *LbNOX* in a new state with molecular oxygen (O₂) bound and the redox active Cys 42 in a reduced form (Cys 42-SH) (fig. S3, see Supplementary Materials for a detailed discussion of the X-ray structure). In conclusion, the high selectivity for NADH over NADPH, negligible H₂O₂ production, and very low K_M for O₂ made *LbNOX* attractive for additional studies in human cells.

To determine if we could express *LbNOX*-FLAG safely and efficaciously in various compartments of human cells we used lentiviral infection to generate HeLa cells that expressed untargeted or mitochondria-targeted human codon-optimized *LbNOX*-FLAG (referred to as *LbNOX* and *mitoLbNOX* henceforth) under the control of a doxycycline-inducible promoter (TRE3G) (Fig. 2A, fig. S1A). We used fluorescence microscopy and cell fractionation to confirm diffuse localization of *LbNOX* and mitochondrial localization of *mitoLbNOX* (Fig. 2B and C). Cells appeared grossly normal without any impact on cell proliferation or reactive oxygen species (ROS) production (fig. S4A, B). Expression of *LbNOX* and *mitoLbNOX* in HeLa cells increased oxygen consumption by 1.6- and 2.4-fold, respectively (Fig. 2D, fig. S4C). The increase was resistant to ETC inhibitors, which indicates that it resulted from *LbNOX* oxidase activity and not from the increased ETC activity. Despite similar expression levels (Fig. 2A), *mitoLbNOX* induced a larger increase in oxygen consumption than *LbNOX* (Fig. 2D), likely because of the higher concentration of NADH within mitochondria (16–18). It is important to remember that in converting NADH to NAD⁺, *LbNOX* also consumes protons and oxygen and, therefore, could affect cellular pH or oxygen levels depending on experimental context.

We determined the impact of expressing *LbNOX* or *mitoLbNOX* on cellular concentrations of NAD⁺ and NADH (Fig. 3 and fig. S5). We used a genetic sensor, SoNar (19), to measure cytosolic NADH. SoNar is a fusion of circularly permuted Yellow Fluorescent Protein and a modified NADH binding protein Rex from *Thermus aquaticus*. Binding of NADH to SoNar leads to an increase in fluorescence. Expression of *LbNOX* or *mitoLbNOX* decreased the fluorescence signal from SoNar indicating that both *LbNOX* and *mitoLbNOX* decrease cytosolic NADH (Fig. 3A, fig. S5B). Consistent with this result, intracellular and secreted lactate/pyruvate ratios, traditionally used proxies for the cytosolic NADH/NAD⁺ ratio (17), decreased in cells expressing *LbNOX* or *mitoLbNOX* (Fig. 3B, fig. S5D). The ratio of total cellular NAD⁺ to total NADH, based on HPLC measurements, was increased 2-fold by *mitoLbNOX*, whereas *LbNOX* did not have a significant effect (Fig. 3C, fig. S5C). Perturbation of the total NAD⁺/NADH ratio likely reflects changes in amounts of mitochondrial NADH because most of the effect on the ratio resulted from changes in NADH concentration (fig. S5A), and most of the NADH inside the cell is present in mitochondria. The latter is supported by fractionation experiments (20) and by the observation that the majority of NAD(P)H autofluorescence in cells comes from mitochondria (21). In summary, *LbNOX* and *mitoLbNOX* can be used to perturb the NAD⁺/NADH ratio and our compartment-specific measurements of HeLa cells (Fig. 3A–C) indicate that although perturbation of the mitochondrial NAD⁺/NADH ratio leads to changes in the cytosolic NAD⁺/NADH ratio, the converse is not true.

We performed metabolic profiling on medium in which cells expressing *LbNOX* or *mitoLbNOX* had been grown (Fig. 3D and fig. S6A–B). We identified pyruvate, aspartate and succinate as three metabolites whose consumption or release was changed more than two-fold (Student's *t*-test; *P* < 0.01) by either enzyme. These changes are attributable to compartment-specific changes of NAD⁺/NADH by *LbNOX* or *mitoLbNOX* (see Supplementary Materials for discussion). It is notable that *LbNOX* and *mitoLbNOX* did not have a significant effect on the uptake of glucose and release of lactate (fig. S6C).

In vitro phosphorylation of mitochondrial pyruvate dehydrogenase (PDH) is regulated by the NAD^+/NADH ratio (22), but this has never been shown in intact cells. Treatment of HeLa cells with dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinases (PDKs), inhibits phosphorylation of PDH, and antimycin treatment, which blocks the ETC and decreases the mitochondrial NAD^+/NADH ratio, increases PDH phosphorylation. In agreement with *in vitro* studies, PDH was almost completely dephosphorylated in the presence of mito*Lb*NOX but not *Lb*NOX (Fig. 3E and fig. S6D). The data on PDH phosphorylation are consistent with our observation that mito*Lb*NOX, but not *Lb*NOX, increases mitochondrial NAD^+/NADH ratio in HeLa cells (Fig. 3C).

We expressed *Lb*NOX and mito*Lb*NOX in primary rat hepatocytes to study gluconeogenesis. The cytosolic NAD^+/NADH ratio has been reported to affect gluconeogenesis, though classical approaches relied on indirect methods for manipulating the redox state (23, 24). In our hepatocyte system, rates of gluconeogenesis were significantly higher if pyruvate rather than lactate was used as a substrate, which we attribute to the NAD^+/NADH ratio-dependent inhibition of lactate to pyruvate conversion (23). Consistent with this hypothesis rates of gluconeogenesis from lactate were increased to those seen with pyruvate when primary hepatocytes expressed *Lb*NOX, whereas gluconeogenesis from pyruvate was not affected (Fig. 3F). Gluconeogenesis from lactate was also increased by mito*Lb*NOX. Gluconeogenesis from pyruvate, however, was inhibited by mito*Lb*NOX, perhaps because strong oxidation of mitochondrial NADH prevents formation of malate (25). In assays of gluconeogenesis from lactate expression of either *Lb*NOX or mito*Lb*NOX decreased the ratio of secreted β -hydroxybutyrate/acetoacetate (Fig. 3G), the classical proxy for the mitochondrial NADH/NAD^+ ratio (17). Although the NAD^+/NADH ratio appears to be increased we cannot exclude the possibility that *Lb*NOX or mito*Lb*NOX induce hypoxia. *Lb*NOX did increase mitochondrial NAD^+/NADH ratio in rat hepatocytes (Fig. 3G) but not in HeLa cells (Fig. 3C), which might reflect differences in cell type or media conditions.

Mammalian cells lacking a functional ETC require the addition of exogenous pyruvate and uridine for cell proliferation (26–28). Uridine is required because one of the enzymes in *de novo* uridine biosynthesis (dihydroorotate dehydrogenase) is coupled to the ETC through coenzyme Q (CoQ). The requirement of pyruvate, however, has been less clear because it participates in many reactions but has been proposed to rescue cell growth by recycling NAD^+ from NADH through cytosolic lactate dehydrogenase (26, 29). If the NAD^+ recycling hypothesis is correct then supplementation with oxaloacetate should have the same effect as pyruvate because it can be reduced by malate dehydrogenase while recycling NAD^+ . Oxaloacetate, like pyruvate, rescued the proliferation defect induced by piericidin whereas malate and lactate did not (Fig. 4A). Alpha-ketobutyrate also rescues the proliferative defect induced by ETC inhibition (30). Furthermore, a large number of α -keto acids can rescue pyruvate dependence of proliferation in cells with intact ETC (31). These findings support the NAD^+ recycling hypothesis, though they are still indirect as α -keto acids have many metabolic roles.

We used *Lb*NOX to directly test whether NAD^+ recycling is an essential function of the ETC that is required for cell proliferation. We inhibited ETC function, with piericidin (a complex

I inhibitor), antimycin (a complex III inhibitor), ethidium bromide (a mtDNA replication inhibitor) and chloramphenicol (an inhibitor of mitochondrial translation) in HeLa cells supplemented with uridine but lacking pyruvate. HeLa cells cannot proliferate in these conditions (Fig. 4B and fig. S7). Expression of either *LbNOX* or *mitoLbNOX* rescued cell proliferation in the presence of these ETC inhibitors indicating that regeneration of NAD^+ in either cytosol or mitochondria is sufficient to complement ETC activity that is required for cell proliferation (Fig. 4B). Metabolic profiling showed that of the nine metabolites whose uptake or release is affected greater than two-fold by antimycin (Student's *t*-test; $P < 0.01$), all could be reversed by either *LbNOX* or *mitoLbNOX*, reflecting a metabolic rescue (fig. S8, see Supplementary Materials for discussion). Our metabolic profiling data is complementary to recent studies demonstrating an inhibition of aspartate biosynthesis in cells with dysfunctional ETC (30, 32, 33). As a control, we also showed that the rescue by *LbNOX* or *mitoLbNOX* was not attributable to an effect on mitochondrial membrane potential (fig. S9A–C), nor was it due to a rescue of ETC-derived ATP synthesis (fig. S9D–G).

Collectively, these studies (Fig. 4, fig. S7–9) underscore the importance of NAD^+ recycling by the ETC to support proliferation. In healthy cells, the ETC produces ATP and simultaneously recycles mitochondrial NADH to NAD^+ , with a secondary oxidation of cytosolic NADH via shuttles. In the absence of a functional ETC, glycolysis is capable of compensating for the lack of ATP production, but it is net redox neutral. NAD^+ recycling is likely key for cell proliferation because many biosynthetic pathways produce NADH as a byproduct (34). These insights confirm the longstanding hypothesis (26, 29) that pyruvate supplementation rescues proliferation in cells with disrupted ETC by restoring NAD^+ /NADH balance via the LDH reaction.

In the future, *LbNOX* and engineered or naturally occurring variants may become valuable tools for studying compartmentalization of redox metabolism. These constructs will allow for a dissection of the relative contributions of redox imbalance and ATP insufficiency to mitochondrial disease pathogenesis. If a substantial amount of the organ pathology of mitochondrial disease stems from reductive stress or pseudohypoxia, then expression of this single polypeptide holds promise as a “protein prosthesis” for the large number of disorders characterized by ETC dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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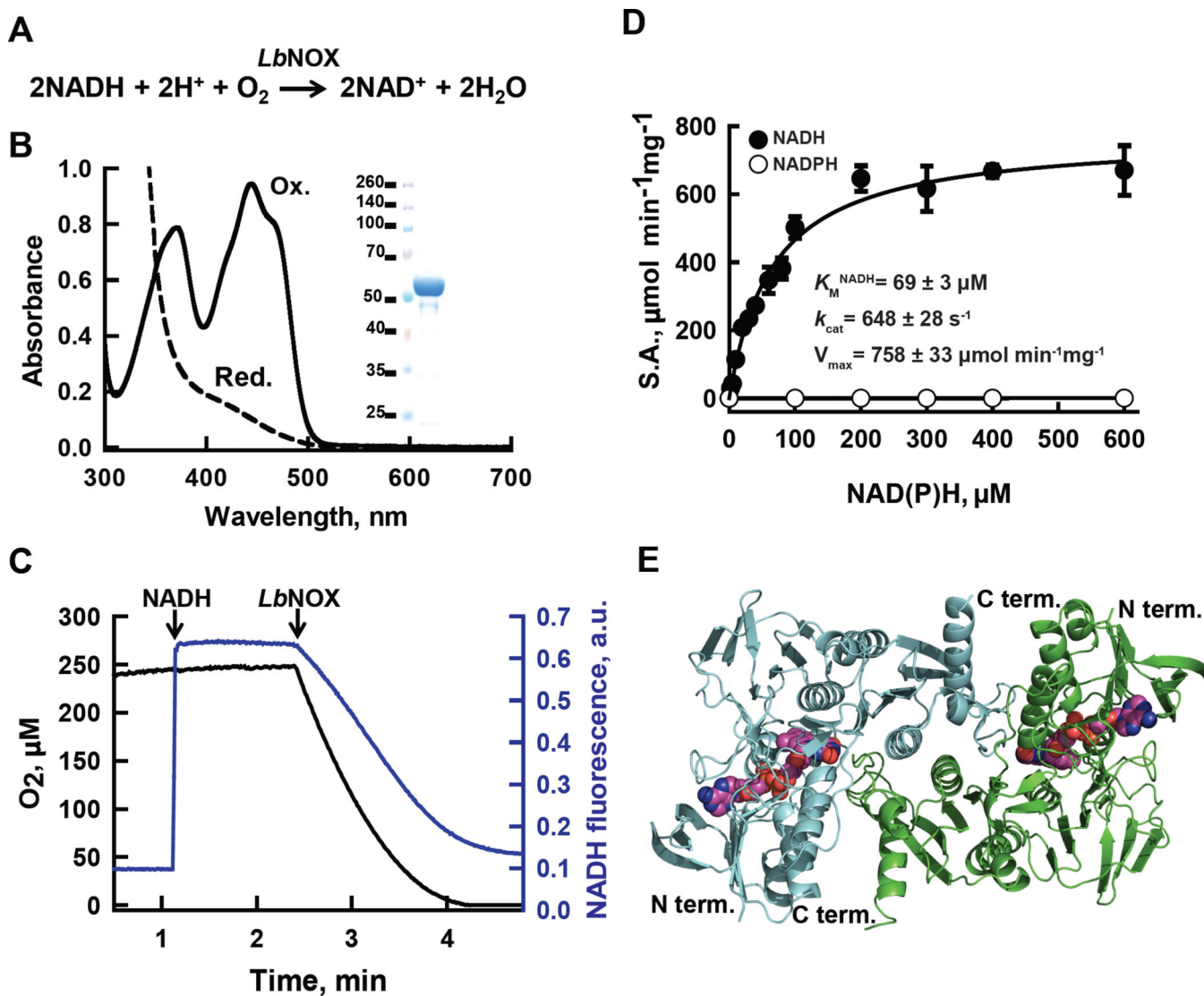


Figure 1. H₂O-forming NADH oxidase from *L. brevis* (*LbNOX*)

(A) Reaction catalyzed by *LbNOX*. (B) UV-visible spectrum of purified *LbNOX*. Protein (83 μM FAD active sites) in oxidized form (solid line) and after addition of excess of sodium dithionite, reduced form (dashed line). *Inset*: SDS-PAGE of purified *LbNOX*. (C) Simultaneous measurement of NADH and oxygen consumption by *LbNOX*. NADH and *LbNOX* were added as indicated by arrows. (D) Dependence of the specific activity of recombinant *LbNOX* on the concentration of NADH and NADPH. Reported values for V_{\max} , k_{cat} and K_M for NADH represent the mean \pm S.D. from $n=4$ independent experiments. (E) Crystal structure of the catalytic dimer of *LbNOX*. Each of the two-fold symmetry related monomers (cyan and green ribbons) contain bound FAD, shown here in sphere (CPK) representation. Details of the catalytic center on the *si*-face of FAD and of the substrate selectivity loop are shown in fig. S3A–C.

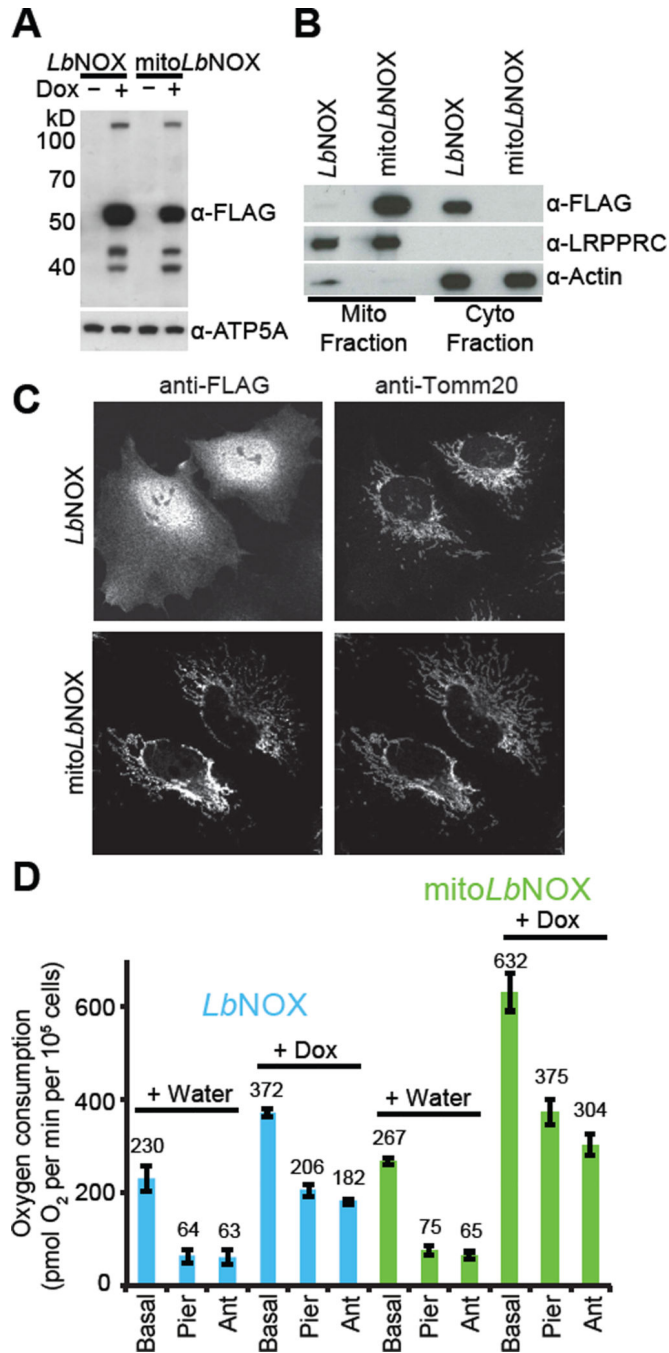


Figure 2. Expression and activity of *LbNOX* in human cells

(A) Western blot of *LbNOX* and *mitoLbNOX* in HeLa cells after 24-hour induction with water or doxycycline (300 ng/ml). Representative gel from one of three independent experiments. (B) Subcellular localization of *LbNOX* and *mitoLbNOX* in HeLa cells determined by cell fractionation. LRPPRC is a mitochondrial marker and Actin is a cytosolic marker. Representative gel from one of three independent experiments. (C) Subcellular localization of *LbNOX* and *mitoLbNOX* in HeLa cells determined using fluorescence microscopy. Tomm20 is a marker of mitochondria. (D) Effect of *LbNOX* and *mitoLbNOX*

expression in HeLa cells on basal, piericidin-resistant and antimycin-resistant oxygen consumption measured with a XF24 extracellular flux analyzer. Mean \pm S.E., $n=3$ independent experiments.

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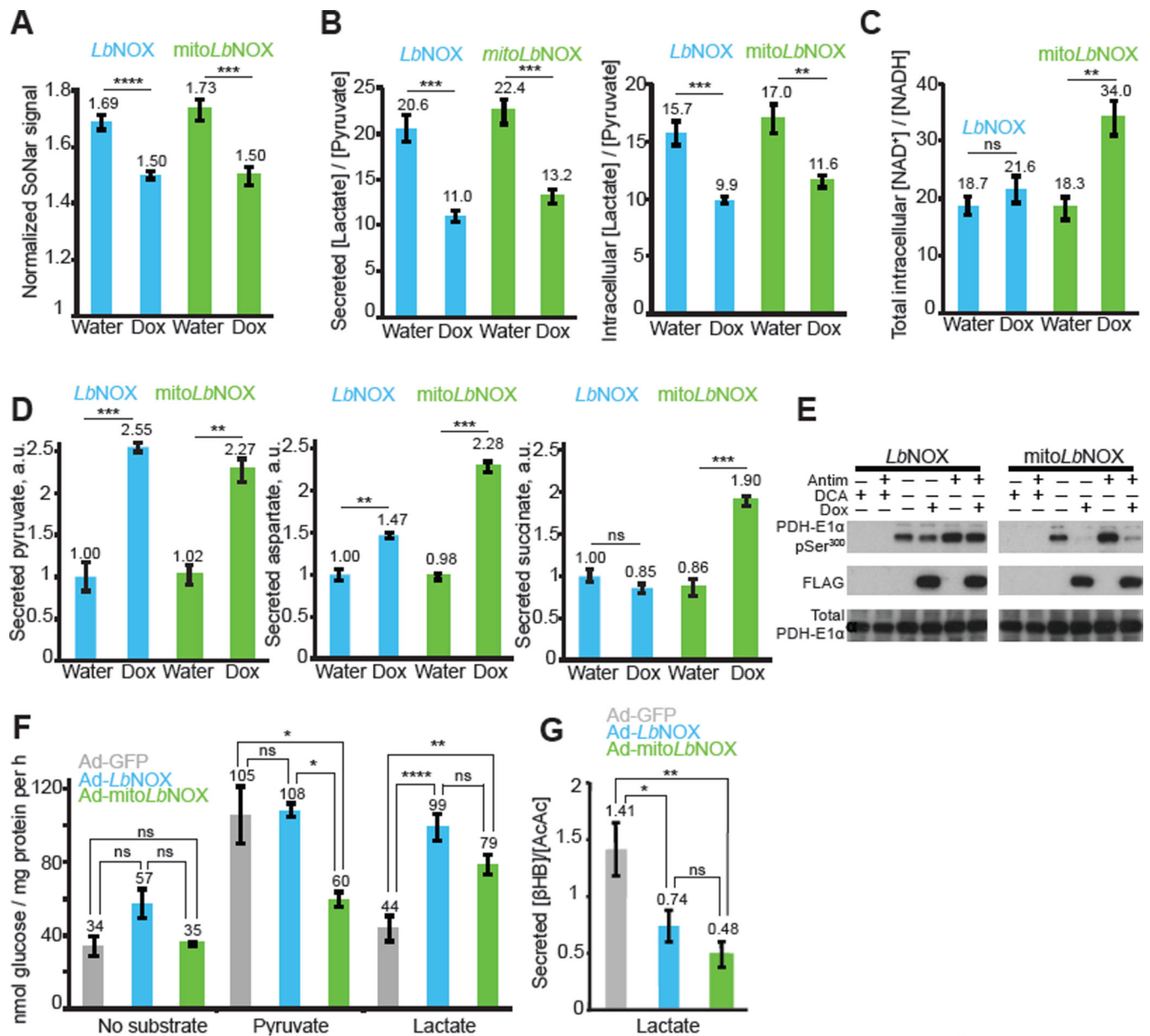


Figure 3. Effect of *LbNOX* and *mitoLbNOX* on NAD⁺/NADH ratios, metabolic fluxes, PDH phosphorylation and gluconeogenesis

(A–C) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on (A) cytoplasmic NADH concentrations determined with fluorescence microscopy using SoNar expressing cells ($n=7$), (B) intracellular and secreted lactate/pyruvate ratio determined by LC-MS ($n=4$), and (C) intracellular NAD⁺/NADH ratios determined by HPLC ($n=4$). Student's *t*-test. ns $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Mean \pm S.E. (D) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on release rate of pyruvate, aspartate and succinate, determined by comparing concentrations in spent versus fresh media. Student's *t*-test. ns $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$. Mean \pm S.E., $n=3$ replicates from one experiment. (E) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on PDH phosphorylation. Representative gel from one of three independent experiments. (F) Effect of adenoviral

transduction of GFP, *LbNOX* or *mitoLbNOX* on primary rat hepatocyte gluconeogenesis in DMEM containing no glucose, no glutamine and no pyruvate using either no substrate, 5mM pyruvate, or 5mM lactate. One-way ANOVA followed by Tukey's multiple comparisons test. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. Mean \pm S.E., $n=3$ (no substrate, pyruvate) or $n=7$ (lactate) independent experiments. **(G)** Effect of *LbNOX* and *mitoLbNOX* on secreted β -hydroxybutyrate/acetoacetate ratio in rat hepatocytes performing gluconeogenesis from lactate as a substrate. Metabolite levels determined using LC-MS. One-way ANOVA followed by Tukey's multiple comparisons test. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$. Mean \pm S.E., $n=10$ independent experiments.

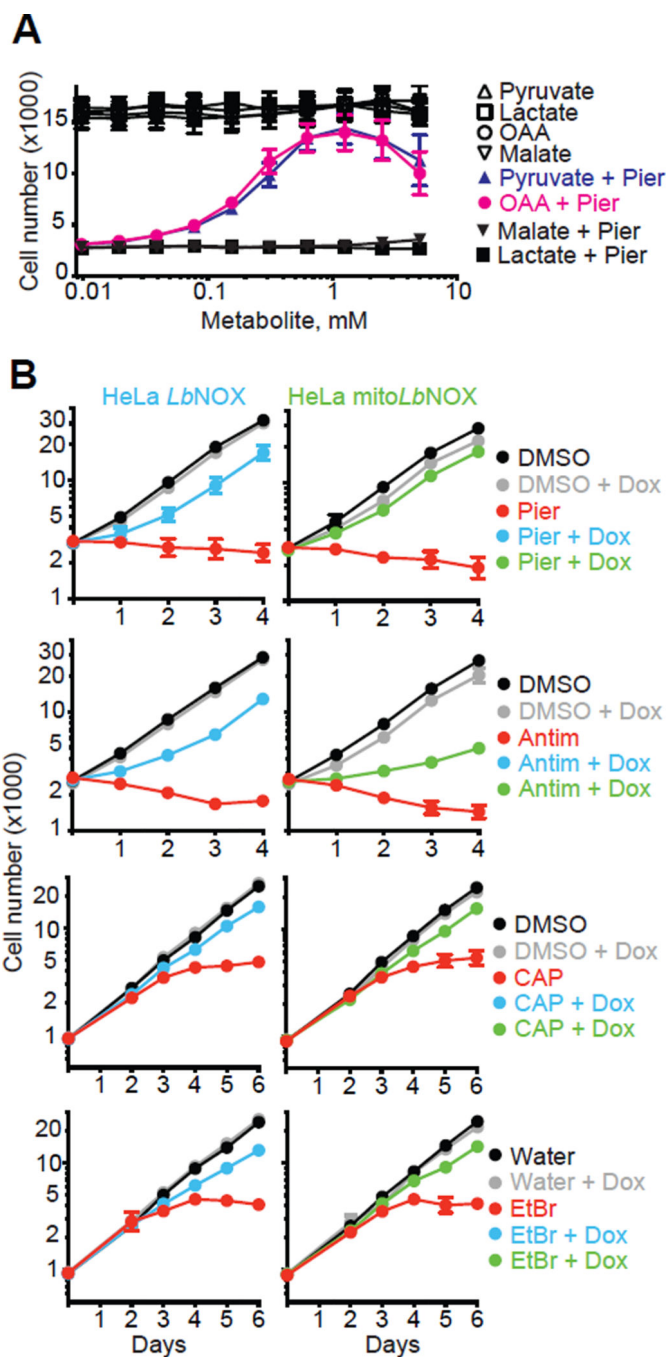


Figure 4. NAD⁺ recycling rescues proliferation in cells with impaired ETC

(A) Effect of pyruvate, oxaloacetate, lactate and malate addition on proliferation of HeLa Tet3G Luciferase cells in the presence of 200 μ M uridine and in the presence or absence of 1 μ M piericidin. Mean \pm S.E., $n=5$ independent experiments. (B) Effect of *LbNOX* and *mitoLbNOX* expression in HeLa cells on inhibition of cell proliferation by 1 μ M piericidin, 1 μ M antimycin, 10 μ g/ml chloramphenicol and 30 ng/ml ethidium bromide in the presence of 200 μ M uridine. Mean \pm S.E., $n=3$ independent experiments.