Supporting Information

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SI Methods

Reagents. Hygromycin (Gold Biotechnology) and kanamycin (Gold Biotechnology) were used at concentrations of 75 mg/L and 20 mg/L for mycobacteria and 150 mg/L and 40 mg/L for *Escherichia coli*, respectively. PBS (phosphate buffer saline) was obtained from Corning cellgro. All other chemicals were obtained from Sigma or Fisher.

Construction and Unmarking of the Deletion Strains. The *ndh, ndhA*, and the full operon *nuoAN* were deleted from *Mtb* CDC1551 and mc^{2}6230 (H37Rv Δ*RD1*Δ*panCD*) using the specialized transduction system (1). Table S3 lists all of the plasmids and phages used for the construction of the knockout strains. Briefly, the left and right flank of each gene or operon were amplified by PCR (see primers in Table S2) and cloned into pYUB1471 using the indicated enzymes (Table S2). The cosmids were linearized with *pac*I, ligated into *pac*I-digested phAE159, and packaged into lambda phage particles in vitro (GigapackII; Stratagene), which were used to transduce *E. coli* HB101 cells. The resulting phasmids phAE237, phAE804, and phAE805 were electroporated into mc^{2}155, and high titer phage lysates were prepared to transduce the *Mtb* strains. The hygromycin cassette was excised from the deletion strains using the phage phAE280 and sucrose selection as previously described (1). The deletion strains were confirmed by Southern analysis and whole-genome sequencing performed on a MiSeq Instrument (Illumina) following the protocol provided by Illumina.

Complementation of Deletion Strains. The complementing plasmid pYUB2722 (Table S3) was obtained by PCR amplification of the *ndhA* gene from *Mtb* H37Rv using the primers listed in Table S2. The PCR product was cloned downstream of the *hsp60* promoter of the *E. coli*–mycobacteria shuttle plasmid integrative vector pMV361, using the cloning sites indicated in Table S2, and sequenced. The plasmids were introduced into the deletion strains by electroporation. Briefly, the cultures (10 mL) were grown at 37 °C to log phase ($OD_{600\ nm} \approx 1$) and spun down. The cell pellets were washed twice with a 10% aqueous glycerol solution (10 mL) and resuspended in 0.2 mL of 10% aqueous glycerol solution. The cell suspensions (0.17 mL) with the appropriate complementing plasmid (2 μL) were electroporated using the following settings: 2.5 V, 25 μF, and 1,000 Ω. The cell suspensions were added to 1 mL of Middlebrook 7H9 media (see above) and incubated at 37 °C for 24 h before plating on kanamycin-containing plates.

Quantitative Real-Time PCR. The relative expression of *ndh, ndhA*, and *nuoH* was measured by RT-qPCR. Triplicate cultures (10 mL) of *Mtb* CDC1551, CDC1551 Δ*ndh*, CDC1551 Δ*ndhA*, CDC1551 Δ*nuoAN*, CDC1551 Δ*ndh*Δ*nuoAN*, CDC1551 Δ*ndhA*Δ*nuoAN*, CDC1551 Δ*ndh* pMV361::*ndh*, and CDC1551 Δ*ndhA* pMV361::*ndhA* were grown to an $OD_{600\ nm} \approx 0.1$ at 37 °C and centrifuged, and the cell pellets were resuspended in 1 mL Qiagen RNA Protect reagent (Qiagen) for 24 h. The suspensions were centrifuged and resuspended in 1 mL buffer RLT from a Qiagen RNeasy kit. The suspension was transferred to Fast-Prep Blue Cap tubes and processed for 45 s at speed 6 in a Fast-Prep apparatus (MP Bio). After cooling on ice, the suspensions were centrifuged, and the supernatant (~750 μL) was removed to a fresh microcentrifuge tube containing 0.25 mL of absolute ethanol. The samples were applied to RNeasy columns, and RNA was purified, as recommended by the Qiagen protocol. RNA yield, purity, and integrity were checked on a Nanodrop spectrophotometer (Nanodrop Products). Contaminating DNA was removed with Ambion TURBO DNA-free (Ambion), according to the manufacturer's instructions. The DNA-free RNA samples were reverse-transcribed in 20-μL reactions containing ~400 ng RNA, 3 μg random hexamers, and 500 μM final dNTPs. Reactions were incubated for 5 min at 65 °C and for 1 min on ice. The first strand buffer, 5 mM DTT, 1 μL RNaseOUT, and 1 μL SuperScript III RT, were then added and left for 5 min at room temperature, followed by a 1-h incubation at 50 °C. For the real-time reaction, each primer (250 nM final concentration; Table S2) and 7.5 μL of template reaction (1:20 dilution) in 25 μL volume with Applied Biosystems Power SYBR Green PCR master mix (Applied Biosystems) were used. Samples (8 μL) were loaded in triplicate in 384-well plates and run on an ABI 7900 HT quantitative thermocycler with the following program: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 10 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C, followed by a dissociation stage of 15 s at 95 °C, 15 s at 60 °C, and 15 s at 95 °C to check specificity of the products. Threshold cycles were normalized to those for 16S rRNA.

Pathology. Pathology analysis was done on the left lungs at the indicated time points and was performed at the Histopathology and Comparative Pathology facility of the Albert Einstein College of Medicine.

Statistical Analysis. All in vitro experiments were performed in triplicate, and graphs show data expressed as mean ± SD. Student's unpaired *t* test (two-tailed) was used for pairwise comparisons. *P* values were calculated using GraphPad Prism 7.01 software (GraphPad Software, Inc.).

1. Jain P, et al. (2014) Specialized transduction designed for precise high-throughput unmarked deletions in *Mycobacterium tuberculosis*. *MBio* 5:e01245-14.

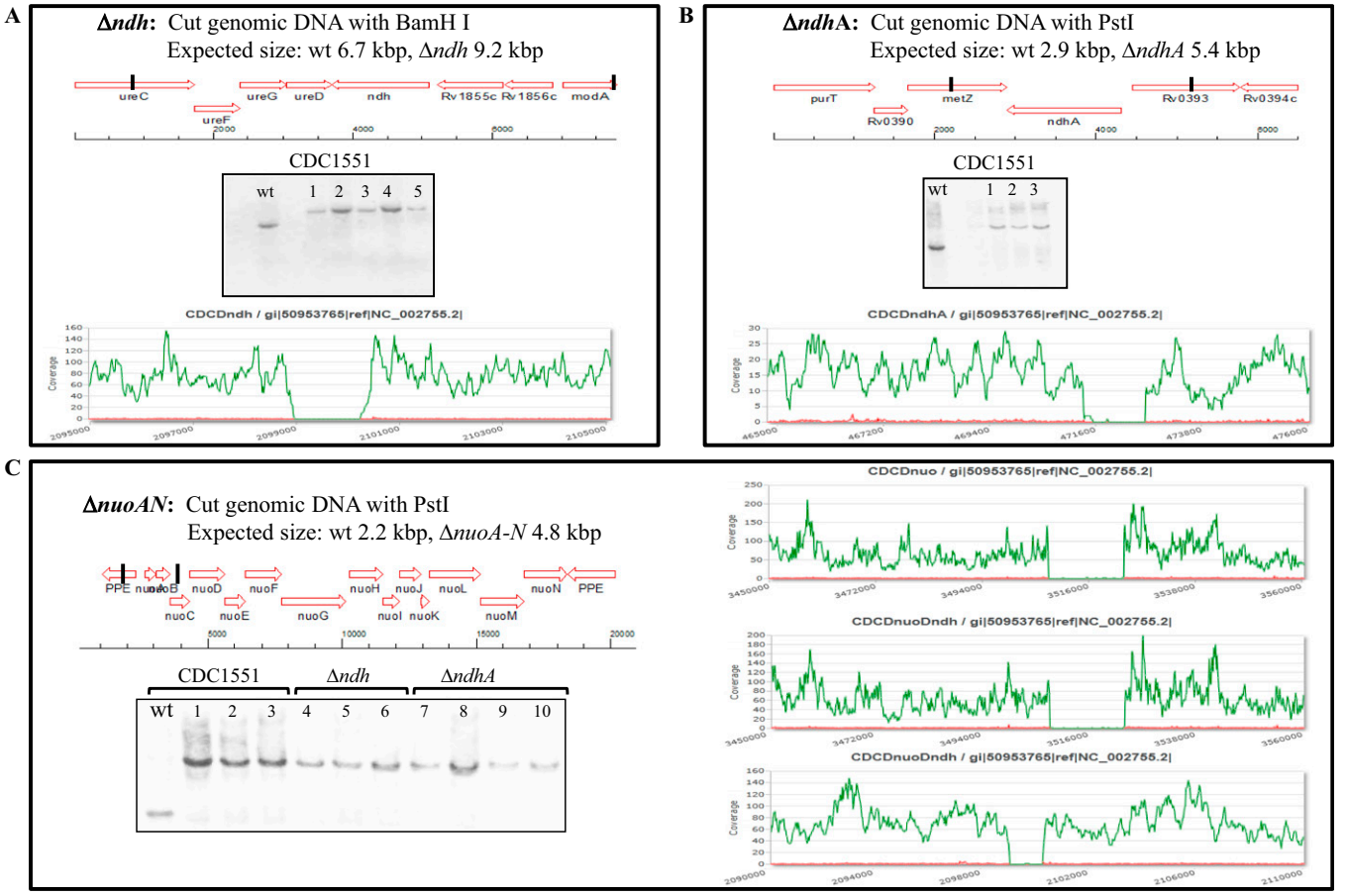

Fig. S1. The single- and double-NADH dehydrogenase knockouts were confirmed by Southern and whole-genome sequencing analyses. The transductants were grown in Middlebrook 7H9 as described in *Methods* to stationary phase. Genomic DNA was extracted following published protocols (1) and cut with *Bam*HI (*A*) or PstI (*B* and *C*). Southern analysis was performed on the cut fragments using ECL Direct Nucleic acid labeling and detection System (GE Healthcare Health Sciences). Numbers on the Southern represent the different transductants tested. WT stands for wild type (CDC1551). Whole-genome sequencing of CDC1551 Δ*ndh,* CDC1551 Δ*ndhA,* CDC1551 Δ*nuoAN,* and CDC1551 Δ*ndh*Δ*nuoAN* was performed on a MiSeq instrument. The regions shown in *A*, *B*, and *C* correspond to the deletion described. The region deleted in Δ*ndh* ranges from 2098970 to 2100234 (1,264 bp), 471576–472656 (1,080 bp) in Δ*ndhA*, and 3507304–3522873 (15,569 bp) in Δ*ndh*Δ*nuoAN*.

1. Larsen MH, Biermann K, Tandberg S, Hsu T, Jacobs WR, Jr (2007) Genetic manipulation of *Mycobacterium tuberculosis*. *Curr Protoc Microbiol* Chapter 10:Unit 10A.2.

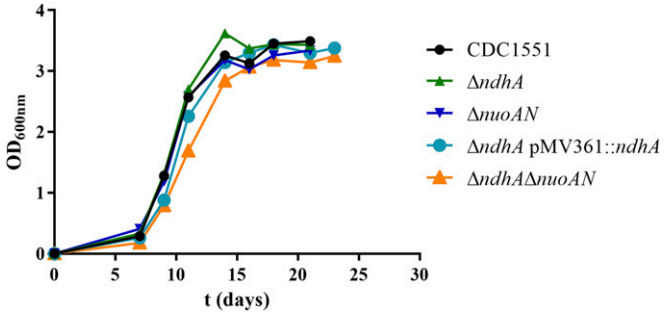

Fig. S2. Δ*ndhA,* Δ*nuoAN*, and Δ*ndhA*Δ*nuoAN* have no growth defect in vitro. CDC1551, Δ*ndhA*, Δ*nuoAN*, and Δ*ndhA*Δ*nuoAN* were grown in Middlebrook 7H9, supplemented with ADS, glycerol, and tyloxapol to midlog ($OD_{600\ nm} \approx 0.8$) and diluted 1/50. Growth was followed by measuring $OD_{600\ nm}$ over time. The graph shows a single replicate, which is representative of two independent experiments.

Table S1. *Mtb* strains used in this study

Strain	Description	Construction	Source
Parental strain mc²6230 (H37Rv ΔRD1ΔpanCD)			
mc²5871	Δ*ndh* γδ(*sacB-hyg*) γδ	ST* of mc²6230with phAE237	1
mc²5872	Δ*ndhA* γδ(*sacB-hyg*) γδ	ST* of mc²6230with phAE804	1
mc²8453	Δ*nuoAN* γδ(*sacB-hyg*) γδ	ST* of mc²6230with phAE805	This work
mc²8454	Δ*ndh*	Unmarking of mc²5871 with phAE280	This work
mc²8455	Δ*ndhA*	Unmarking of mc²5872 with phAE280	This work
mc²8456	Δ*nuoAN*	Unmarking of mc²8453 with phAE280	This work
mc²8457	Δ*ndh*Δ*nuoAN* γδ(*sacB-hyg*) γδ	ST* of mc²8456 with phAE237	This work
mc²8458	Δ*ndhA*Δ*nuoAN* γδ(*sacB-hyg*) γδ	ST* of mc²8456 with phAE804	This work
mc²8459	Δ*ndh*Δ*nuoAN*	Unmarking of mc²8457 with phAE280	This work
mc²8460	Δ*ndhA*Δ*nuoAN*	Unmarking of mc²8458 with phAE280	This work
mc²8461	Δ*ndh*::P_{hsp60} *ndh*	pYUB2721 transformed in mc²8454	This work
mc²8462	Δ*ndhA*::P_{hsp60} *ndhA*	pYUB2722 transformed in mc²8455	This work
Parental strain CDC1551[†]			
mc²5877	Δ*ndh* γδ(*sacB-hyg*) γδ	ST* of CDC1551 with phAE237	This work
mc²5878	Δ*ndhA* γδ(*sacB-hyg*) γδ	ST* of CDC1551 with phAE804	This work
mc²5879	Δ*nuoAN* γδ(*sacB-hyg*) γδ	ST* of CDC1551 with phAE805	This work
mc²5880	Δ*ndh*	Unmarking of mc²5877 with phAE280	This work
mc²5881	Δ*ndhA*	Unmarking of mc²5878 with phAE280	This work
mc²5882	Δ*nuoAN*	Unmarking of mc²5879 with phAE280	This work
mc²5883	Δ*ndh*Δ*nuoAN* γδ(*sacB-hyg*) γδ	ST* of mc²5880 with phAE805	This work
mc²5884	Δ*ndhA*Δ*nuoAN* γδ(*sacB-hyg*) γδ	ST* of mc²5881 with phAE805	This work
mc²5885	Δ*ndh*Δ*nuoAN*	Unmarking of mc²5883 with phAE280	This work
mc²5887	Δ*ndhA*Δ*nuoAN*	Unmarking of mc²5884 with phAE280	This work
mc²5888	Δ*ndh*::P_{hsp60} *ndh*	pYUB2721 transformed in mc²5880	This work
mc²5889	Δ*ndhA*::P_{hsp60} *ndhA*	pYUB2722 transformed in mc²5881	This work

*Specialized transduction.
[†]Colorado State University.

1. Hartman T, et al. (2014) Succinate dehydrogenase is the regulator of respiration in *Mycobacterium tuberculosis*. *PLoS Pathog* 10:e1004510.

Table S2. Primers used in this study

Primer	Sequence
AES construction	
ndhLL	TTTTTTTTCCATAAATTGGCGTGGTTAGCGCCGGTCGAG [Van91I]
ndhLR	TTTTTTTTCCATTTCTTGGGCGGCAAAGAAGCTCAAGCG [Van91I]
ndhRL	TTTTTTTTCCATAGATTGGGCTCAGCGGCAAGCGCTAAG [Van91I]
ndhRR	TTTTTTTTCCATCTTTTGGGGCCGGGGGTGACCTATTCG [Van91I]
ndhALL	TTTTTTTTGCATAAATTGCCGTCGGTGGCATACAACTGG [BstAp1]
ndhALR	TTTTTTTTGCATTTCTTGCATACAGTGTCACAGCCTCTTGATGTCTCACTGAGGTCTCTCGCCGAATCCACTACCGATG [BstAp1]
ndhARL	TTTTTTTTCCATAGATTGGGAAGTCCCTTACGGTTAATCCGAGTGTCTGGTCTCGTAGTGGAACAGCAGGCACAAGGA [Van91I]
ndhARR	TTTTTTTTCCATCTTTTGGACCAAGCACATCGACGGTCA [Van91I]
nuoLL	TTTTTTTTCCATAAATTGGTCGCCGATGCCCCGGAGTAG [Van91I]
nuoLR	TTTTTTTTCCATTTCTTGGCCAGCGCCGCCAGTACCAGG [Van91I]
nuoRL	TTTTTTTTCACAGAGTGCACCGTGGTGCTGGGGATCG [DraIII]
nuoRR	TTTTTTTTCACCTTGTGCCTCGGCCTCGGGAACAATG [DraIII]
pYUB2722 construction	
ndhA F	TTTCAGCTGCGAGGACCATGACGCTCTCATC [Pvu2)]
ndhA R	TTTAAGCTTCGATCGGGCGTTGAGCTAACC [Hind3]
Real-time PCR	
ndh RTF	GCTCGGTCACACCTACCAAA
ndh RTR	CTCAATATGCGGCCACGCAA
ndhA RTF	CAGGCGCGTTTAGGACCATC
ndhA RTR	TCGCGTTGAGTTGAACCTCG
nuoH RTF	CGCTCAACGGCTTTCGCAACA
nuoH RTR	GGCGCTGGGCTGGAAGTTATT

Restriction sites are indicated in brackets.

Table S3. Plasmids and phages used in this study

Strains/plasmids/phages	Genotype or description	Source
pMV361^{Kan}	Integrative *E.coli* mycobacterial shuttle vector with *hsp60* promoter	1
pYUB2721	*M. tuberculosis ndh* cloned downstream of the *hsp60* promoter of pMV361	2
pYUB2722	*M. tuberculosis ndhA* cloned downstream of the *hsp60* promoter of pMV361	This work
pYUB1471	Allelic Exchange Substrate (AES) cloning vector for specialized transduction	3
pYUB2723	pYUB1471 containing the flanking regions of *ndh*	4
pYUB2724	pYUB1471 containing the flanking regions of *ndhA*	4
pYUB2725	pYUB1471 containing the flanking regions of the *nuoAN* operon	This work
phAE237	phAE159 with pYUB2723	This work
phAE280	Unmarking phage	3
phAE804	phAE159 with pYUB2724	This work
phAE805	phAE159 with pYUB2725	This work

1. Stover CK, et al. (1991) New use of BCG for recombinant vaccines. *Nature* 351:456–460.
2. Vilchèze C, et al. (2005) Altered NADH/NAD+ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria. *Antimicrob Agents Chemother* 49:708–720.
3. Jain P, et al. (2014) Specialized transduction designed for precise high-throughput unmarked deletions in *Mycobacterium tuberculosis*. *MBio* 5:e01245-14.
4. Hartman T, et al. (2014) Succinate dehydrogenase is the regulator of respiration in *Mycobacterium tuberculosis*. *PLoS Pathog* 10:e1004510.