Supplementary Materials for

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

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Materials and Methods
Supplementary Text
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Other Supplementary Materials for this manuscript includes the following:

Table S2

Materials and Methods

Chemical reagents

Oligomycin A, antimycin A, doxycycline, FAD, NAD^+, NADH, $NADP^+$, NADPH, AMP, ADP, ATP, malate, sodium dithionite and oxaloacetate were purchased from Sigma. Piericidin A was purchased from Santa Cruz Biotechnlogy. Hoechst 33345 was purchased from Life Technologies.

Nucleotide sequences of constructs used in the study

Sequences of mitoNOXes

Below are *H. sapiens* codon optimized sequences of NOXes with added MTS and a C-terminal linker containing a FLAG-tag. For clarity corresponding regions of each sequence are highlighted in different colors: NotI, MTS, $(GGS)_3M$ linker, FLAG tag, Stop codon, MluI.

Lactococcus lactis:

GCGGCCGCATGCTGGCCACCAGGGTCTTTAGCCTGGTGGGCAAAAGAGC
CATCTCCACATCCGTCTGCGTGAGAGCCCATAAGATTGTCGTCATCGGCACCA
ATCACGCCGGCATTGCTACCGCCAACACACTCCTGGAGCAGTACCCCGGCCA
TGAAATTGTCATGATTGATAGAAACTCCAATATGAGCTACCTGGGCTGTGGC
ACCGCTATCTGGGTGGGCAGGCAGATCGAGAAACCCGACGAGCTGTTTTACG
CCAAGGCCGAAGACTTTGAGGCCAAGGGCGTGAAGATCCTCACCGAGACCG
AAGTCTCCGAGATTGACTTCGCCAACAAGAAGGTCTATGCCAAGACCAAGAG
CGATGACGAGATCATCGAGGCCTACGATAAACTCGTGCTCGCCACAGGCAGC
AGACCTATCATCCCCAACCTCCCCGGAAAGGACCTGAAGGGAATCCACTTCC
TCAAGCTGTTCCAGGAAGGACAGGCCATCGACGCCGAGTTCGCCAAGGAGA
AGGTCAAGAGGATTGCTGTGATCGGCGCCGGCTATATCGGCACCGAGATCGC
CGAGGCCGCTAAAAGGAGGGGCAAGGAAGTCCTGCTGTTCGACGCCGAAAA
CACCTCCCTGGCCAGCTACTACGACGAAGAGTTTGCCAAAGGCATGGACGAG
AACCTCGCCCAACACGGAATTGAACTCCACTTCGGCGAGCTGGCCAAAGAGT
TCAAGGCCAACGAGGAGGGCTACGTGTCCCAGATCGTGACCAACAAGGCCA
CATACGATGTCGACCTGGTGATCAACTGCATCGGCTTTACAGCCAACTCCGCT
CTGGCCTCCGACAAGCTGGCCACCTTCAAGAACGGCGCCATCAAAGTGGACA
AGCACCAGCAGAGCTCCGACCCCGATGTCTATGCTGTGGGCGACGTGGCCAC
CATCTACAGCAACGCCCTCCAGGACTTCACATATATCGCCCTGGCCAGCAAC
GCTGTGAGATCCGGCATCGTCGCTGGCCATAATATCGGAGGCAAGGAGCTGG
AGAGCGTCGGAGTCCAGGGCAGCAACGGCATCTCCATCTTCGGCTATAACAT
GACCAGCACAGGCCTGTCCGTGAAGGCCGCCAAGAAACTCGGCCTGGAAGT
GAGCTTCAGCGACTTTGAGGACAAGCAGAAGGCCTGGTTCCTGCACGAGAAC
AACGATAGCGTGAAGATCAGAATCGTGTATGAGACAAAAAGCAGAAGGATC
ATCGGCGCCCAGCTGGCCTCCAAGTCCGAAATCATCGCTGGCAACATCAACA
TGTTTAGCCTGGCCATCCAGGAGAAGAAAACCATCGACGAGCTCGCCCTGCT
GGATCTCTTCTTCCTGCCCCACTTCAACAGCCCCTACAACTACATGACCGTGG
CCGCTCTCAACGCTAAAGGTGGATCTGGTGGATCTGGTGGATCTATGGATTAC
AAGGATGACGATGACAAGTAAACGCGT

Lactobacillus brevis:

GCGGCCGCATGCTCGCTACAAGGGTCTTTAGCCTCGTCGGAAAGAGAGC
TATCAGCACCTCCGTCTGCGTGAGAGCTCATAAGGTCACCGTGGTCGGATGC
ACCCATGCCGGCACCTTCGCCATCAAGCAAATCCTCGCTGAGCACCCTGACG
CCGAGGTCACCGTCTACGAGAGGAACGATGTGATCTCCTTCCTGTCCTGTGGC
ATCGCCCTCTACCTGGGCGGAAAAGTGGCCGATCCCCAAGGCCTCTTCTACA
GCTCCCCTGAAGAACTGCAGAAGCTGGGCGCTAATGTGCAGATGAACCACAA
CGTGCTGGCCATCGACCCTGACCAAAAGACCGTCACAGTCGAGGACCTCACC
AATCACGCCCAGACCACCGAGTCCTACGACAAACTGGTGATGACCTCCGGAA
GCTGGCCTATCGTGCCCAAAATCCCCGGCATCGACAGCGATAGGGTGAAGCT
CTGCAAGAATTGGGCCCACGCCCAGGCTCTGATTGAGGACGCCAAGGAGGCC
AAGAGGATCACCGTCATCGGCGCCGGATACATCGGAGCCGAACTGGCCGAG
GCCTACTCCACAACAGGCCACGACGTCACCCTGATTGACGCCATGGCTAGGG
TCATGCCCAAGTACTTCGATGCCGACTTCACCGACGTCATCGAACAGGACTA
CAGGGACCATGGCGTGCAACTCGCTCTGGGCGAGACAGTGGAGAGCTTCACC
GACAGCGCCACCGGCCTCACAATCAAGACAGACAAGAACTCCTATGAGACC
GACCTGGCCATCCTCTGCATTGGCTTTAGGCCCAACACAGACCTGCTGAAAG
GCAAAGTGGACATGGCCCCTAACGGCGCCATCATTACCGACGACTACATGAG
GTCCAGCAACCCTGATATTTTCGCTGCTGGCGACTCCGCCGCCGTCCATTACA
ACCCCACACACCAAAACGCCTACATTCCCCTCGCTACCAACGCCGTCAGGCA
GGGAATCCTCGTCGGAAAGAACCTCGTCAAGCCCACAGTGAAGTACATGGGA
ACCCAGTCCAGCTCCGGACTGGCCCTCTATGACAGGACAATTGTCTCCACAG
GCCTCACACTGGCCGCCGCCAAGCAACAAGGCCTCAATGCCGAGCAGGTCAT
CGTGGAGGACAACTATAGGCCCGAGTTCATGCCTTCCACCGAGCCCGTCCTC
ATGAGCCTGGTCTTCGACCCCGATACACACAGAATCCTGGGAGGCGCCCTGA
TGTCCAAATACGACGTGTCCCAGAGCGCTAATACCCTGTCCGTCTGCATCCAG
AACGAGAACACCATCGATGACCTGGCCATGGTGGACATGCTGTTCCAGCCCA
ATTTCGACAGGCCCTTCAACTACCTGAACATTCTCGCCCAGGCTGCCCAAGCT
AAAGTGGCCCAATCCGTCAACGCTGGTGGATCTGGTGGATCTGGTGGATCTA
TGGATTACAAGGATGACGATGACAAGTAAACGCGT

Serpulina hyodysenteriae:

GCGGCCGCATGCTGGCCACAAGAGTGTTCTCCCTCGTCGGCAAAAGAGC
CATCAGCACAAGCGTCTGCGTGAGGGCCCACAAAGTGATCGTGATCGGCTGC
AACCACGCTGGAACATGGGCCGCTAAAACCCTGAAGGCCACAGACCCCAACT
GTCAGGTGGTGACCTACGACAGGAACGACAACATCTCCTTCCTCGCCTGTGG
AATCGCTCTGTGGGTCGGCGGCGTGGTGAAGGACCCTAAAGGCCTCTTTTAT
GCCAGCCCTGAGTCCCTGAGGGGAGAGGGAATTGACGTCTATATGGGCCACG
ACGTGACCAAGATCGACTGGGCCAACAAGAAGCTCTGCGTGAAGGAGCTCA
AGACCGGCAAGGAGTTCGAGGACACCTATGACAAGCTGATCCTGGCTACAGG
ATCCTGGCCCGTGACCCCTCCCATCGAAGGACTGAAGCAGGAGGGCACCACC
TACGGACTGAAGAAGGGAATCTTCTTTAGCAAGCTGTACCAACAGGGCCAGG
AGATTATCGACGAGATCGCCAAGCCTGACGTGAAGAAGGTGATGGTGGTGGG
AGCCGGATATATCGGAGTCGAGCTCATCGAGGCCTTCAAGAACCATGGCAAG

GAGGTGATTCTGATGGAGGCCATGCCTAGGGTGATGGCCAATTACTTCGACA
AGGAGATCACCGACGAGGCCGAAAAAAGGATCAAGGAGGCCGGCATCGAGA
TGCATCTGGGCGAGACAGTGAAGAAGTTCGAAGGCGACGACAGAGTGAAGA
AGGTCGTCACCGACAAGGGCTCCTATGACGTGGACATGGTCGTCATGAGCGT
CGGCTTCAGGCCTAACAACGAGCTGTATAAAGACTATCTGGAGACCCTGCCC
AATGGCGCCATCGTCGTCGATACCACCATGAAGACCACCAAGGATCCCGACG
TGTTCGCCATTGGAGATTGCGCCACCGTGTATAGCAGGGCCTCCGAGAAGCA
AGAATACATCGCCCTGAGCACCAACGCTGTGAGGATGGGCATTGTGGCTGCT
AACAACGCCCTGGGAAAGCACGTCGAGTACTGTGGCACCCAAGGCTCCAACG
CTATCTGCGTCTTCGGCTACAACATGGCCTCCACAGGCTGGAGCGAAGAGAC
AGCCAAGAAGAAGGGACTCAAGGTGAAGTCCAACTTCTTCAAGGACAGCGA
GAGGCCTGAGTTCATGCCCACAAATGAGGACGTCCTCGTCAAGATCATCTAC
GAAGAGGGCTCCAGGAGGCTGCTGGGAGCTCAGATTGCCAGCAAGCACAAC
CACGCCGAAGCTATTCACGCCTTCAGCCTCGCCATCCAGAACGGAATGACCG
TGGATCAGTTCGCCCTGTCCGACTTCTTCTTCCTGCCCCACTACAATAAGCCC
CTGTCCTGGATGACAATGGTGGCCTACACAGCTAAAGGTGGATCTGGTGGAT
CTGGTGGATCTATGGATTACAAGGATGACGATGACAAGTAAACGCGT

Streptococcus mutans:

GCGGCCGCATGCTGGCCACCAGAGTGTTTAGCCTGGTCGGAAAGAGGGC
CATCAGCACCAGCGTCTGCGTCAGGGCCCATAGCAAGATCGTGATCGTCGGA
GCTAACCACGCTGGCACCGCTGCCATCAATACCGTCCTCGACAACTACGGCA
GCGAGAACGAAGTCGTGGTGTTTGACCAGAACTCCAACATCTCCTTCCTCGG
CTGTGGAATGGCTCTGTGGATTGGCAAGCAGATCAGCGGCCCCCAGGGACTC
TTCTACGCCGACAAGGAATCCCTGGAGGCTAAGGGCGCCAAAATCTATATGG
AGTCCCCCGTCACAGCTATTGACTACGACGCCAAGAGGGTCACAGCCCTGGT
GAACGGCCAAGAGCATGTGGAGAGCTACGAAAAGCTGATCCTCGCCACCGG
CAGCACACCTATTCTGCCCCCCATTAAAGGCGCCGCTATCAAGGAGGGCTCC
AGGGACTTCGAAGCTACCCTGAAGAACCTGCAGTTCGTGAAGCTCTACCAGA
ACGCCGAGGACGTGATCAATAAGCTGCAGGACAAGACCCAGAACCTGAATA
GGATCGCCGTGGTCGGCGCTGGCTACATCGGAGTCGAGCTGGCTGAGGCCTT
TAAGAGACTGGGCAAGGAGGTCATCCTCATCGATGTCGTGGACACATGCCTG
GCCGGCTACTACGATCAGGACCTCTCCGAGATGATGAGACAGAACCTGGAGG
ACCACGGCATCGAGCTCGCCTTCGGCGAAACCGTGAAAGCCATTGAGGGCGA
CGGAAAGGTCGAGAGGATCGTCACCGATAAAGCTAGCCACGACGTGGACAT
GGTCATCCTGGCTGTGGGCTTTAGGCCCAACACAGCTCTGGGCAATGCCAAG
CTGAAGACCTTTAGGAACGGCGCCTTCCTCGTCGACAAGAAGCAGGAGACAT
CCATTCCTGACGTGTACGCCATCGGCGACTGCGCCACCGTGTATGACAACGC
CATCAACGACACCAACTATATCGCCCTGGCTTCCAACGCCCTCAGAAGCGGA
ATTGTGGCCGGCCATAATGCCGCTGGCCACAAGCTCGAGTCCCTGGGAGTGC
AGGGCTCCAACGGAATTAGCATCTTCGGCCTCAACATGGTGTCCACAGGCCT
GACCCAAGAGAAGGCCAAAAGATTCGGCTACAATCCCGAGGTGACCGCTTTT
ACCGACTTTCAGAAGGCCTCCTTCATCGAGCACGACAACTATCCTGTGACCCT
GAAGATCGTCTACGACAAGGACTCCAGACTGGTCCTGGGAGCTCAGATGGCC
TCCAAGGAGGACATGTCCATGGGAATCCACATGTTTTCCCTGGCCATCCAGG
AAAAGGTGACCATCGAGAGACTCGCCCTCCTCGACTATTTCTTCCTCCCCCAT

TTCAACCAACCTTATAACTACATGATTAAGGCCGCCCTCAAGGCCAAAGGTG
GATCTGGTGGATCTGGTGGATCTATGGATTACAAGGATGACGATGACAAGTA
AACGCGT

Streptococcus pneumoniae:

GCGGCCGCATGCTCGCCACCAGAGTGTTCAGCCTGGTGGGCAAAAGGGC
CATTTCCACCTCCGTGTGCGTGAGAGCCCACAGCAAGATCGTGGTGGTGGGC
GCTAACCACGCTGGCACCGCCTGCATCAACACCATGCTGGACAATTTCGGCA
ACGAGAACGAGATTGTCGTGTTCGACCAGAACAGCAATATCAGCTTCCTGGG
ATGCGGCATGGCCCTGTGGATCGGCGAGCAAATCGATGGCGCCGAGGGACTG
TTCTACAGCGACAAGGAGAAGCTGGAGGCCAAGGGCGCCAAGGTGTACATG
AACAGCCCCGTGCTCTCCATCGACTATGACAATAAGGTGGTGACCGCCGAGG
TCGAGGGCAAGGAGCATAAGGAGAGCTACGAGAAGCTGATCTTTGCCACAG
GATCCACCCCCATCCTGCCTCCCATCGAGGGAGTGGAGATCGTCAAGGGCAA
CAGAGAGTTCAAGGCCACCCTGGAAAACGTCCAATTCGTCAAACTGTACCAG
AACGCCGAGGAAGTGATCAACAAGCTGAGCGACAAGTCCCAGCACCTGGAT
AGAATCGCCGTGGTCGGAGGCGGCTATATCGGCGTGGAGCTCGCCGAAGCCT
TCGAGAGGCTGGGCAAGGAGGTGGTGCTGGTGGACATCGTGGACACCGTGCT
GAATGGATACTACGACAAGGACTTCACCCAGATGATGGCCAAGAACCTGGAG
GACCATAACATCAGGCTCGCTCTGGGCCAAACCGTGAAGGCTATCGAGGGCG
ACGGCAAGGTCGAGAGACTGATTACAGATAAGGAAAGCTTCGACGTGGACA
TGGTGATTCTGGCCGTGGGCTTTAGGCCCAACACCGCTCTGGCCGACGGCAA
GATCGAACTCTTCAGGAACGGCGCCTTCCTGGTGGACAAGAAGCAGGAAACC
AGCATCCCCGGAGTGTACGCCGTCGGCGATTGCGCTACCGTGTATGACAACG
CCAGGAAAGACACCAGCTACATCGCTCTGGCCAGCAATGCCGTGAGGACCGG
CATTGTCGGCGCTTACAACGCCTGCGGCCACGAGCTGGAGGGCATTGGAGTC
CAGGGATCCAATGGAATCAGCATCTACGGCCTGCACATGGTGTCCACCGGCC
TGACACTGGAAAAGGCCAAAGCCGCTGGCTATAATGCCACCGAGACAGGCTT
CAATGATCTCCAGAAGCCCGAGTTCATGAAGCACGACAACCACGAAGTCGCC
ATCAAGATCGTCTTCGACAAGGACTCCAGAGAGATCCTGGGCGCCCAGATGG
TGAGCCACGACATCGCCATCTCCATGGGCATCCACATGTTTAGCCTGGCCATC
CAAGAGCACGTGACAATCGACAAGCTGGCCCTGACCGACCTGTTTTTCCTGC
CCCACTTTAACAAGCCCTACAACTACATTACCATGGCTGCCCTGACCGCCGA
GAAGGGTGGATCTGGTGGATCTGGTGGATCTATGGATTACAAGGATGACGAT
GACAAGTAAACGCGT

Sequence of *S. cerevisiae* Ndi1

In the construct obtained from GENEWIZ the sequence encoding a FLAG-tag was added after the endogenous MTS of NDI1. Therefore the resulting construct encodes Ndi1 with an N-terminal FLAG-tag. For clarity corresponding regions of each sequence are highlighted in different colors:NotI, NheI, Endogenous MTS, AgeI,FLAG tag, $(GGS)_2GG$ linker, XmaI, Stop codon,EcoRI.

GCGGCCGCGCTAGCATGCTGTCCAAGAACCTGTACAGCAACAAGAGGCT
GCTCACCTCCACCAACACCCTCGTCAGATTTGCTTCCACAAGGAGCACAACC
GGTGATTACAAGGATGACGATGACAAGGGTGGATCTGGTGGATCTGGTGGAC

CCGGGGGCGTCGAAAACAGCGGAGCTGGCCCTACCAGCTTCAAGACCATGA
AGGTGATCGACCCCCAACACTCCGACAAACCCAATGTCCTCATCCTCGGCAG
CGGATGGGGAGCTATCTCCTTCCTGAAGCACATCGACACCAAGAAGTACAAC
GTGAGCATCATCTCCCCCAGGAGCTATTTCCTCTTCACACCCCTGCTCCCTAG
CGCTCCTGTGGGAACCGTGGACGAGAAGAGCATCATCGAGCCCATTGTGAAC
TTCGCTCTGAAGAAGAAAGGCAACGTGACCTACTATGAGGCTGAGGCCACCA
GCATCAACCCTGACAGGAATACCGTGACCATCAAGTCCCTCAGCGCCGTGAG
CCAACTCTACCAGCCCGAAAACCATCTCGGCCTGCATCAAGCCGAACCCGCC
GAGATCAAGTACGACTACCTGATCTCCGCTGTGGGCGCCGAGCCCAATACCT
TCGGCATCCCCGGCGTCACCGATTACGGCCACTTCCTGAAGGAAATCCCCAA
CTCCCTGGAGATTAGGAGGACCTTCGCCGCCAATCTCGAGAAGGCCAACCTG
CTCCCCAAGGGCGACCCCGAAAGGAGGAGACTCCTGAGCATTGTGGTCGTCG
GCGGAGGACCTACAGGAGTGGAGGCTGCCGGAGAACTGCAGGATTACGTCC
ACCAGGACCTGAGAAAGTTCCTGCCCGCCCTCGCTGAGGAAGTCCAGATCCA
TCTGGTGGAGGCCCTCCCTATCGTGCTCAACATGTTTGAGAAAAAGCTCAGCT
CCTATGCCCAAAGCCACCTGGAGAACACCAGCATCAAGGTGCACCTGAGAAC
AGCCGTGGCCAAGGTGGAGGAAAAGCAACTGCTGGCCAAGACCAAGCACGA
GGACGGAAAAATCACCGAGGAGACCATCCCCTATGGCACACTCATCTGGGCC
ACCGGCAACAAAGCTAGACCTGTGATCACCGACCTGTTCAAGAAGATCCCCG
AGCAAAATAGCAGCAAGAGGGGCCTGGCCGTGAACGACTTCCTGCAGGTCA
AGGGCAGCAACAACATTTTCGCCATCGGCGACAACGCTTTCGCTGGACTCCC
TCCTACAGCTCAAGTCGCCCACCAGGAGGCTGAGTACCTGGCCAAGAACTTC
GACAAGATGGCCCAGATTCCCAACTTTCAGAAGAATCTGTCCTCCAGGAAGG
ACAAGATCGACCTGCTCTTTGAGGAGAATAATTTCAAGCCTTTCAAGTACAA
CGACCTCGGAGCTCTGGCCTATCTCGGCTCCGAGAGGGCCATCGCTACAATC
AGGAGCGGCAAGAGGACCTTCTACACAGGAGGCGGCCTCATGACCTTCTACC
TGTGGAGGATCCTGTACCTCTCCATGATTCTCTCCGCTAGGAGCAGGCTGAAG
GTGTTTTTCGACTGGATTAAGCTGGCTTTCTTCAAAAGGGACTTCTTCAAGGG
CCTCTAGGAATTC

Cell lines and cell culture

Formulations of cell culture media used in this study are listed in Table S3. HeLa cells were purchased from ATCC (CCL-2) and were cultured in Dulbecco's modified Eagle's medium [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)]. HEK293T cells were purchased from ATCC (CRL-11268) and were cultured in DMEM [High glucose DMEM containing $NaHCO_3$ (Life Technologies, 11995) and 10 % FBS (Sigma, F2442)]. Lentiviral-infected HeLa cells were cultured in DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] supplemented with 200 μg/mL geneticin (Life Technologies, 10131-035) and 1 μg/mL puromycin (Life Technologies, A1113803). All of the experiments were performed in the absence of geneticin and puromycin.

Preparation of DNA constructs

Cloning of NOXes into pLVX-TRE3G vector

Human codon-optimized genes encoding H_2O-forming NADH oxidases (NOXes) from *Lactobacillus brevis*, *Lactococcus lactis*, *Streptococcus pneumoniae*, *Streptococcus mutans* and *Serpulina hyodysenteriae*, flanked by NotI and MluI restriction sites in the pUC57 vector, were custom synthesized by GENEWIZ. The above mentioned mitoNOX constructs also included: *(i)* a mitochondrial targeting sequence (MTS) of subunit IV of human cytochrome c oxidase (23 amino acids long) added upstream of the NOX coding sequence and *(ii)* a linker sequence with a FLAG-tag added in-frame and downstream of the *nox* gene. Only the MTS and the NOX coding regions were codon optimized. After digestion with NotI and MluI, inserts were directly ligated into the pLVX-TRE3G vector (Clontech, CA). To remove the MTS and produce constructs of untargeted NOXes corresponding *nox* genes were amplified using the following primers containing the NotI and MluI restriction sites which are underlined: *Lactobacillus brevis* 5'-TTA ATT GCG GCC GCATGA AGGTCA CCGTGGTCG-3'; *Lactococcus lactis* 5'-TTA ATT GCG GCC GCATGA AGATTG TCG TCA TCG-3'; *Streptococcus pneumoniae* 5'-TTA ATT GCG GCC GCATGA GCA AGATCGTGGTGG-3'; *Streptococcus mutans* 5'-TTA ATT GCG GCC GCATGA GCA AGATCG TGA TCG TC-3'; *Serpulina hyodysenteriae* 5'-TTA ATT GCG GCC GCATGA AAGTGA TCG TGA TCG-3' and the reverse primer 5'-TTA ATT ACG CGT TTA CTT GTC ATC GTC ATC CTT GT-3'. After digestion with NotI and MluI the PCR products were ligated into the pLVX-TRE3G vector (Clontech, CA).

Cloning of *Lb*NOX into pET30a

L. brevis nox gene was amplified from the pUC57 vector (containing a *H. sapiens* codon-optimized sequence) using the following primers containing the BamHI and XhoI restriction sites which are underlined: 5'-TTA ATT GGA TCC ATG AAG GTC ACC GTG GTC GG-3' and 5'-TTA ATT CTC GAG TCA CTT GTC ATC GTC ATC C-3'. After digestion the PCR product was ligated into the pET30a vector (EMD Millipore). The resulting construct encodes *Lb*NOX with both a N-terminal Hisx6-tag and a C-terminal FLAG-tag.

Cloning of *S. cerevisiae* Ndi1 into pLVX-TRE3G vector

In the construct ordered from GENEWIZ, the Ndi1 coding sequence was *H. sapiens* codon-usage optimized and a coding sequence for a FLAG-tag was inserted right after the endogenous MTS cleavage site preceded by AgeI restriction site. The insert was cut from pUC57, and directly ligated into the pLVX-TRE3G vector (Clontech, CA) using NotI and EcoRI restriction sites.

Lentivirus production

Half a million HEK293T cells were seeded per well in a 6-well plate (one plate per lentivirus) in 2 mL of DMEM (High glucose DMEM (Life Technologies, 11995), 10 % FBS (Sigma, F2442)). The next evening the medium was replaced with fresh DMEM and cells were transfected with 100 µl of transfection mixture per well. The transfection mixture contained 3 µl X-treme Gene 9 reagent (Roche, 06365787001), 500 ng psPAX2

(psPAX2 was a gift from Didier Trono, Addgene plasmid # 12260), 50 ng pMD2.G (pMD2.G was a gift from Didier Trono, Addgene plasmid # 12259), 500 ng of the pLVX-TRE3G vector of interest (including pLVX-TRE3G-Luc control vector, expressing Luciferase, obtained from Clontech) and Opti-MEM medium (Life Technologies, 31985-070) up to 100 µl. To make the transfection mixture, 50 µl solutions of X-treme Gene 9 and DNA mixtures were prepared separately and the DNA solution was added dropwise to the X-treme Gene 9 solution. The mixture was incubated at room temperature for 30 min before adding it to cells. Two days after transfection, medium was collected, centrifuged at 500 x *g* for 5 min to pellet cells and the supernatant was aliquoted and stored at -80^{0}C.

Adenovirus production

Two custom adenoviruses (Adenovirus Type 5 (dE1/E3)) were produced by Vector Biolabs. The same *Lb*NOX and mito*Lb*NOX nucleotide sequences were used as in the lentiviral pLVX-TRE3G constructs. *Lb*NOX or mito*Lb*NOX adenoviruses co-express eGFP and either *Lb*NOX or mito*Lb*NOX, respectfully, each gene driven by its own CMV promoter. Adenovirus with eGFP driven by the CMV promoter was used as a control (Vector Biolabs, 1060-HT).

Generation of stable cells using lentiviral infection

Fifty thousand HeLa cells were seeded in 2 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] per well in a 6-well plate. Twenty four hours after seeding, 200 µl of solution containing lentivirus was added per well. Twenty four hours postinfection medium was replaced. After an additional twenty four hours, medium was replaced with 2 mL of DMEM ± 200 µg/mL geneticin (Life Technologies, 10131-035) ± 1 µg/mL puromycin (Life Technologies, A1113803). Stable cells were selected for at least a week before performing experiments and were cultured in the presence of the indicated concentrations of antibiotics.

Oxygen consumption rate (OCR) of cell lines overexpressing NOXes and Luciferase

Oligomycin- and antimycin-resistant OCR by cell lines overexpressing Luciferase and NOXes from *L. brevis*, *L. lactis*, *S. pneumoniae*, *S. mutans* and *S. hyodysenteriae*

Oxygen consumption rates (OCR) of HeLa cells expressing 5 bacterial NOXes (untargeted and targeted to mitochondria) and Luciferase under the control of the doxycycline-inducible promoter (TRE3G) were measured with the XF24-3 Extracellular Flux Analyzer (Seahorse Bioscience, manufactured in 2008). Cells were seeded at 30-40 x 10^3 cells per well in XF24 24-well cell culture microplates in 200 µl of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$ and 10 % dialyzed FBS (Life Technologies, 26400-044] and were incubated at 37^{o}C in 5 % CO_2 incubator. Medium was replaced the next day with 1 mL of fresh medium per well and doxycycline (final concentration – 300 ng/mL, prepared in water) or water was added to the corresponding wells to induce protein expression. Twenty four hours later, medium was replaced with 950 µl of the assay medium [DMEM (US Biological, D9800), 10 % dialyzed FBS (Life Technologies, 26400-044) and 25 mM HEPES-KOH, pH 7.4] and plates were placed in the X24 Extracellular Flux Analyzer for measurements of OCR. Each measurement was

performed over 4 min after a 2-min mix and a 2-min wait period. Basal measurements were collected 8 times and 4 measurements were collected after injection of oligomycin (final concentration 1 μM), followed by 4 measurements after addition of antimycin A (final concentration 1 μM) from the XF24 ports in 50 μl of the assay medium.

Antimycin- and piericidin-resistant OCR by cell lines overexpressing Luciferase, *Lb*NOX, and mito*Lb*NOX

Cells were seeded at 30–40 x 10^3 per well in XF24 24-well cell culture microplates in 200 μl of normal growth medium [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$ and 10 % dialyzed FBS (Life Technologies, 26400-044)] and were incubated at 37 °C in 5 % CO_2 incubator. For all experiments the next day medium was renewed with 1 mL per well and doxycycline (final concentration – 300 ng/mL) or water was added. Twenty four hours later, medium was changed to no pyruvate media [no pyruvate DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$ and 10 % dialyzed FBS (Life Technologies, 26400-044)]. Two hours later, medium was replaced with 950 μl of the assay medium [no pyruvate DMEM (US Biological, D9802), 15.9 mg/L phenol red, 10 % dialyzed FBS (Invitrogen, 26400-044) and 25 mM HEPES-KOH, pH 7.4)] and OCR was measured. Each measurement was performed over 4 min after a 2-min mix and a 2-min wait period. Basal measurements were collected 8 times and 4 measurements were collected after injection of each drug: antimycin (final concentration 1 μM) or piericidin (final concentration 1 μM). Three measurements before and after addition of the drug were averaged and normalized by the cell number.

Expression and purification of *Lb*NOX

E. coli BL21 (DE3) cells (Life Technologies, C6010-03), harboring the pET30a vector with the *L. brevis nox* gene were grown at 37 °C in six 2.8-L flasks, each containing 1 L of LB medium supplemented with 50 μg/mL kanamycin to an A_{600} of 0.4-0.6. At that point, the temperature was decreased to 15 °C and cells were grown for additional 2 hours. After induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), cells were grown for 14-16 hours at 15 °C. Harvested cells were resuspended in ~150 mL of lysis buffer (50 mM Na_2HPO_4, pH 8.0, 500 mM NaCl, 20 mM imidazole) containing six protease inhibitor tablets (Roche Applied Science, 05 056 489 001), 60 μl of benzonase nuclease (EMD Millipore, 71205-3), 4 mM phenylmethylsulfonyl fluoride (PMSF) (prepared in ethanol), 100 μM FAD (Sigma, F6625-100MG) and disrupted by sonication on ice (output setting of 50 % for 20 min with 30 sec bursts and 59 sec breaks). Following centrifugation, the cell lysate was filtered through a 0.4 μm filter, diluted to ~600 mL with lysis buffer and loaded onto a 25-mL Nickel Sepharose 6 Flast Flow column (GE Healthcare). After washing with 15 column volumes (CV) of lysis buffer, the protein was eluted with a gradient of 20–300 mM imidazole in lysis buffer over 8 CV. Fractions containing *Lb*NOX were pooled, and exchanged into 50 mM Na_2HPO_4, pH 7.5 buffer, and applied to a 30-mL Source 15Q column equilibrated with 50 mM Na_2HPO_4, pH 7.5, 50 mM NaCl buffer at flow rate of 6 mL/min. Source 15Q resin was obtained from GE Healthcare (17-0947-01) and packed into an Omnifit glass column (Sigma, 56009-U). After the sample was applied, the column was washed with 5 CV of equilibration buffer and eluted with 15 CV gradient of 50-300 mM NaCl in 50 mM Na_2HPO_4, pH 7.5 buffer. The ion-exchange chromatography step on Source 15Q allows

separation of apo*Lb*NOX (*Lb*NOX which is devoid of FAD cofactor) and *Lb*NOX partially loaded with FAD from fully reconstituted *Lb*NOX (holo*Lb*NOX). The fractions from the peak which corresponded to fully reconstituted *Lb*NOX with the highest specific activity (~550 μmol $min^{-1}mg^{-1}$) were pooled, concentrated and loaded onto a 120 mL HiPrep 16/60 Sephacryl S-400 column (GE Healthcare, 28-9356-04) equilibrated with 50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl buffer at flow rate of 1.2 mL/min. Fractions containing *Lb*NOX were pooled, concentrated, flash-frozen in liquid nitrogen, and stored at -80 °C. Ion-exchange chromatography and gel-filtration steps were performed on an AKTA pure FPLC system (GE Healthcare). To remove extra amino acids arising from the N-terminal Hisx6 tag *Lb*NOX samples (0.5-1.5 mg) were incubated with 18-25 U of recombinant enterokinase (EMD Millipore, 69066-3) at 4 °C overnight. After cleavage with enterokinase samples were passed through a His GraviTrap column (GE Healthcare, 11-0033-99), equilibrated with 50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl buffer and the flow-through containing cleaved protein was collected and used in the experiments. This step provided the most active protein with a specific activity of ~750 μmol min^{-1} mg^{-1}.

Determination of the oligomerization state

The oligomerization state of *Lb*NOX was determined by size-exclusion chromatography. Protein was injected (0.1 mL of 10 mg/mL protein) onto a HiPrep 16/60 Sephacryl S-400 HR column (GE Healthcare) equilibrated with 50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl buffer. The column was operated at a flow rate of 1.2 mL/min. *Lb*NOX was injected onto the column 6 times, along with each size-exclusion standards (GE Healthcare, 28-4038-41) 3 times that included aldolase (158 kD), conalbumin (75 kD), ovalbumin (44 kD) and carbonic anhydrase (29 kD).

Enzyme Assays

Recombinant *Lb*NOX activity was monitored by following the decrease of the absorbance of NAD(P)H at 340 nm using a Cary 100 spectrophotometer (Agilent, CA). A typical reaction mixture contained 2 μM FAD in 0.5 mL of the assay buffer (50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl) and was incubated for 4 min at 37 ^oC before NAD(P)H (2-600 μM) and enzyme (0.08-6.8 μg) were added. An extinction coefficient (ε_{340} = 6.2 $mM^{-1}cm^{-1}$) was used to calculate NAD(P)H oxidase activity. The k_{cat} values for *Lb*NOX were calculated per monomer of the protein.

Simultaneous monitoring of oxygen consumption and NADH fluorescence was performed using a custom-built fluorimeter. In our setup the RedEye oxygen patch (OceanOptics) was installed in a cuvette and the optical probe was connected to a fluorimeter. Oxygen causes quenching of the RedEye patch fluorescence, which allows monitoring of the partial pressure of oxygen. In our experiments 0.5 mL of the assay buffer was preincubated at 28 ^oC before NADH (40-1500 μM) and *Lb*NOX (0.1-2 μg) were added.

UV-visible Spectroscopy

UV-visible spectra were recorded on a Cary 100 spectrophotometer (Agilent, CA). *Lb*NOX (40-80 μM FAD active sites) in the assay buffer was incubated at 24 ^oC and (0.3-3 mM) sodium dithionite was added under aerobic conditions. An extinction coefficient (ε_{450} =11.3 $mM^{-1}cm^{-1}$) was used to quantify FAD.

Determination of H_2O_2 production by recombinant *Lb*NOX

H_2O_2 production was monitored in a continuous assay followed by the increase in absorbance at 570 nm upon resorufin formation (ε_{570} = 54 mM^{-1} cm^{-1}). It has been shown previously that presence of NADH in the assay mixture results in the oxidation of Amplex Red dye in the HRP-dependent manner and this process is greatly diminished by supplementation of superoxide dismutase (SOD) (*35*). Therefore in our continuous assay we used SOD to minimize the background rate of resorufin production, which is not related to H_2O_2 production by *Lb*NOX. The assay mixture contained 350 µM NADH, 10 µl of HRP (Abcam, ab102500), 10 µl of Amplex Red (Abcam, ab102500), 100 µM D-glucose, 130 U SOD (Sigma, S9697-75 KU) in 0.5 mL of the assay buffer (50 mM Na_2HPO4, pH 7.5, 150 mM NaCl). The reaction was preincubated at 37 ^oC for 3 min before *Lb*NOX was added (0.15 µg) and, subsequently the *Lb*NOX-dependent rate of H_2O_2 production was recorded, 4 U of glucose oxidase (Sigma, G2133-10 KU) was added (Fig. S2A). Glucose oxidase was used to validate the assay (Fig. S2A). The H_2O_2-forming activity of *Lb*NOX was compared to the total NADH oxidase activity, which was determined in a parallel experiment in which the decrease of NADH absorbance at 340 nm was monitored under identical conditions.

Enzyme-monitored turnover experiments

The reaction of *Lb*NOX with oxygen was studied by the enzyme-monitored turnover method (*36, 37*). Rapid reaction studies were carried out at 4 °C in a SX20 stopped flow instrument (Applied Photophysics, UK) equipped with a diode array detector. To follow the redox state of the FAD cofactor, *Lb*NOX enzyme (1.9, 2.9, 5.6 and 9.18 µM FAD active sites after mixing) was mixed with NADH (2 mM after mixing), both prepared in air-saturated assay buffer (50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl) and absorbance at 455 nm was monitored over time. The solubility of oxygen at 4 °C was calculated from an oxygen solubility table at corresponding salinity of the assay buffer which is ~ 380 µM. Results represent (n=3) independent experiments. The traces at 455 nm reflect the conversion of oxidized to reduced enzyme forms and are treated as previously described (*36*), where the area under the curve is proportional to the concentration of oxygen, which is a limiting substrate under these conditions. From the traces generated at different enzyme concentrations it can be seen that the absorbance at 455 nm drops rapidly to the steady state where the signal remains constant until the rapid drop at the point where oxygen is depleted and the enzyme becomes fully reduced (Fig. S2B). This last phase is very abrupt even at 1.9 µM enzyme, demonstrating that concentration of oxygen is significantly higher than its apparent K_m even during the very last turnover. We therefore estimated that apparent K_m for O_2 is less than 2 µM. Based on the enzyme concentration (1.9-9.18 µM), oxygen concentration (380 µM) and the time it took to consume O_2 (8.12-1.67 sec), the apparent k_{cat} can be calculated at 4 °C (Fig. S2B, *inset*).

Protein crystallization and structure determination

Crystals of *Lb*NOX were grown by the vapor diffusion technique. For the initial screening of crystallization conditions 0.1 µl of protein solution (10 mg/mL *Lb*NOX in 10 mM HEPES, pH 7.5, 100 mM NaCl) and 0.1 µl of precipitant solution (MCSG Suite, Microlytic, MA) were mixed using the NanoTransfer NT8 pipetting robot (Formulatrix,

MA) and incubated over a 50 µl reservoir in the sitting drop plates. Progress of protein crystallization was monitored with Rock Maker (Formulatrix, MA). Optimal crystals were formed in 20 % PEG 3350, 0.2 M NH_4Cl at 21 ^oC. For harvesting, crystals were cryoprotected by adding 20 % (v/v) of ethylene glycol to the precipitant solution. X-ray diffraction data were collected at beamline 8.2.2 at the Advanced Light Source (Berkeley, CA). Data collected at λ=1.000Å were indexed, integrated, and scaled using the software HKL-2000 (*38*). Four molecules of *Lb*NOX in the asymmetric unit of P1 crystal were found by molecular replacement with Phaser-MR program of PHENIX package (*39, 40*) using the structure of H_2O-forming NAD(P)H oxidase from *Lactobacillus sanfranciscensis* (PDB ID 2CDU) as a model. The structure was refined using Phenix.refine (*40*) and/or refmac5 in the CCP4 suite (*41*), and manually using COOT (*42*). Oxygen and FAD molecules were added in later rounds of refinement. The final protein model contained all residues except for the N-terminal Hisx6 tag and the C-terminal FLAG tag. All data collection and refinement statistics are summarized in Table S1. All protein structure figures were generated using PyMOL program (*43*).

Cell proliferation assays

Rescue of chloramphenicol and ethidium bromide-induced inhibition of cell proliferation (Fig. 4B and fig. S7)

Five hundred HeLa Tet3G NDI1, Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded in 200 µl of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] per well in a black 96-well plate with a clear bottom (Corning, 3904). Twenty four hours after seeding, medium was exchanged to DMEM without pyruvate [DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)], 200 µM uridine ± 10 µg/mL chloramphenicol ± 30 ng/mL ethidium bromide ± 1 mM pyruvate ± 300 ng/mL doxycycline. After 0, 2, 3, 4, 5 and 6 days, medium was aspirated and cells were fixed by adding 100 µl of 4 % paraformaldehyde in PBS and incubating at room temperature for at least 30 min. Paraformaldehyde solution was aspirated and cells were stained with 200 µl of 1 µg/mL Hoechst 33345 in PBS. Plates were covered with sealing aluminum foil and stored at 4 ^oC before counting cells in each well with Molecular Dynamics ImageXpress Ultra (see imaging protocol below).

Rescue of piericidin and antimycin-induced inhibition of cell proliferation (Fig. 4B and fig. S7)

One thousand HeLa Tet3G NDI1, Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded in 200 µl of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] per well in a black 96-well plate with a clear bottom (Corning, 3904). Twenty four hours after seeding, 10 µl of 6 µg/mL doxycycline (300 ng/mL final concentration) or water was added to each well. Twenty four hours after addition of doxycycline, medium was exchanged to DMEM without pyruvate [DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)], 200 µM uridine ± 1 µM piericidin, ± 1 µM antimycin, ± 1 mM pyruvate and ± 300 ng/mL doxycycline. After 0, 1, 2, 3 and 4 days, medium was aspirated and cells were fixed by adding 100 µl of 4 %

paraformaldehyde in PBS and incubating at room temperature for at least 30 min. Paraformaldehyde solution was aspirated and cells were stained with 200 µl of 1 µg/mL Hoechst 33345. Plates were covered with sealing aluminum foil and stored at 4 °C before counting cells in each well with Molecular Dynamics ImageXpress Ultra (see imaging protocol below).

Rescue of piericidin-induced inhibition of cell proliferation with pyruvate, lactate, malate and oxaloacetate (Fig. 4A)

Two thousand HeLa Tet3G Luciferase cells were seeded in 200 µl of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] per well in a black 96-well plate with a clear bottom (Corning, 3904). Twenty four hours after seeding, medium was exchanged to DMEM without pyruvate [DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)], 200 µM uridine ± 1 µM piericidin, ± indicated concentrations of pyruvate, lactate, malate and oxaloacetate. After 3 days, medium was aspirated and cells were fixed by adding 100 µl of 4 % paraformaldehyde in PBS and incubating at room temperature for at least 30 min. Paraformaldehyde solution was aspirated and cells were stained with 200 µl of 1 µg/mL Hoechst 33345. Plates were covered with sealing aluminum foil and stored at 4 ^{o}C before counting cells in each well with Molecular Dynamics ImageXpress Ultra (see imaging protocol below).

Nuclei counting using Molecular Devices ImageXpress

Images of 96-well plates with fixed cells stained with Hoechst 33345 were collected using Molecular Devices ImageXpress Micro XLS. Four images were taken to cover the whole well. Images were analyzed and nuclei number per well was counted using CellProfiler 2.0 image analysis software(*44, 45*). The cell counting method had a linear range from 500 to 40000 cells per well as determined by counting plates with known number of cells seeded 6 hours prior to fixation.

Glucose concentration-dependent cell survival (fig. S9)

Ten thousand HeLa Tet3G NDI1, Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded in 200 µl of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] per well of a black 96-well plate with a clear bottom (Corning, 3904). Twenty four hours after seeding, 10 µl of 6 µg/mL doxycycline (300 ng/mL final concentration) or water was added to each well. Twenty four hours after addition of doxycycline, medium was exchanged to DMEM without glucose [DMEM (US Biological, D9800-02), 4 mM glutamine, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)], 200 µM uridine ± 1 µM piericidin, ± 300 ng/mL doxycycline ± indicated glucose concentration. After 24 hours, medium was aspirated and cells were fixed by adding 100 µl of 4 % paraformaldehyde in PBS and incubating at room temperature for at least 30 min. Paraformaldehyde solution was aspirated and cells were stained with 200 µl of 1 µg/mL Hoechst 33345. Plates were covered with sealing aluminum foil and stored at 4 ^{o}C before counting cells in each well with Molecular Dynamics ImageXpress Ultra (see imaging protocol above).

PDH phosphorylation

Two hundred thousand HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded in 2 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] per well of a 6-well plate. Twenty four hours after seeding, medium was exchanged to DMEM without pyruvate [DMEM (US Biological (D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] ± 300 ng/mL doxycycline. Twenty four hours after doxycycline addition, medium was exchanged to DMEM without pyruvate [DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] ± 300 ng/mL doxycycline ± 1 µM piericidin ± 5 mM dichloroacetate (DCA). One hour later, cells were rinsed with 2 mL of ice cold PBS and lysed in the well by adding 400 µl of ice cold 1X Laemmli Sample Buffer with 1X protease/phosphatase inhibitor cocktail (Cell Signaling, 5872S), incubated for 5 min on ice, transferred to an Eppendorf tube and heated for 5 min at 95°C. Protein levels were detected using western blot with anti-FLAG antibody (Cell Signaling, 2368) for *Lb*NOX and mito*Lb*NOX detection, anti-PDH-E1α antibody (Invitrogen, 459400) and anti-PDH-E1α phospho-Ser^{300} antibody (EMD Millipore, AP1064).

Metabolite extraction and media preparation for determination of lactate, pyruvate, NAD^+ and NADH

One million HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded per 10 cm plate in 10 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)]. Twenty four hours and 1 hour before extraction, medium was exchanged with 10 mL of DMEM without pyruvate [DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] with or without 300 ng/mL doxycycline. The media that were used 1 hour before extraction were preincubated overnight in tissue culture incubator without cells. For NAD^+ and NADH measurements, all solutions (i.e. PBS and 80% methanol: 20% water) were degassed by bubbling with argon to remove oxygen. Media were quickly aspirated, cells rinsed with 15 mL of ice-cold PBS, transferred to dry ice and 4 mL of dry ice cold solution of 80 % methanol: 20 % water (spiked with 5 µM Sodium L-Lactate-3,3,3-D3 and 0.2 µM Sodium [3-^{13}C]Pyruvate) was added and quickly spread around by tilting the plate. The whole procedure from aspiration of media to adding 80 % methanol solution took about ~15 seconds. The plate was transferred on dry ice to -80 ^{o}C freezer and incubated for 15 min. Cells were collected with cell scraper while the plate was kept on dry ice and the solution was centrifuged at 2,000 x *g* at 4 ^{o}C for 5 min. Supernatant was collected and stored on dry ice. The pellet was resuspended in 0.5 mL of 80 % Methanol: 20 % Water (spiked with 5 µM Sodium L-Lactate-3,3,3-D3 and 0.2 µM Sodium [3-^{13}C]Pyruvate) at 4 ^{o}C, vortexed for 10 sec, incubated for 15 min on wet ice and spun at 2000 x g for 5 min. The pellet was re-extracted one more time and all the supernatants were combined and stored at -80 ^{o}C overnight. Supernatants were evaporated using a Speedvac (without heating) down to 1 mL, deproteinated by centrifuging through 10 kD cutoff filter membrane and further evaporated down to 200 µl using a Speedvac. Water was used to bring all the samples up to the same volume of 200 µl. Before analysis, the solutions were centrifuged at 20,000 x *g* for 10 min to remove insoluble particles. These water solutions were used for determination of total

NAD^+/NADH ratio using HPLC equipped with a UV-vis detector. Samples were diluted with methanol to 80 % methanol: 20 % water solution for LC-MS detection of lactate and pyruvate. Culture media samples were prepared for LC-MS detection of lactate and pyruvate by diluting culture media with methanol to 80 % methanol: 20 % water solution (spiked with 50 µM Sodium L-Lactate-3,3,3-D3 and 2 µM Sodium [3-^{13}C]Pyruvate), the 80 % methanol solution was incubated at room temperature for 30 min and centrifuged at 20,000 x *g* to pellet the protein precipitate. The supernatant was used for LC-MS analysis.

Determination of total NAD^+/NADH ratio by HPLC

Samples from the metabolite extraction step (see above) were used. Metabolites were analyzed by ion exchange chromatography using µBondapak NH_2 300 × 3.9-mm column (Waters, WAT084040) attached to an Agilent 1260 HPLC system. The column was maintained at 24 ^oC during runs. Typically 80-100 µl of the sample was injected, while the rest of the samples were kept at 4 ^oC in the autosampler module. Initial conditions were 96 % of Buffer A (10 mM KH_2PO_4, pH 4.4) and 4 % Buffer B (1000 mM KH_2PO_4, pH 4.5) and a flow rate of 1.0 mL/min. Between 10 and 40 min, Buffer B was increased to 45 %. Between 40 and 41 min Buffer B was increased to 100 % and kept at that concentration for 5 min. Between 46 and 47 min, Buffer B was decreased to 4 % and held for 20 min at that composition to equilibrate the column between injections. Absorbance was monitored at 255 nm for NAD^+ and 255 nm and 340 nm for NADH. Under these conditions, the retention time for NAD^+ was 5.8 min and for NADH was 12.7 min. Calibration curves were generated using known amounts of NAD^+ (0.3-2.4 nmoles) and NADH (0.05-0.3 nmoles). In this method we were also able to detect other nucleotides with the following retention times: AMP- 6.7 min, ADP- 18.6 min and ATP- 27.5 min. The energy charge calculated ([ATP]+ 0.5 [ADP])/([ATP]+[ADP]+[AMP]) was typically ~0.95.

LC-MS method for detection of lactate and pyruvate

A Dionex UltiMate 3000 UHPLC system coupled to a Q Exactive Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA) was used to perform measurements of lactate and pyruvate in both the cell extract and cell media samples. A ZIC-cHILIC 150 x 2.1-mm, 3 µm particle size column (EMD Millipore, 1.50658.0001) was used and kept at room temperature. Initial conditions were: 15 % of Buffer A (20 mM ammonium acetate, pH adjusted to 7.7 using ammonium hydroxide) and 85 % Buffer B (acetonitrile) and a flow rate of 0.22 mL/min. Between 0.5 and 3.5 min, Buffer B was decreased to 75%. Between 3.5 and 9 min Buffer B was decreased to 40%. Between 9 and 10.5 min Buffer B was decreased to 2 % and kept at that composition for 1 min. Between 11.5 and 12.5 Buffer B was increased to 85 % and held for 12 min at that composition to equilibrate the column between injections. Needle washing solution was 75 % acetonitrile. Ten microliters of the sample was injected into LC-MS. Negative mode ionization was applied. Targeted SIM method was used to acquire the MS data. The following MS conditions were used: microscan 1; resolution 140,000; AGC target 5E5; maximum 120 ms; msx 4; isolation window 1.0 m/z. The inclusion list was 87.0088 (pyruvate), 89.0244 (lactate), 90.0188 (sodium [3-^{13}C]pyruvate), 92.0429 (sodium L-lactate-3,3,3-D3). The ratio of the peak intensity of lactate or pyruvate to peak intensity

of isotope labeled lactate or pyruvate was calculated. The isotope ratio method was used to quantify the lactate and pyruvate in the cell extract and media.

Metabolomics experiments

One million HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded per a T-75 flask in 10 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)]. Twenty four hours after seeding, medium was exchanged to 10 mL of DMEM without pyruvate [DMEM (US Biological, D9802), 10 mM Glucose, 15.9 mg/L phenol red, 200 µM uridine, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] with or without 300 ng/mL doxycycline. Twenty four hours after adding doxycycline, medium was exchanged to 10 mL of DMEM without pyruvate [DMEM (US Biological, D9802), 10mM Glucose, 15.9 mg/L phenol red, 200 µM uridine, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] with or without 300 ng/mL doxycycline, 1 µM antimycin. One mL of media was collected after 24 hours and stored at -80 ºC.

A Q Exactive Plus Orbitrap Mass Spectrometer coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA) was employed to perform metabolome profiling. One hundred µL of cell medium sample was aliquoted into an 1.5 mL Eppendorf tube, and 900 µL of acetonitrile:methanol (75 %:25 % vol/vol) was used to extract it. After 15 sec of vortexing, it was transferred to 4 °C for 20 min. After centrifugation for 20 min at 4 °C at maximum speed, 150 µL of supernatant was transferred into LC-MS glass vial. Ten µL of supernatant was injected into LC-MS. Two HILIC (hydrophilic interaction liquid chromatography) columns were used for metabolome profiling. One column was Xbridge Amide HILIC 100 x 2.1-mm, 2.5 µm particle size column (Waters, 186006091) and the MS was acquired under the negative ionization mode. The column was maintained at 27 ^{o}C during runs. The mobile phase A was 20 mM ammonium acetate, 0.25 % ammonium hydroxide pH adjusted to 9.0. The mobile phase B was 100 % acetonitrile. The flow rate was 220 µL/min. The gradient elution was as follows: 0 min: 85 % B; 0.5 min: 85 %B; 9 min: 35 %B; 11 min: 2 %B; 12 min: 85 %B; 25 min: 85 %B. The other column was Atlantics Silica HILIC 150 x 2.1-mm, 3 µm particle size column (Waters, 186002015) and the MS was acquired under the positive ionization mode(*46*). The MS data acquisition was full scan mode in a range of 70–1000 m/z, with the resolution set at 140,000, the AGC target at 3E6, and the maximum injection time at 400 ms. The pooled medium sample was used as the quality control sample and was injected between every 6 samples to monitor the data quality of all LC-MS runs. The injection order of samples was randomized. Progenesis QI software (Waters, NC) was used to perform the peaks picking, peaks alignment, deconvolution of adduct peaks and peak intensity integration. Metabolite reference standards in a 96 well-plate format were purchased from IROA Technologies (Bolton, MA). An in-house metabolite retention time library of 520 reference standards was established. The metabolites were identified based on matching the accurate mass within a 2 ppm tolerance window and a retention time within 0.25 min tolerance window. For Fig. 3D, fig. S6 and S8, metabolite peak intensity present in the media before addition of cells were subtracted from final metabolite peak intensity at 24 hours to produce the amount of metabolite that was consumed or secreted by cells during that time.

Measurement of mitochondrial membrane potential with flow cytometry using TMRM

One hundred thousand HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded per well of 6-well plate in 2 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)]. Twenty four hours after seeding, medium was exchanged to 2 mL of DMEM without pyruvate [DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] with 300 ng/mL doxycycline. Twenty four hours after doxycycline addition, cells were treated with the indicated combination 10 nM TMRM and one of the following drugs – 1 μM antimycin, 1 μM oligomycin, 5 μM CCCP or 0.1 % DMSO. One hour after TMRM addition, medium was aspirated and 0.5 mL of trypsin solution was added for 5 min followed by addition of 0.5 mL of DMEM without pyruvate, fluorescent vitamins and phenol red [DMEM (US Biological, D9802), 5 mM glucose, 25 mM HEPES, pH 7.4, 1 % dialyzed FBS (Life Technologies, 26400-044)] containing the same combination of 10 nM TMRM, 1 μM antimycin, 1 μM oligomycin, 5 μM CCCP and DMSO as the first treatment before trypsinization. Cells were centrifuged 5 min at 500 x *g* and resuspended in 1 mL of DMEM without pyruvate, fluorescent vitamins and phenol red [DMEM (US Biological, D9800-17), 5 mM glucose, 25 mM HEPES, pH 7.4, 1 % dialyzed FBS (Life Technologies, 26400-044)] containing the same combination of 10 nM TMRM, 1 μM antimycin, 1 μM oligomycin, 5 μM CCCP and DMSO as the first treatment before trypsinization. Cells were analyzed on BD LSRII flow cytometer within 20 min and the data was analyzed using FlowJo software.

Measurement of cytoplasmic NAD^+/NADH ratio with SoNar

Fifty thousand HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded per well of 12-well glass bottom MatTek dish (Part number: P12G-1.5-14-F) in 2 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)]. Twenty four hours after cell seeding, medium was replaced with fresh DMEM and cells were transfected with 10 μl of the transfection mixture. The transfection mixture contained 15 μl X-treme Gene 9 Reagent (Roche, 06365787001), 5 μg pCDNA3.1-SoNar (pCDNA3.1-SoNar was a gift from Joseph Loscalzo) and Opti-MEM media (Life Technologies, 31985-070) up to 500 μl. Twenty four hours after transfection, medium was exchanged to 2 mL of DMEM without pyruvate [DMEM (US Biological, D9802), 15.9 mg/L phenol red, 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)] with 300 ng/mL doxycycline. Twenty four hours after doxycycline addition, cells were washed twice with 2 mL of imaging medium – DMEM without pyruvate, fluorescent vitamins and phenol red (DMEM (US Biological, D9800-17), 5 mM glucose, 25 mM HEPES, pH 7.4, 1 % dialyzed FBS (Life Technologies, 26400-044)) and incubated in 2 mL of imaging medium. Cells were equilibrated on a microscope stage equipped with 37^{o}C incubation chamber for at least 1 hour and imaged using a 40x Plan Fluor 1.3NA oil objective, 405 nm excitation laser and 525/50 nm emission filter. Nikon NSTORM microscope with Andor iXon3 EMCCD camera was used for imaging in widefield mode. Cells were imaged every 30 sec for 5 min in imaging medium followed by washing twice with 2 mL of imaging medium (without glucose, with 10 mM pyruvate) and imaged every 30 sec for 5 min in imaging medium (without glucose, with 10 mM pyruvate). Data analysis was performed using ImageJ software by performing flat field correction using images of empty wells, manually

drawing ROI around cells and subtracting background using areas with no cells. Measurements for each cell were normalized to average value of that cell in imaging medium (without glucose, with 10 mM pyruvate) to account for variability in reporter expression. Experiments presented in Fig. 3A and fig. S5B are average of 6 (Luciferase) or 7 (*Lb*NOX, mito*Lb*NOX) experiments performed on different days. Each experiment consisted of imaging 2 different wells with 3-10 cells each.

Determination of *Lb*NOX and mito*Lb*NOX localization using immunofluorescence

One hundred thousand HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded per well of 6-well plate (with a No 1.5 coverslip in each well) in 2 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)]. Twenty four hours after seeding, medium was exchanged to 2 mL of DMEM (DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % dialyzed FBS (Life Technologies, 26400-044)) with 300 ng/mL doxycycline. Twenty four hours after doxycycline addition, the coverslip was removed from the well, rinsed in warm PBS (37^{o}C), fixed in 4 % paraformaldehyde in warm PBS (37^{o}C) for 5 min, rinsed in PBS and quenched with TBS, 0.1 %Triton X-100 for 10 min. Coverslips were blocked with PBS, 2 % BSA, 0.1 % Triton X-100 for 10 min, stained with mouse anti-Tomm20 (Santa Cruz, sc-17764) in PBS, 2 % BSA, 0.1 % Triton X-100 for 1 hour, washed 3 times for 5 min with PBS, then stained with both Alexa Fluor 645-conjugated anti-FLAG (Cell Signaling, 3916) and Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Cell Signaling, 4408) in PBS, 2 % BSA, 0.1 % Triton X-100 for 1 hour and washed 3 times for 5 min with PBS. Coverslips were mounted on slides using Prolong Gold (Life Technologies, P10144). Fluorescent images were acquired using Leica SPS AOBS Scanning Laser Confocal Microscope (HCX PL APO CS 40x 1.25NA Oil UV objective, Argon 488 nm laser for Alexa 488 and HeNe 633 nm laser for Alexa 645).

Cell fractionation experiments

HeLa Tet3G *Lb*NOX and mito*Lb*NOX cells were seeded in ten 15 cm cell culture dishes in 20 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10 % non-dialyzed FBS (Sigma, F2442-500ML)] and doxycycline was added (300 ng/mL final concentration) when cells were close to confluency. Twenty four hours after doxycycline addition medium was removed and cells were washed once with 10 mL of ice-cold PBS. One mL of PBS was added to cells and cells were harvested using a cell scraper. Subsequently cells were resuspended in 0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 1 mM EGTA-Tris buffer with protease inhibitor cocktail (Roche Applied Science, 05 056 489 001), lysed using a Parr bomb and mitochondria were purified using Percoll density gradient as previously described (*47, 48*). In order to prepare the cytosolic fraction cells from one 15 cm dish were resuspended in 1 mL of PBS with protease inhibitor cocktail, lysed by passing through a 27.5 g needle attached to 1 mL syringe 12 times. Intact cells and nuclei were pelleted by centrifugation for 10 min at 800 x *g* at 4 ^{o}C. The supernatant was transferred to fresh Eppendorf tubes and centrifuged for 10 min at 8000 x *g* at 4 ^{o}C. The resulted supernatant was the cytosolic fraction. Antibodies against FLAG sequence (Cell Signaling, 2368S), β-actin (Cell Signaling, 8457S) and LRPPRC (Sigma, SAB2700419-100UL) were used.

Primary rat hepatocyte experiments

Primary hepatocytes were obtained from the MGH Cell Resource Core, where they were freshly isolated by collagenase perfusion of livers from 24-hour fasted Sprague-Dawley rats and plated at a density of 1.2 x 10^6 cells/well onto 6-well collagen-coated plates in hepatocyte medium obtained from the resource core (DMEM with high glucose and pyruvate (Life Technologies, 11995), supplemented with 0.5 units/mL insulin, 20 ng/mL epidermal growth factor, 14 ng/mL glucagon, 7.5 µg/mL hydrocortisone, 10 % FBS, 1 % penicillin-streptomycin). Twenty-four hours after plating, medium was refreshed and cells were transduced with the indicated adenoviral vector (MOI 20). Twenty-four hours after transduction the cells were washed twice with PBS and placed in glucose, glutamine and pyruvate-free DMEM (Life Technologies, A14430) for two hours. This medium was removed and replaced with fresh glucose, glutamine, pyruvate-free DMEM supplemented with the indicated substrate to a final concentration of 5 mM. Media were sampled two hours later and glucose was measured using the Amplex Red Glucose Assay (Molecular Probes, A22189) and normalized to total protein levels from hepatocytes lysed in 1% SDS as measured by the BCA assay. Separate glucose standardization curves for the Amplex Red assay were created in the presence of the specific gluconeogenic substrate used to compensate for H_2O_2 quenching observed in the presence of pyruvate. Media from the lactate gluconeogenesis experiments were additionally collected for LC-MS measurements as described below. Each replicate reflects hepatocytes isolated from a different rat on an experiment performed on a separate day.

LC-MS method for detection of acetoacetate and β-hydroxybutyrate

One hundred µL of the media sample was pipetted into a 1.5 mL Eppendorf tube, and 20 µL of water (containing 250 µM 2,4-^{13}C2 β-hydroxybutyrate) was added. The samples were vortexed for 10 sec and spun down for 20 sec. Subsequently 400 µL of acetonitrile was added to the tube, vortexed for 10 sec, and transferred to 4 °C for 30 mins. The samples were spun down at 4 °C for 10 mins at maximum speed. Ten µL of the supernatant was injected into the LC-MS. Beta-hydroxybutyrate was quantified based on the isotopic ratio of peak of isotopic labeled standards versus the peak of unlabeled in the real sample. There is no commercially available isotopic labeled standard of acetoacetate. A series of standard solutions of acetoacetate at 6 different concentrations (0.5, 1, 5, 10, 20, 100 µM) were prepared. Then the media samples and standard solutions were prepared the same way as in the above protocol. Acetoacetate was quantified based on the external calibration method. An Xbridge Amide HILIC 100 x 2.1-mm, 2.5 µm particle size, column (Waters, 186006091) was used and the MS was acquired under the negative ionization mode. The LC-MS running condition was the same as for the method used for metabolomics (see below).

Measurements of Reactive Oxygen Species (ROS) in cell lines overexpressing Luciferase, *Lb*NOX and mito*Lb*NOX

Ten-fifteen thousand HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded in 0.2 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10% non-dialyzed FBS (Sigma F2442-500ML)] in a black 96-well plate with clear bottom (Corning, 3904). Twenty four hours after seeding, 10 µl of 6 µg/mL doxycycline (300

ng/mL final concentration) was added to corresponding wells. Twenty four hours after addition of doxycycline, cells were washed in the assay medium (DMEM free from phenol red, pyruvate, folic acid, niacinamide, pyridoxal, riboflavin and thiamine [US Biological, D9800-17), 25 mM HEPES-KOH, pH 7.4, 1 % non-dialyzed FBS (Sigma, F2442-500ML), and 5 mM glucose]. CM-H_2DCFDA Dye (Life Technologies, C6827) was dissolved in DMSO and was added in the assay medium to cells (10 µM final concentration) and cells were incubated at 37 °C in a 5 % CO_2 incubator for 1.5 hour. Cells were then washed once in the assay medium and selected perturbations were added (5 µM antimycin A or 200 µM menadione (vitamin K_3)). Fluorescence (Ex/Em 485 nm/530 nm) was recorded over 3 hours and end-point values were used in the data analysis.

Measurements of H_2O_2 production by cell lines overexpressing Luciferase, *Lb*NOX and mito*Lb*NOX

Ten-fifteen thousand HeLa Tet3G Luciferase, *Lb*NOX or mito*Lb*NOX cells were seeded in 0.2 mL of DMEM [DMEM (US Biological, D9800), 3.7 g/L $NaHCO_3$, 10% undialyzed FBS (Sigma F2442-500ML)] per well in a black 96-well plate with a clear bottom (Corning, 3904). Twenty four hours after seeding, 10 µl of 6 µg/mL doxycycline (300 ng/mL final concentration) was added to corresponding wells. Twenty four hours after addition of doxycycline, cells were washed in the assay medium [DMEM free from phenol red, pyruvate, folic acid, niacinamide, pyridoxal, riboflavin and thiamine (US Biological, D9800-17), 25 mM HEPES-KOH, pH 7.4, 1 % non-dialyzed FBS (Sigma, F2442-500ML), and 5 mM glucose]. To each well, containing cells in 50 µl of the assay medium (± 5 µM antimycin, ± 200 µM menadione (vitamin K_3)) another 50 µl were added (48 µl of the assay buffer, 1 µl of Amplex Red (Abcam, ab102500) and 1 µl of HRP (Abcam, ab102500)). In addition, in separate wells H_2O_2 standards were prepared in the assay medium (0, 1, 2, 3, 4, 5 µM final concentration). Fluorescence (Ex/Em 535 nm/587 nm) was recorded over 3 hours and end-point values were used in the data analysis.

Supplementary Text

Crystal structure of *Lb*NOX

We report here the crystal structure of NADH-specific water-forming oxidase from *L. brevis* at 2.4 Å resolution (Fig. 1E, fig. S3 and Table S1). The overall structure of *Lb*NOX is similar to other reported structures of H_2O-forming NAD(P)H oxidases from *L. sanfranciscensis* (PDB ID 2CDU) and *S. pyogenes* (PDB ID 2BC0) (*14, 15*). Two molecules of *Lb*NOX related by non-crystallographic two-fold symmetry formed a tightly associated dimer [2682 $Å^2$ of buried surface area (BSA)] in which the C-terminal dimerization domain of each monomer penetrated deep into its pair-mate active site containing the isoalloxazine moiety of FAD and the redox active Cys 42 (Fig. 1E, fig. S3). The main chain carbonyl oxygen of Phe 422 in each monomer was hydrogen bonded to the N3 nitrogen of FAD in a pair-mate molecule, an interaction that likely contributes to the catalytic function of *Lb*NOX.

Biochemically, H_2O-forming NAD(P)H oxidases (NOXes) as well as NADH peroxidases (NPXes) are quite similar (*49*). Both enzymes in addition to FAD, contain a non-flavin redox center, a conserved cysteinyl residue (Cys 42 in *Lb*NOX as well as in both NOX and NPX from *E. faecalis*). In all previously reported structures of NOXes (PDB ID 2CDU, 2BC0) and NPX from *E. faecalis* (PDB ID 1NPX, 1JOA) this redox active Cys 42 is found in an oxidized form (a cysteine-sulfenic acid, Cys 42-SOH or a cysteine-sulfonic acid, Cys 42-SO_3H) (*14, 15, 50*). However, in the *Lb*NOX structure we present here, the redox active Cys 42 is found in the reduced form (Cys 42-SH), facing away from the isoalloxazine ring, suggesting that recombinant *Lb*NOX was purified in the reduced state [EH_2 (FAD, Cys 42-SH)] (*51*). In addition, we have directly observed molecular oxygen (O_2) bound in the active site of each monomer (fig. S3A, D). Within the asymmetric unit of the triclinic crystal lattice, two dimers of *Lb*NOX, are linked into a weakly associated tetramer (769 $Å^2$ BSA), consistent with the tetramer found in solution. Thus, the presence of a FLAG tag epitope in our construct did not alter the oligomerization state of the protein which is in agreement with our size-exclusion chromatography experiments.

The substrate specificity of *Lb*NOX towards NADH likely results from the presence of Asp 177, which would clash with the phosphate moiety of NADPH, and the lack of positively charged residues in positions 178, 179 and 184, which are required to stabilize NADPH binding (fig. S3B, C).

Metabolic profiling

We have measured the concentration of 100 metabolites in fresh media and in media incubated for 24 hours with HeLa Tet3G *Lb*NOX or HeLa Tet3G mito*Lb*NOX cells treated with DMSO or 1 μM antimycin in the presence or absence of doxycycline. Under our experimental conditions, we could reliably measure consumption and production rate of 40 metabolites because their concentration after 24-hour incubation with cells was significantly different from their concentration in fresh media (Student's *t*-test; $P < 0.01$).

We identified pyruvate, aspartate, succinate, asparagine, xylitol (or ribitol) and N-acetylputrescine as 6 out of 40 metabolites whose consumption or production rate was significantly affected (Student's *t*-test; $P < 0.01$) by either *Lb*NOX or mito*Lb*NOX (Fig. 3D, fig. S6A-B and Table S2). Xylitol and Ribitol are stereoisomers and were not

separated using our LC-MS method. Production of pyruvate, aspartate and succinate was impacted more than 2-fold (Fig. 3D). The effect of *Lb*NOX and mito*Lb*NOX on the rate of pyruvate, aspartate and succinate production can be readily explained be the effect of *Lb*NOX and mito*Lb*NOX on cytosolic and mitochondrial NAD^+/NADH ratios. Pyruvate concentration is controlled by cytosolic NAD^+-dependent lactate dehydrogenase and secretion of pyruvate was increased to the same degree in cells expressing *Lb*NOX or mito*Lb*NOX, in agreement with their effect on cytosolic NAD^+/NADH ratios. Both *Lb*NOX and mito*Lb*NOX increased aspartate secretion, most likely because it is produced from oxaloacetate using aspartate aminotransferase and amounts of oxaloacetate are controlled by the NAD^+-dependent enzyme malate dehydrogenase. Furthermore, aspartate production was increased in cells expressing mito*Lb*NOX significantly more (One way ANOVA followed by Tukey's multiple comparisons test; $P < 0.0001$) than it was in cells expressing *Lb*NOX. This may occur because there are mitochondrial and cytosolic paralogs of malate dehydrogenase and aspartate aminotransferase, and because mito*Lb*NOX but not *Lb*NOX can increase NAD^+/NADH ratios, and thus activate aspartate production, in both compartments. Succinate is produced downstream of the mitochondrial NAD^+-dependent enzyme α-ketoglutarate dehydrogenase and its secretion was only increased in cells expressing mito*Lb*NOX, in agreement with our observation that only mito*Lb*NOX perturbs mitochondrial NAD^+/NADH ratios.

We identified pyruvate, asparagine, aspartate, malate, fumarate, α-ketoglutarate, serine, xylitol (or ribitol), galactitol (or mannitol) and deoxyuridine as 10 out of 40 metabolites whose consumption or production rate was significantly affected (Student's *t*-test; $P < 0.01$) by antimycin in both HeLa Tet3G *Lb*NOX and HeLa Tet3G mito*Lb*NOX cells in the absence of doxycycline (fig. S8, Table S2). Galactitol or Mannitol are stereoisomers and were not separated using our LC-MS method. Production or consumption of pyruvate, asparagine, aspartate, malate, fumarate, α-ketoglutarate, serine, xylitol (or ribitol) and galactitol (or mannitol) was impacted more than 2-fold by antimycin treatment. *Lb*NOX or mito*Lb*NOX rescued consumption or production rate of all of the metabolites affected by antimycin treatment indicating that these changes in metabolic fluxes are caused by the effect of antimycin on NAD^+/NADH ratio and not on proton pumping activity of ETC (fig. S8).

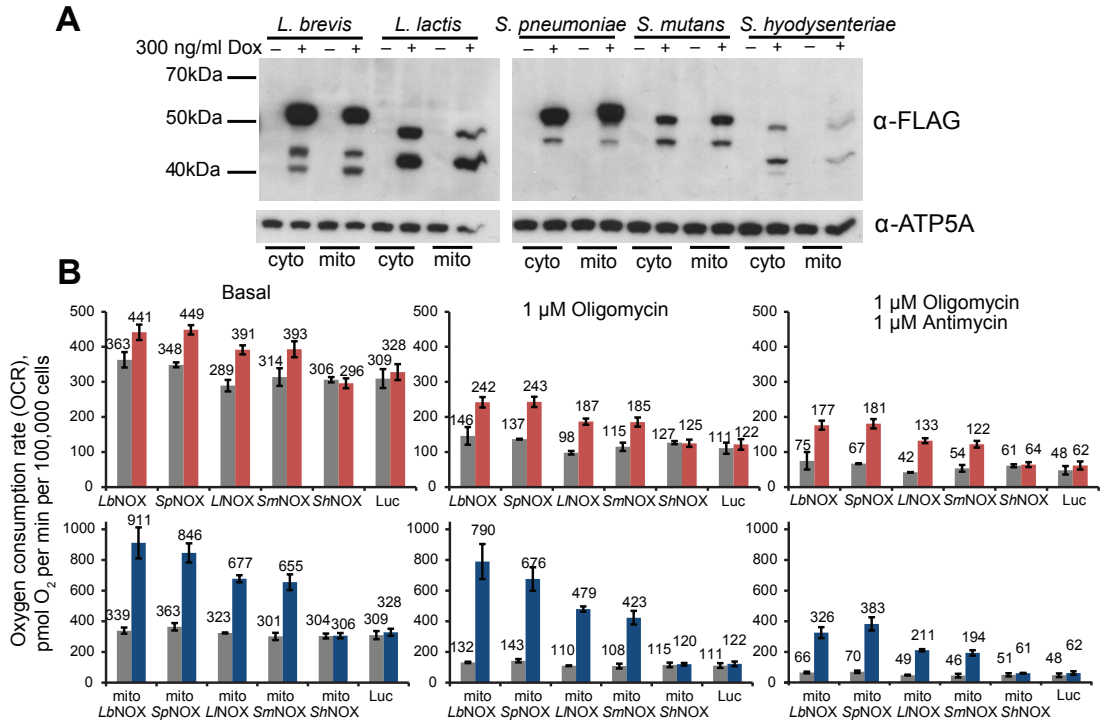

Fig. S1. Heterologous expression and activity of H_2O-forming NADH oxidases in HeLa Tet3G cells.

(A) Doxycycline-inducible expression of H_2O-forming NADH oxidases from *L. brevis*, *L. lactis*, *S. pneumoniae*, *S. mutans* and *S. hyodysenteriae* in HeLa Tet3G cells. **(B)** Oxidase activity of overexpressed NADH oxidases based on their oligomycin and antimycin-resistant oxygen consumption rates. Mean values ± S.E., *n*=4 (for *Lb*NOX and mito*Lb*NOX) or *n*=3 (other NOXs) independent experiments. In grey: cells without doxycycline (Dox) addition. In red, blue: HeLa Tet3G NOX (red) or mitoNOX (blue) cells treated with doxycycline.

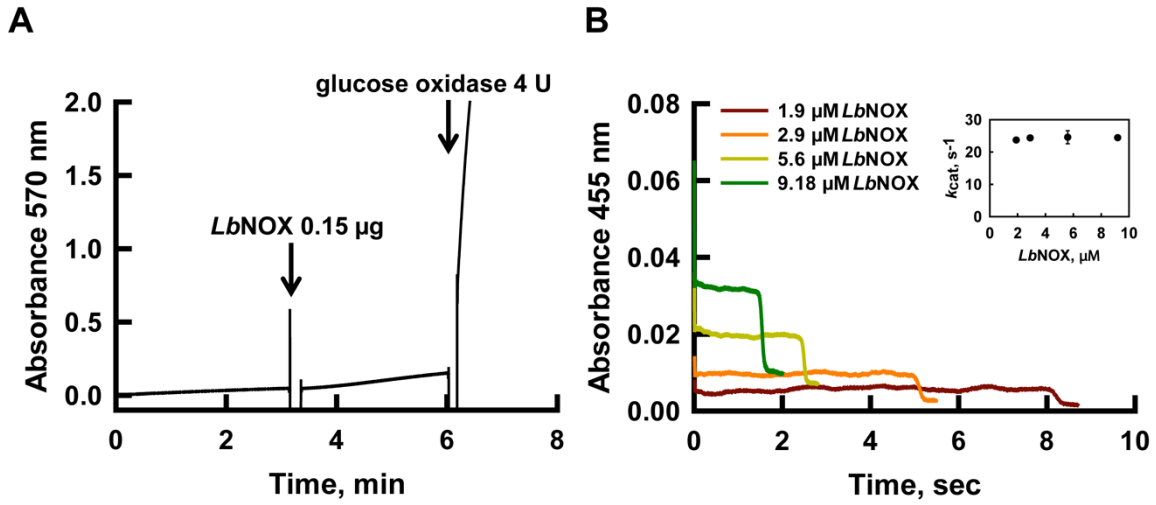

Fig. S2. Biochemical characterization of recombinant *Lb*NOX.

(A) H_2O_2-formation by *Lb*NOX monitored in Amplex Red-based continuous assay at 37°C. Reaction mixture contained 50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl, 350 µM NADH, super oxide dismutase (SOD) 130 U, 100 µM D-glucose and Amplex Red/HRP. At the times indicated *Lb*NOX and glucose oxidase were added. Glucose oxidase was used to validate the assay, since this enzyme produces H_2O_2 from oxygen and D-glucose. Rates of H_2O_2-forming activity of *Lb*NOX were compared to rates of NADH disappearance in parallel experiments. It was estimated that the rate of H_2O_2 formation is 1.7 ± 0.3 % of the rate of NADH conversion to NAD^+ based on four (n=4) independent experiments. A representative trace is shown. **(B)** Determination of K_m for O_2 and k_{cat} based on enzyme-monitored turnover. The experiment was carried out by monitoring the time dependence of FAD oxidation via its absorbance at 455 nm in 50 mM Na_2HPO_4, pH 7.5, 150 mM NaCl at 4 °C, as enzyme (1.9, 2.9, 5.6 and 9.18 µM FAD active sites after mixing) was mixed with a high excess of NADH (2 mM after mixing) in a stopped-flow apparatus. Note that even at the lowest enzyme concentration used (1.9 µM) the steady-state phase of the reaction ends abruptly when all O_2 is consumed. This indicates that apparent K_m for oxygen is significantly lower than 1.9 µM. A representative trace is shown. *Inset:* calculated apparent k_{cat} at 4 °C based on three (n=3) independent experiments.

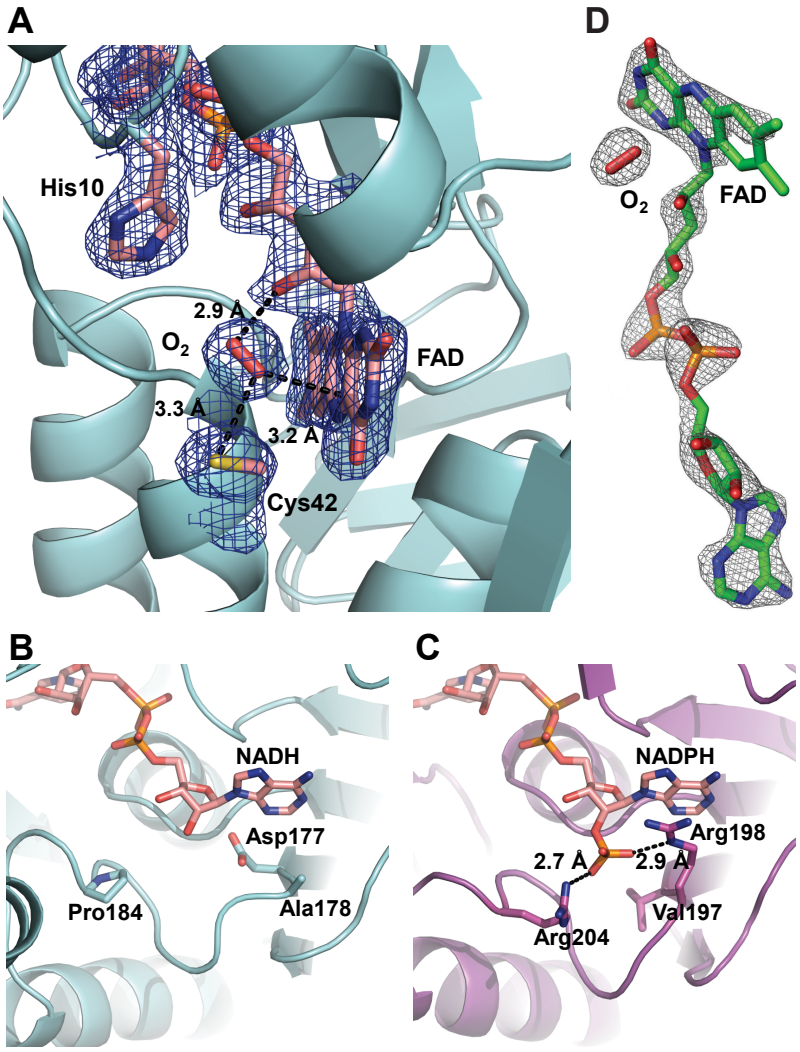

Fig. S3. Substrate binding sites of *Lb*NOX.

(A) Molecular oxygen in the active site of *Lb*NOX (PDB ID 5ER0). $2F_o$-F_c electron density map around FAD contoured at 1.5 σ. His 10, Cys 42, FAD and O_2 are depicted in sticks representation. Dashed black lines depict hydrogen bonds. **(B-C)** A model of NADH-*Lb*NOX complex (B) was generated based on the structural similarity with glutathione reductase bound to NADPH (PDB ID 1GET, shown in **(**C**)**). The NAD(P)H binding domains of each protein (residues 147-247 of *Lb*NOX and 167-268 of glutathione reductase) were superimposed using the least square algorithm of COOT (*42*). Note that Arg 198 and Arg 204 of glutathione reductase, which tightly bind the phosphate moiety in the 2' position of NADPH, are replaced in *Lb*NOX with Ala 178 and Pro 184, respectively. Also, the negatively charged side chain of Asp 177 in *Lb*NOX replaces the neutral Val 197 of glutathione reductase. The combined effects of these substitutions make NADPH an extremely poor substrate for *Lb*NOX. **(D)** Representative sigma-A weighted composite-annealed omit $2F_o$-F_c electron density map for FAD and molecular oxygen bound to *Lb*NOX (monomer A of 5ER0). The map was contoured at 1.5 σ.

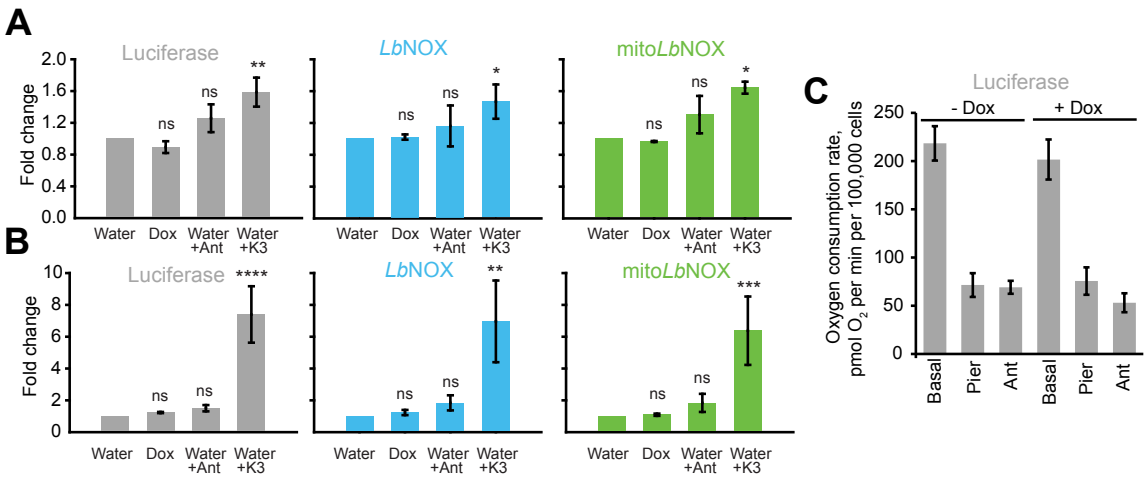

Fig. S4. The effect of overexpression of Luciferase, *Lb*NOX and mito*Lb*NOX in HeLa Tet3G cells on the production of ROS, H_2O_2 and oxygen consumption.

(A) Measurements of reactive oxygen species (ROS) based on reaction with CM-H_2DCFDA**.** Ninety six-well plates with cells expressing Luciferase, *Lb*NOX or mito*Lb*NOX (± doxycycline (Dox)) were incubated in Phenol Red-free medium with 10 µM CM-H_2DCFDA for 1.5 h. After perturbations were added (200 µM menadione (vitamin K3) or 5 µM antimycin A (Ant)) fluorescence was measured (ex/em 485/530 nm). Menadione and antimycin were used as positive controls. One-way ANOVA followed by Dunnett's multiple comparisons test. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$. Mean ± S.D., n=3 independent experiments. **(B)** Measurements of H_2O_2 with Amplex Red. Ninety six-well plates with cells expressing Luciferase, *Lb*NOX or mito*Lb*NOX (± doxycycline (Dox)) were transferred into Phenol Red-free medium and corresponding perturbations (200 µM menadione (K3) or 5 µM antimycin (Ant)) were added. After Amplex Red/HRP was added to cells, fluorescence was measured (ex/em 535/587 nm). Menadione and antimycin were used as positive controls. One-way ANOVA followed by Dunnett's multiple comparisons test. ns $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P <$ 0.0001. Mean ± S.D., n=3 independent experiments. **(C)** Effect of doxycycline-inducible Luciferase expression on basal, piericidin (Pier) -resistant and antimycin (Ant)-resistant oxygen consumption measured with XF24 extracellular flux analyzer. Mean ± S.E., n=3 independent experiments.

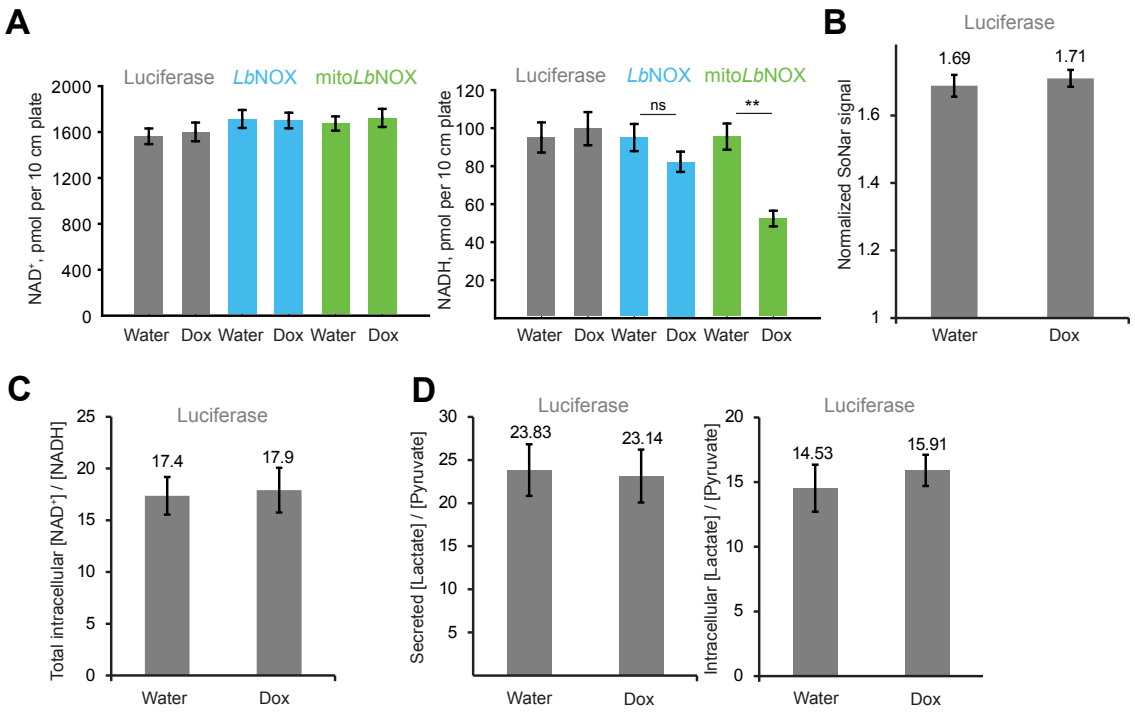

Fig. S5. Effect of expression of Luciferase, *Lb*NOX or mito*Lb*NOX in HeLa Tet3G cells on NAD^+/NADH ratio.

(A) Effect of doxycycline-inducible Luciferase, *Lb*NOX and mito*Lb*NOX expression in HeLa Tet3G Luciferase, *Lb*NOX and mito*Lb*NOX cells on total amounts of NAD^+ and NADH in confluent 10 cm^2 plate of cells determined by HPLC. Mean ± S.E., n=4 independent experiments. Student's t-test. ** $P < 0.01$, ns $P > 0.05$. **(B)** Effect of doxycycline-inducible Luciferase expression in HeLa Tet3G Luciferase cells on fluorescence signal of the cytoplasmic NADH sensor, SoNar. Mean ± S.E., n=6 independent experiments. **(C)** Effect of doxycycline-inducible Luciferase expression in HeLa Tet3G Luciferase cells on NAD^+/NADH ratio determined by HPLC. Mean ± S.E., n=4 independent experiments. **(D)** Effect of doxycycline-inducible Luciferase expression in HeLa Tet3G Luciferase cells on secreted lactate/pyruvate ratio and intracellular lactate/pyruvate ratio. Mean ± S.E., n=4 independent experiments.

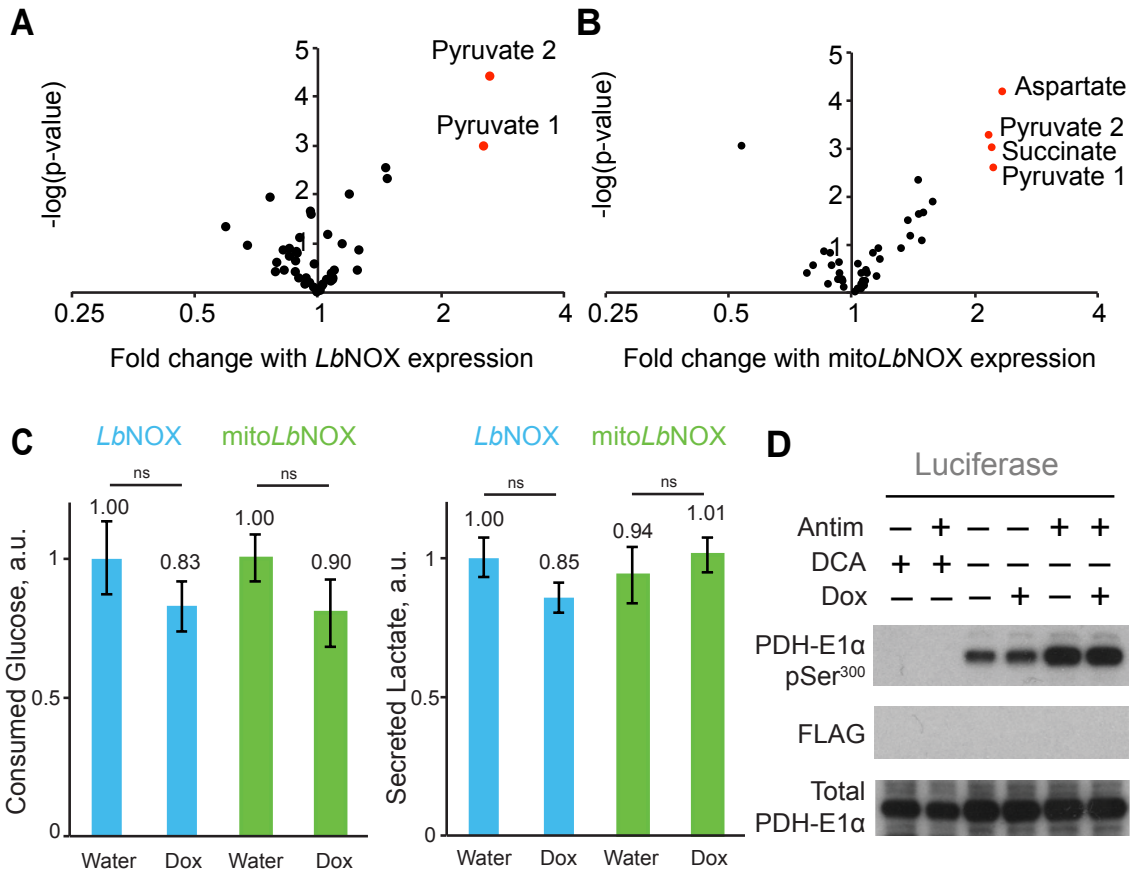

Fig. S6. Effect of Luciferase, *Lb*NOX or mito*Lb*NOX expression on metabolic fluxes and PDH phosphorylation.

(A-B) Effect of doxycycline-inducible *Lb*NOX (A) and mito*Lb*NOX (B) expression in HeLa Tet3G *Lb*NOX and mito*Lb*NOX cells on consumption and production of metabolites in spent media after 24-hour incubation. Volcano plots of the data from Table S2 are shown. Metabolites labeled in red are metabolites that significantly change > 2-fold (Student's *t*-test. $P < 0.01$). Fold change was calculated using consumption and secretion rates determined by subtracting peak intensity of a metabolite present in fresh media from peak intensity of a metabolite after 24-hour incubation with cells. Only 40 metabolites that change significantly compared to fresh media (Student's *t*-test. $P < 0.01$) after 24-hour incubation with cells are shown. Data is average of three replicates from one experiment. **(C-D)** Effect of doxycycline-inducible *Lb*NOX and mito*Lb*NOX expression in HeLa Tet3G *Lb*NOX and mito*Lb*NOX cells on secretion and consumption rates of lactate and glucose. Student's *t*-test. ns $P > 0.05$.

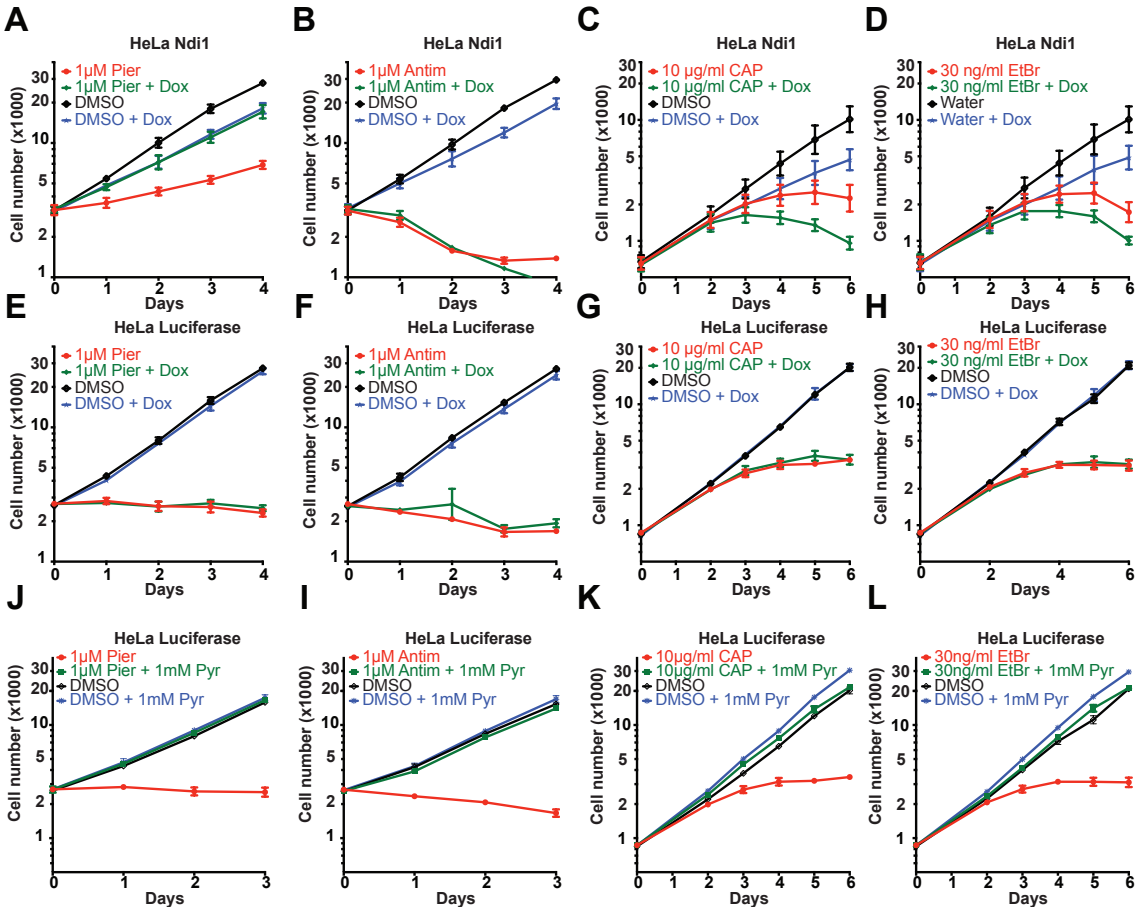

Fig. S7. Effect of Ndi1 and Luciferase expression and pyruvate addition on cell proliferation in the presence of ETC inhibitors.

(A-D) The effect of doxycycline-inducible Ndi1 **(E-H)** and Luciferase expression and **(J-L)** pyruvate addition on piericidin (A, E, J), antimycin (B, F, I), chloramphenicol (C, G, K) and ethidium bromide (D, H, L) induced proliferative defect in HeLa Tet3G Ndi1 or Luciferase cells in the presence of 200 μM uridine. Mean ± S.E., *n*=3 (A, B, E-L) or *n*=5 (C, D) independent experiments.

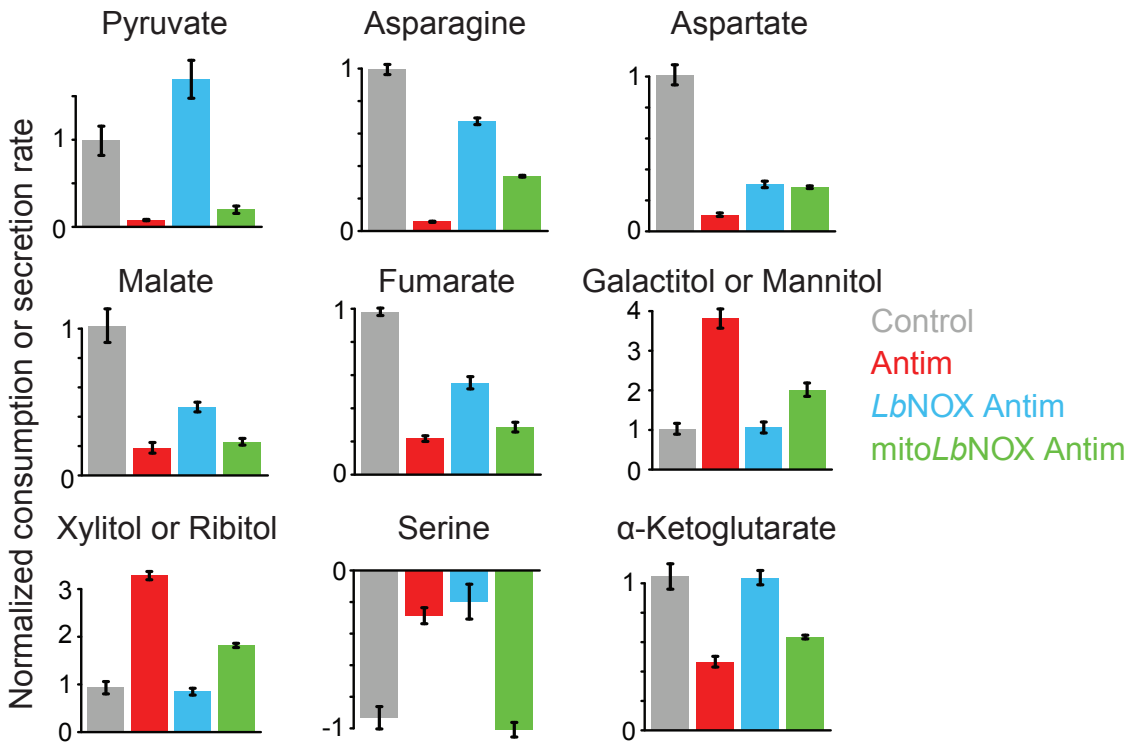

Fig. S8. Effect of antimycin on uptake and release of metabolites in the presence and absence of *Lb*NOX or mito*Lb*NOX.

HeLa Tet3G *Lb*NOX and mito*Lb*NOX cells were pretreated with doxycycline or water for 24 hours, treated with 1 μM antimycin or DMSO for 24 hours in fresh media and spent media was analyzed using LC-MS (Table S2). Only the metabolites whose uptake or secretion is significantly affected by antimycin > 2-fold are shown. (Student's *t*-test. $P < 0.01$). Consumption and secretion rates were calculated by subtracting peak intensity of a metabolite present in fresh media from peak intensity of a metabolite after 24-hour incubation with cells and values were normalized to average of HeLa Tet3G *Lb*NOX and mito*Lb*NOX cells without doxycycline. Control and Antim samples are HeLa Tet3G *Lb*NOX without doxycycline in the absence or presence of 1 μM antimycin. Mean ± S.E., n=3 replicates from one experiment.

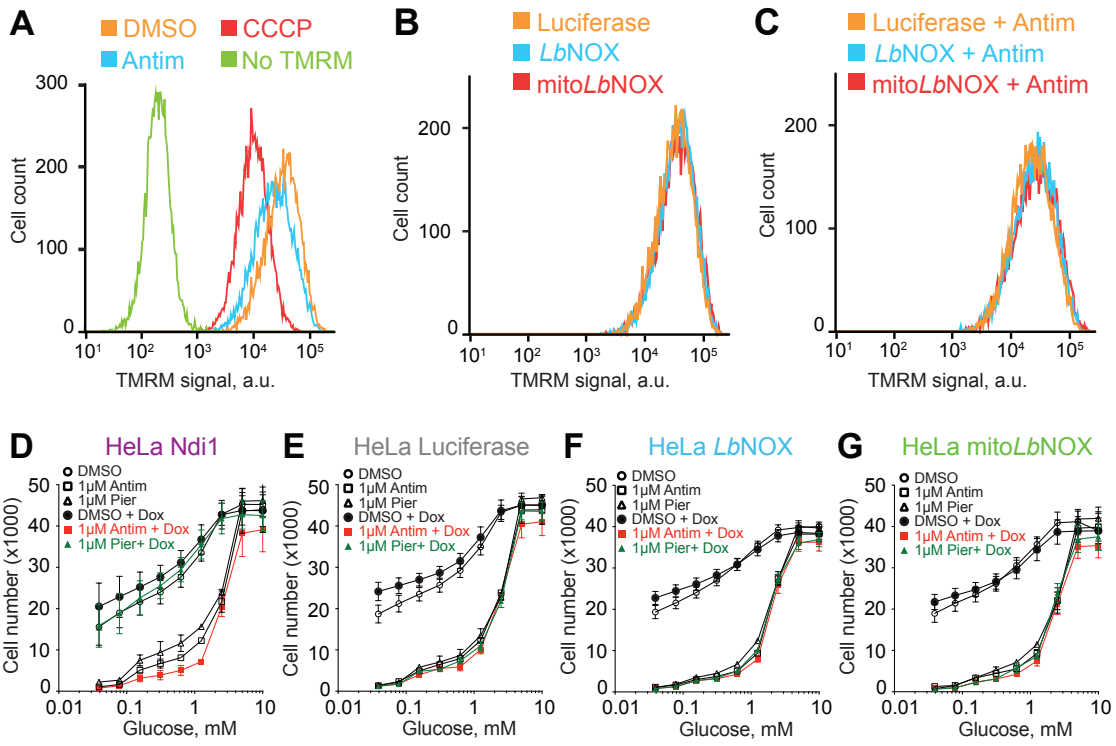

Fig. S9. Effect of *Lb*NOX and mito*Lb*NOX on mitochondrial membrane potential and ETC-derived ATP synthesis.

(A) Effect of CCCP and antimycin on mitochondrial membrane potential measured using flow cytometry and TMRM dye. Representative data from one of the 3 independent experiments. **(B-C)** Effect of doxycycline-inducible *Lb*NOX and mito*Lb*NOX expression in HeLa Tet3G *Lb*NOX and mito*Lb*NOX cells on mitochondrial membrane potential in the presence and absence of 1 µM antimycin determined using flow cytometry with TMRM dye. Representative data from one of the 3 independent experiments. **(D-G)** Effect of doxycycline-inducible Ndi1, Luciferase, *Lb*NOX and mito*Lb*NOX expression on HeLa Tet3G Ndi1, Luciferase, *Lb*NOX and mito*Lb*NOX cell survival, respectively, at various glucose concentrations in the presence of 200 µM uridine and in the presence or absence of 1 µM piericidin or 1 µM antimycin. Mean ± S.E., *n*=3 independent experiments. We expressed piericidin-resistant NADH-quinone oxidoreductase (Ndi1) from *S. cerevisiae* as a positive control. Ndi1 restores NAD^+ recycling by bypassing complex I while also promoting proton pumping at complexes III and IV (*52*). Ndi1 restored survival and proliferation in the presence of piericidin, but not with other ETC inhibitors at low glucose concentration and in the absence of pyruvate, respectively (fig. S7A-D).

Table S1. X-ray Data Collection and Structure Refinement Statistics[a]

Protein	*Lb*NOX (NADH oxidase)
Organism	*Lactobacillus brevis*
PDB ID	5ER0
Data collection	
Space group	P1
Cell dimensions	
a, b, c (Å)	67.9, 86.3, 93.3
α, β, γ (°)	96.9, 94.0, 92.4
Resolution (Å)	48.03-2.40, (2.49-2.40)[b]
Unique reflections	79,832 (7488)
R_{sym} (%)	11.2 (43.0)
R_{meas} (%)	13.0 (50.5)
$R_{p.i.m}$ (%)	6.6 (26.4)
$\langle I/\sigma(I) \rangle$	9.5 (2.1)
Wilson B-factor (Å^2)	25.2
Completeness (%)	98.0 (91.8)
Multiplicity	3.9 (3.5)
Refinement	
R_{work}	0.17 (0.22)
R_{free}	0.21 (0.28)
Reflections used in refinement	79,720 (7483)
Number of non-hydrogen atoms	14,950
protein	13,736
ligands (FAD, OXY)	220
Protein residues	1800
Number of monomers in ASU	4[c]
r.m.s.d. on bond lengths (Å)[d]	0.002
r.m.s.d. on bond angles (°)	0.46
Ramachandran statistics (%)	
favorite	97.0
allowed	3.0
disallowed	0.0
Clashscore	2.92
Average B-factor (Å^2)	28.7
protein	28.6
ligands (FAD, OXY)	22.9
solvent	31.0
Rotamer outliers (%)	0.53

[a] Data were collected from a single crystal.
[b] Values in parentheses represent the highest-resolution shell.
[c] Two noncrystallographic dimers.
[d] r.m.s.d. from ideal values.
Definitions of crystallographic data quality indicators listed in Table S1:

$$R_{sym} = \frac{\sum_{hkl}\sum_{j} | I_{hkl,j} - \langle I_{hkl} \rangle |}{\sum_{hkl}\sum_{j} I_{hkl,j}}$$, where $\langle I_{hkl} \rangle$ is the average of symmetry related observations.

$$R_{meas} = \frac{\sum_{hkl} \sqrt{n/(n-1)} \sum_{j=1}^{n} | I_{hkl,j} - \langle I_{hkl} \rangle |}{\sum_{hkl}\sum_{j} I_{hkl,j}}$$

$$R_{p.i.m} = \frac{\sum_{hkl} \sqrt{1/(n-1)} \sum_{j=1}^{n} | I_{hkl,j} - \langle I_{hkl} \rangle |}{\sum_{hkl}\sum_{j} I_{hkl,j}}$$

R_{work} and R_{free} are calculated for the working and reference sets of reflections, respectively, as follows:

$$R = \frac{\sum_{hkl} | F_{hkl}^{obs} - F_{hkl}^{calc} |}{\sum_{hkl} F_{hkl}^{obs}}$$

Table S2. Metabolomics data.

See the Microsoft Excel file submitted separately.

Table S3. Formulations of cell culture media used in the study.

	DMEM US Biological D9800	DMEM US Biological D9800-02	DMEM US Biological D9802	DMEM US Biological D9800-17	DMEM Life Technolog. 11995	DMEM Life Technolog. A14430
	g/L	g/L	g/L	g/L	g/L	g/L
Inorganic Salts:						
Calcium Chloride•2H2O	0.265	0.265	0.265	0.265	0.200 CaCl2(anhyd.)	0.200 CaCl2(anhyd.)
Ferric Nitrate•9H2O	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Magnesium Sulfate	0.09767	0.09767	0.09767	0.09767	0.09767	0.09767
Potassium Chloride	0.4	0.4	0.4	0.4	0.4	0.4
Sodium Chloride	6.4	6.4	6.4	6.4	6.4	6.4
Sodium Phosphate Monobasic	0.109	0.109	0.109	0.109	0.125 NaH2PO4-H2O	0.125 NaH2PO4-H2O
Sodium Bicarbonate	Absent	Absent	Absent	Absent	3.7	3.7
Amino Acids:						
L-Arginine•HCl	0.084	0.084	0.084	0.084	0.084	0.084
L-Cystine•2HCl	0.0626	0.0626	0.0626	0.0626	0.063	0.063
L-Glutamine	0.584	Absent	0.584	0.584	0.584	Absent
Glycine	0.03	0.03	0.03	0.03	0.03	0.03
L-Histidine•HCl•H2O	0.042	0.042	0.042	0.042	0.042	0.042
L-Isoleucine	0.105	0.105	0.105	0.105	0.105	0.105
L-Leucine	0.105	0.105	0.105	0.105	0.105	0.105
L-Lysine•HCl	0.146	0.146	0.146	0.146	0.146	0.146
L-Methionine	0.03	0.03	0.03	0.03	0.03	0.03
L-Phenylalanine	0.066	0.066	0.066	0.066	0.066	0.066
L-Serine	0.042	0.042	0.042	0.042	0.042	0.042
L-Threonine	0.095	0.095	0.095	0.095	0.095	0.095
L-Tryptophan	0.016	0.016	0.016	0.016	0.016	0.016
L-Tyrosine•2Na•2H2O	0.10379	0.10379	0.10379	0.10379	0.104	0.104
L-Valine	0.094	0.094	0.094	0.094	0.094	0.094
Vitamins:						
Choline Chloride	0.004	0.004	0.004	0.004	0.004	0.004
Folic Acid	0.004	0.004	0.004	Absent	0.004	0.004
myo-Inositol	0.0072	0.0072	0.0072	0.0072	0.0072	0.0072
Niacinamide	0.004	0.004	0.004	Absent	0.004	0.004
D-Pantothenic Acid, Ca	0.004	0.004	0.004	0.004	0.004	0.004
Pyridoxal•HCl	0.004	0.004	0.004	Absent	0.004	0.004
Riboflavin	0.0004	0.0004	0.0004	Absent	0.0004	0.0004
Thiamine•HCl	0.004	0.004	0.004	Absent	0.004	0.004
Other:						
D-Glucose	1	Absent	1	Absent	4.5	Absent
Pyruvic Acid, Sodium	0.11	0.11	Absent	Absent	0.11	Absent
Phenol Red, Sodium	0.0159	0.0159	Absent	Absent	0.015	Absent