Leishmania type II dehydrogenase is essential for parasite viability irrespective of the presence of an active complex I

Margarida Duarte[a,b,1], Cleide Ferreira[a,b], Gurleen Kaur Khandpur[c], Tamara Flohr[d], Jannik Zimmermann[c], Helena Castro[a,b], Johannes M. Herrmann[d], Bruce Morgan[c], and Ana M. Tomás[a,b,e]

[a]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal; [b]Instituto de Biologia Molecular e Celular, Universidade do Porto, 4200-135 Porto, Portugal; [c]Institute for Biochemistry, Zentrum für Human- und Molekularbiologie, Saarland University, 66123 Saarbrücken, Germany; [d]Division of Cellular Biology, University of Kaiserslautern, 67663 Kaiserslautern, Germany; and [e]Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4050-313 Porto, Portugal

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Type II NADH dehydrogenases (NDH2) are monotopic enzymes present in the external or internal face of the mitochondrial inner membrane that contribute to NADH/NAD+ balance by conveying electrons from NADH to ubiquinone without coupled proton translocation. Herein, we characterize the product of a gene present in all species of the human protozoan parasite *Leishmania* as a bona fide, matrix-oriented, type II NADH dehydrogenase. Within mitochondria, this respiratory activity concurs with that of type I NADH dehydrogenase (complex I) in some *Leishmania* species but not others. To query the significance of NDH2 in parasite physiology, we attempted its genetic disruption in two parasite species, exhibiting a silent (*Leishmania infantum*, Li) and a fully operational (*Leishmania major*, Lm) complex I. Strikingly, this analysis revealed that NDH2 abrogation is not tolerated by *Leishmania*, not even by complex I–expressing Lm species. Conversely, complex I is dispensable in both species, provided that NDH2 is sufficiently expressed. That a type II dehydrogenase is essential even in the presence of an active complex I places *Leishmania* NADH metabolism into an entirely unique perspective and suggests unexplored functions for NDH2 that span beyond its complex I–overlapping activities. Notably, by showing that the essential character of NDH2 extends to the disease-causing stage of *Leishmania*, we genetically validate NDH2—an enzyme without a counterpart in mammals—as a candidate target for leishmanicidal drugs.

Leishmania | mitochondria | NADH oxidation | type II NADH dehydrogenase | complex I

Leishmania are protozoan parasites of the Trypanosomatidae family responsible for a set of serious diseases collectively known as the Leishmaniases. Every year, ∼2.0 million people develop symptomatic Leishmaniasis, ranging from self-healing cutaneous lesions to fatal visceralizing disease (1). In the absence of a vaccine, management of human Leishmaniasis (2) is largely dependent on chemotherapy. However, available drugs are highly toxic, and the frequency of resistant parasite strains is increasing worldwide, making the development of innovative therapeutic strategies an urgent desideratum.

Maintenance of a balanced NADH/NAD+ ratio is crucial for cell survival. Because biological membranes are impermeable to NADH, this has to be oxidized in the site of its production or, in alternative, be carried out by specific shuttles to organelles in which it can be utilized as source of reducing equivalents. In mammals, as in most eukaryotes, NADH oxidation depends on the activity of type I NADH dehydrogenases (respiratory chain complex I). These enzymes are large multisubunit complexes that transfer two electrons from NADH to ubiquinone with the concomitant translocation of protons across the inner membrane, thereby contributing not only to NAD+ regeneration but also to ATP production and maintenance of the mitochondrial membrane potential. In eukaryotes, complex I localizes to the mitochondrial inner membrane with the NADH dehydrogenase active site facing the matrix—hence, it oxidizes NADH present within this compartment (3, 4). Unlike mammals, some plants, fungi, and protozoa as well as bacteria are equipped with a different type of dehydrogenase, known as a type II NADH dehydrogenases (or NDH2), to reoxidize NADH (5–8). These enzymes, whose conservation in those organisms is still not fully understood, are single-polypeptide NADH:ubiquinone oxidoreductases, which lack a coupled proton pumping activity. They therefore contribute to NAD+ regeneration and ATP formation but not directly to establish a membrane potential. Like complex I, eukaryotic type II NADH dehydrogenases localize to the inner mitochondrial membrane. However, in contrast to those enzymes, type II NADH dehydrogenases can face either the matrix (internal NDH2) or the intermembrane space (external NDH2) (5–8); accordingly, they either regenerate NADH present in mitochondria, complementing or replacing complex I activity, or formed in the cytosol, respectively. Incidentally, type II NADH dehydrogenases often display essential roles in human and animal pathogens

Significance

Leishmaniasis is one of the most dangerous, neglected tropical diseases. Oxidative phosphorylation is a known target of antipathogenic therapeutics. Herein, we addressed the contribution of type II NADH dehydrogenase (NDH2) and complex I activities to *Leishmania* physiology. Our results provide evidence that *Leishmania* NDH2 is essential throughout the parasites' life cycle, independently of the presence of a functional complex I. Furthermore, sustained expression of NDH2 renders complex I dispensable. This indicates that NDH2 has unique and essential function(s) in *Leishmania* parasites and validates the enzyme as a promising drug target. Future studies may benefit from the *Leishmania* model to unravel the metabolic advantage of type II NADH dehydrogenases, enlightening the reason for their persistence across evolution.

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[1]To whom correspondence may be addressed. Email: mduarte@ibmc.up.pt.

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(9–12). This, added to the fact that these respiratory components are absent from mammalian hosts, renders them promising targets for drug development (13).

Leishmania are predicted to express a type II dehydrogenase raising the possibility that selective inhibition of this enzyme could be exploited for therapy. For this to be successful, however, this NDH2, be it an internal or an external enzyme, should carry out function(s) essential to parasites, which cannot be bypassed by other systems. The current state of knowledge regarding *Leishmania* NADH oxidation does not permit to foretell whether this is the case. An internal NDH2 may be dispensable in case of complex I expression. Although this activity could not be detected in some parasite species (14–16), the fact is that all *Leishmania* genomes have the potential to encode the subunits that compose this multienzyme complex (17). Similarly, the compartmentalization of *Leishmania* glycolysis in glycosomes (18, 19) may render superfluous a cytosolic NDH2 as, contrary to most eukaryotic cells, no glycolytic NADH is expected to be generated in the cytosol of *Leishmania*. Of note, abrogation of *Trypanosoma brucei* NDH2, a protein whose subcellular location is still unclear, entails no gross phenotype to parasites, and, as such, has no value for therapy (20, 21).

Having as main objectives to characterize *Leishmania* NDH2 and to address its functional significance to parasites, this manuscript brings important and unique insights in the process of NADH oxidation in *Leishmania*. We argue that 1) NDH2 is an internal type II NADH dehydrogenase, whose activity can substitute for that of complex I, 2) NDH2 function is required for *Leishmania* survival with complex I apparently being unable to meet all NDH2 functions, and 3) the essentiality of NDH2 renders *Leishmania* type II NADH dehydrogenase a valid drug target, a fact reinforced by the inability of NDH2 single mutants to prosper in animal models of infection.

Results

***Leishmania* Express a Putative Internal NDH2.** A gene coding for a putative NDH2 enzyme is present in all sequenced *Leishmania* genomes (https://tritrypdb.org/). Sequence alignment of NDH2 polypeptides reveals a high degree of conservation within the *Leishmania* genus (e.g., 97% identity between the proteins of *Leishmania infantum* [Li] and *Leishmania major* [Lm]) as well as with functional NDH2 enzymes of other organisms (49% similarity to *Saccharomyces cerevisiae* NDI1 [ScNDI]). Importantly, *Leishmania* NDH2 sequences conserve the domains that in active type II NADH dehydrogenases are implicated in protein interaction with the flavin cofactor and with the NADH substrate (*SI Appendix*, Fig. S1). Compared with other type II NADH dehydrogenases, NDH2 enzymes of *Leishmania* (and related trypanosomatid parasites) exhibit short N termini—the length from the N terminus to the first conserved region is only 10 amino acids—that, unlike their ortholog enzymes, do not specify any obvious mitochondrial targeting sequence (MTS) according to common in silico prediction tools (MitoprotII and TargetP).

The *NDH2* gene is expressed throughout the life cycle of Li, as confirmed by Western blot (WB) analysis of parasite lysates (Fig. 1*A*). Using a polyclonal antiserum raised against purified recombinant Li NDH2, we could detect a polypeptide of 56 kDa (corresponding to the predicted molecular weight of the Li NDH2 enzyme) in both logarithmic (Log) and stationary (Stat) phase promastigotes (Pro). Expression of NDH2 was also detected in the mammalian (clinically relevant) amastigote (Ama) stage of Li, grown either as axenic (i.e., without host cells) or as intramacrophagic forms (IntraMł).

To ascertain the subcellular location of NDH2 in *Leishmania*, we resorted to indirect immunofluorescence analysis with an anti-NDH2 antiserum. Labeling of Li promastigotes overexpressing NDH2 from an episome (the immunofluorescent signal of NDH2 was barely detected in wild-type parasites) showed that NDH2 colocalized with that of the mitochondrial peroxiredoxin mTXNPx (22) (Fig. 1*B*). This observation indicates that NDH2 is targeted to mitochondria, possibly by means of a short MTS, as occurs with other *Leishmania* mitochondrial proteins (23).

To investigate whether NDH2 is an internal or external type II NADH dehydrogenase we assessed its pattern of digestion by proteinase K (PK) in the presence of increasing concentrations of digitonin. We found that NDH2 remains intact in digitonin concentrations that expose the mitochondrial intermembrane space (IMS) proteins essential for respiration and viability (ERV) (24) and small translocase of the inner membrane (TIM) (25) and only becomes accessible to PK proteolysis in detergent concentrations that also expose the matrix protein mTXNPx (>1 mg digitonin/mg protein) (Fig. 1*C*). These results support the conclusion that NDH2 is an enzyme facing the mitochondrial matrix.

Finally, we used alkaline carbonate extraction to address the membrane topology of Li NDH2. We observed that the protein extracts mostly in the soluble fraction, with only a small fraction in the insoluble pellet, an elution profile that matches that of peripheral proteins (Fig. 1*D*). Association of Li NDH2 with the mitochondrial inner membrane should occur through its C-terminal domain acquiring a monotopic topology, as described for NDH2 homologs from yeast (27) and bacteria (28). Such membrane association for Li NDH2 is also compatible with the absence of transmembrane domains as predicted by in silico tools (http://www.cbs.dtu.dk/services/TMHMM/).

Heterologous Complementation Support That NDH2 Is an Active NADH Dehydrogenase. To test for NADH-oxidizing activity of *Leishmania* NDH2, we carried out heterologous complementation assays in a *S. cerevisiae* strain deleted for the gene encoding internal (Δ*ndi1*) (29) NADH dehydrogenase, as GFP-split analysis (30) indicated that the enzyme retained its endogenous location when expressed in the yeast system (*SI Appendix*, Fig. S2). Our results demonstrate that complementation with the Li *NDH2* sequence rescued the growth defect of Δ*ndi1* on glycerol plates, conditions that induce production of NADH in both the cytosol and the mitochondrial matrix, thus providing indirect evidence that the *Leishmania* NDH2 enzyme is an active NADH-oxidizing enzyme (Fig. 2*A*). Likewise, a chimera of Li NDH2 directed to the mitochondrial matrix, by fusion to the MTS of the *Neurospora crassa* ATP synthase subunit 9 (Su9-NDH2), also complemented the growth defect of Δ*ndi1* (Fig. 2*A*). In contrast, no growth effect was observed when Li NDH2 was expressed in either the wild-type or in cells devoid of external NADH dehydrogenases (Δ*nde1*Δ*nde2*) (31) (Fig. 2*A*).

Next, to query the substrate specificity of Li NDH2, we monitored oxygen consumption by intact mitochondria isolated from Δ*ndi1* and Δ*nde1*Δ*nde2* yeast strains complemented or not with Li NDH2 in the presence of bolus NADH. These assays showed that the rate of oxygen consumption of Δ*ndi1* mitochondria was not altered by the presence of the Li *NDH2* gene (*SI Appendix*, Fig. S3). On the contrary, these measurements revealed that complementation of Δ*nde1*Δ*nde2* mitochondria with the *Leishmania* NDH2 enzyme significantly enhanced oxygen consumption by 2.3-fold ($P < 0.05$) in the presence of NADH (Fig. 2*C*). This increment was absent when NADPH was supplied as a source of reducing equivalents (Fig. 2*C*), confirming the specificity of Li NDH2 for the NADH substrate. The activity recorded in Δ*nde1*Δ*nde2* yeast cells transformed with Li NDH2 suggests that a small fraction of the parasite enzyme, owing to its short MTS, inserted into the inner mitochondrial membrane in the opposite orientation (i.e., with the NADH-oxidizing active site facing toward the IMS) and hence accessible to exogenously added substrates. Validating this premise, mitochondria isolated from Δ*nde1*Δ*nde2* cells complemented with the chimera of Li NDH2

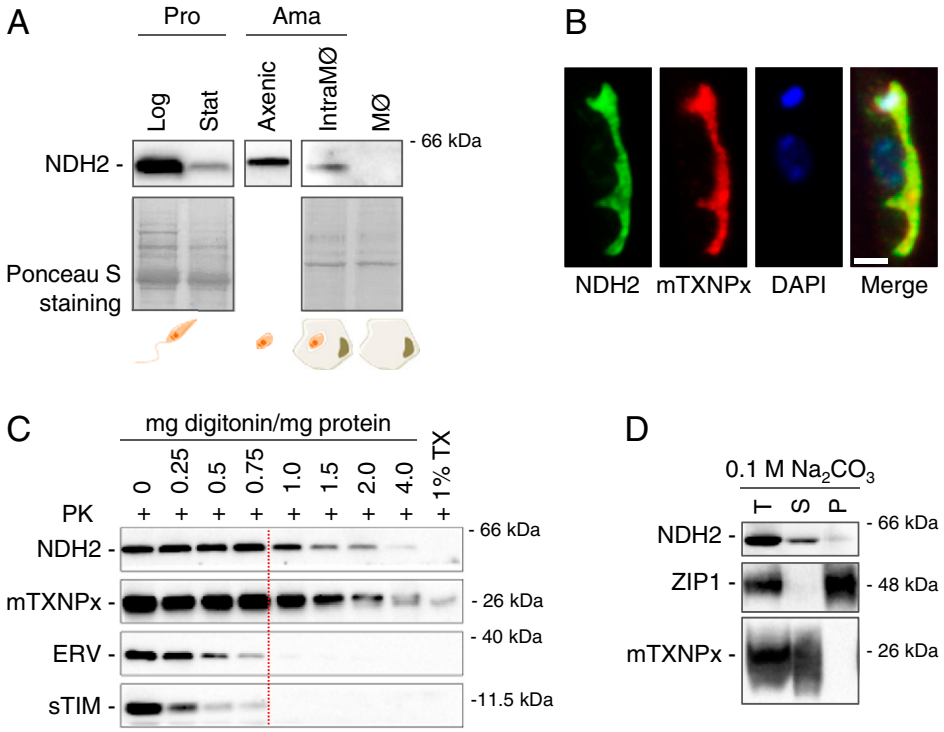

Fig. 1. The *Leishmania* NDH2 is a type II NADH dehydrogenase of the mitochondrial matrix. (*A*) NDH2 is expressed along the *L. infantum* (Li) life cycle. Western blot (WB) analysis of protein extracts from Li promastigotes (Pro), either in logarithmic (Log) and stationary (Stat) phases of growth, as well as from amastigotes (Ama), obtained either from axenic cultures (Axenic) or from monolayers of infected bone marrow derived macrophages (IntraMØ). Also included are protein extracts of naïve macrophages (MØ). Membranes are decorated with a polyclonal antiserum raised against purified recombinant Li NDH2. Ponceau S staining of the membranes is shown as loading control. (*B*) NDH2 localizes to *Leishmania* mitochondria. Indirect immunofluorescence analysis of Li promastigotes expressing NDH2 from an episome (Li *NDH2*+), using antisera against NDH2 (green channel) and against the mitochondrial mTXNPx protein (red channel), in the presence of the DNA-stain DAPI (blue channel). Merging of all three channels is shown on the *Right* panel. (Scale bar, 2.5 μm.) (*C*) NDH2 is present in the matrix of *Leishmania* mitochondria. The protein content of Li amastigotes was gradually exposed to proteinase K (PK) digestion by permeabilization of plasma and organelle membranes with incremental amounts of digitonin. The resulting protein extracts were subsequently analyzed by WB using antibodies directed against NDH2, mTXNPx (located in the mitochondrial matrix), ERV, and sTIM (located in the intermembrane space). The red vertical line signals the concentration of digitonin in which ERV and sTIM are fully exposed to PK, but NDH2 and mTXNPx are shielded from the action of this protease by means of the inner mitochondrial membrane. The right lane ("1% TX") refers to parasite lysates obtained upon coincubation with 1% Triton X-100 and PK—it controls for maximal PK digestion of the proteins under analysis. (*D*) Li NDH2 is a peripheral protein. Alkaline carbonate extraction was performed on *L. infantum* promastigotes. Parasites were disrupted in 0.1 M Na_2CO_3 pH 11.5 and fractionated by ultracentrifugation. Total protein extracts (T) as well as supernatants (S) and membrane pellets (P) resulting from fractionation were analyzed by WB using anti-NDH2 antibody. Anti-ZIP1 (26) and anti-mTXNPx (22) antibodies were used to control for membrane and hydrophilic proteins, respectively. Uncropped membranes are shown in *SI Appendix*, Fig. S8.

(Su9-NDH2) did not show increased NADH-driven oxygen consumption (Fig. 2*C*).

NDH2 Feeds the Electron Transport Chain of *Leishmania*. Confirmation that NDH2 functions as an active NADH dehydrogenase, supplying the electron transport chain (ETC) of *Leishmania* with reducing equivalents, was obtained from oxygen consumption assays of intact wild-type (Li) and NDH2-overexpressing promastigotes (Li *NDH2*+) (*SI Appendix*, Fig. S4, *Left*). These measurements revealed that upregulation of NDH2 significantly increases (1.3 times, $P < 0.01$) basal oxygen consumption by intact parasites (Fig. 2*D*). The presence of KCN (complex IV inhibitor; Fig. 2*B*) dramatically decreases oxygen consumption, confirming that this parameter is monitoring the activity of the mitochondrial respiratory chain.

There are two major points for entry of electrons in the mitochondrial respiratory chain: NADH (via complex I and NDH2) and succinate (via complex II) (Fig. 2*B*). To distinguish NADH- from succinate-driven respiration, we followed up oxygen consumption in the absence of a functional complex II (i.e., under complex II inhibition by TTFA) (Fig. 2*D*). We also used KCN as complex IV inhibitor to exclude non–ETC-related consumption of oxygen from these calculations (Fig. 2*D*). Following this approach, we estimated oxygen consumption rates dependent on oxidation of NADH (Fig. 2*D*, black vertical typing; Fig. 2*E*, black columns) or succinate (Fig. 2*D*, violet vertical typing; Fig. 2*E*, violet columns). The results show that NADH oxidation increases (even if not reaching statistical significance) in response to NDH2 overexpression (Fig. 2*E*, black columns), suggesting that this enzyme is involved in the transfer of electrons from NADH to the ETC of *Leishmania*. Importantly, upregulation of NDH2 also accelerated succinate-dependent oxygen consumption (Fig. 2*E*, purple columns), suggesting that *NDH2*+ parasites display increased metabolic activity of pathways that generate succinate (i.e., the Krebs cycle). Together, data obtained in this section identify NDH2 as a rate-limiting enzyme of *Leishmania* respiration.

In summary, we conclude that NDH2 is a bona fide, active, internal type II mitochondrial NADH dehydrogenase.

NDH2 Is Essential for *Leishmania* Survival along the Parasite Life Cycle. To investigate the functional relevance of NDH2 in *Leishmania*, we attempted inactivation of this gene in *L. infantum* promastigotes resorting to classical homologous recombination

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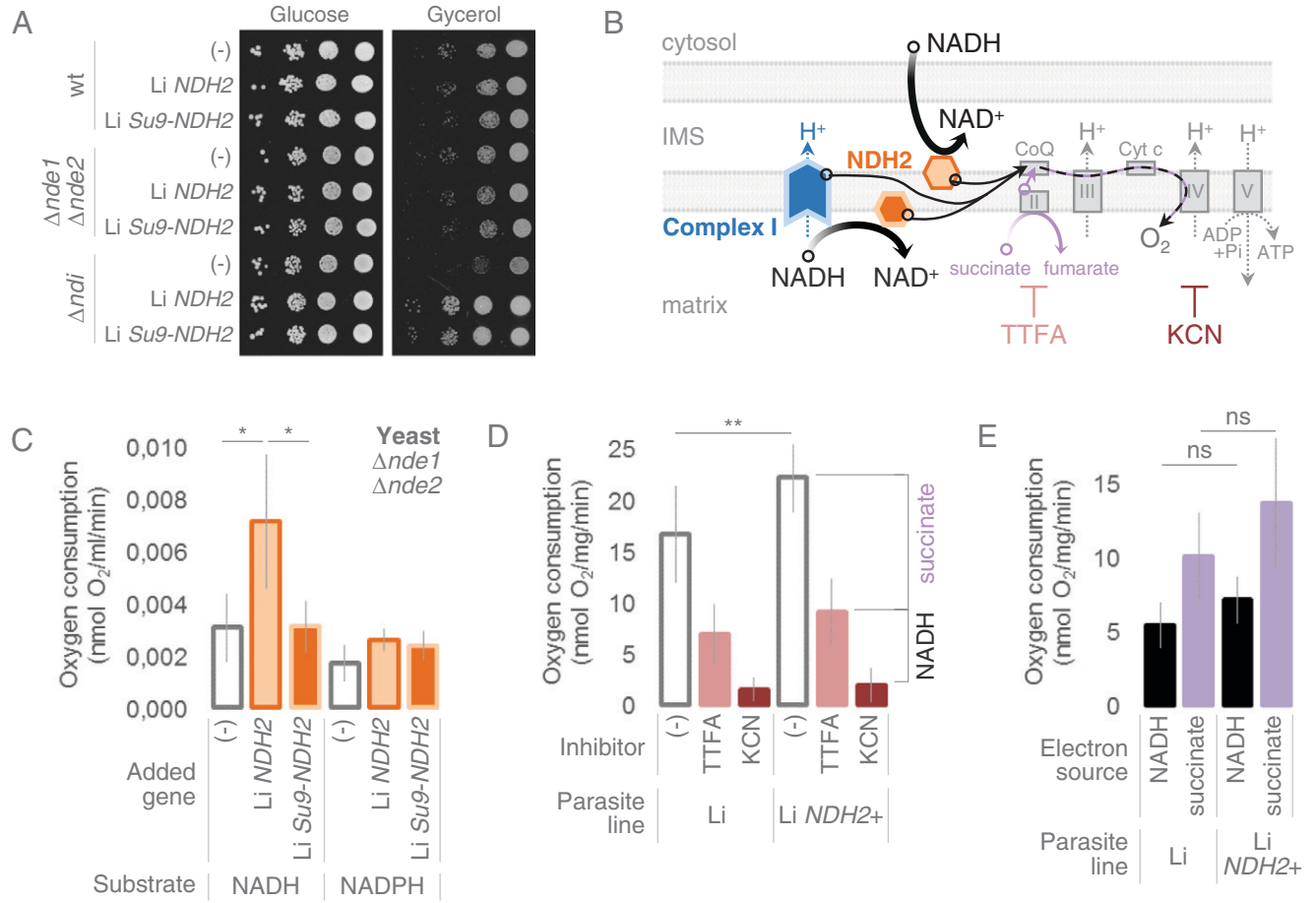

Fig. 2. The *Leishmania* NDH2 is an active NADH dehydrogenase. (*A*) The *L. infantum* NDH2 enzyme rescues the defective phenotype of yeast devoid of the inner NADH dehydrogenase NDI (Δ*ndi*) growing on glycerol. Yeast Δ*ndi* knockouts were transformed with either the p415TEF multicopy vector [(-)] or the p415TEF vector carrying the *L. infantum NDH2* gene (Li *NDH2*) or the Li *NDH2* gene fused N-terminally to the MTS of the *N. crassa* ATP synthase subunit 9 (Li *Su9-NDH2*). Cells were grown on galactose-based selective media to midlog phase. Then, 10-fold serial dilutions were dropped onto glycerol plates (oxidative phosphorylation-dependent metabolism) and incubated at 30 °C for 4 d, prior to being photographed. Yeast grown on glucose-rich plates (oxidative phosphorylation-independent metabolism) exhibited no growth defect. The same experimental protocols were applied to cells devoid of external (i.e., intermembrane space [IMS]) NADH dehydrogenases (Δ*nde1*Δ*nde2*) and to the parental wild-type (wt) BY4742 yeast strain. (*B*) Key features of the mitochondrial ETC. The ETC is supplied with reducing equivalents derived from NADH (black arrows) generated either in the mitochondrial matrix or in the cytosol. NADH feeds the ETC at the level of the multisubunit complex I (highlighted in blue; this dehydrogenase links NADH oxidation to proton [H^+] pumping to the IMS) or at single-subunit NDH2 (highlighted in orange) enzymes. *Leishmania* NDH2 is an inner mitochondrial enzyme (dark orange fill), as shown in Fig. 1 *B* and *C*. An alternative, NADH-independent way of feeding the ETC is via succinate reduction (violet arrow) at the level of succinate dehydrogenase (complex II). The scheme also depicts coenzyme Q (CoQ), cytochrome *c* reductase (complex III), cytochrome *c* (Cyt c), cytochrome *c* oxidase (complex IV), and ATP synthase (complex V). The mixed black/violet arrow shows the transfer of electrons between complexes up to O_2 reduction by complex IV. The gradient of protons pumped to the IMS throughout the ETC can be reversed with coupled ATP production by complex V. Complex II and complex IV inhibitors (TTFA and KCN, respectively) are also shown. (*C*) The *L. infantum* NDH2 enzyme utilizes NADH but not NADPH as reducing substrate. Oxygen consumption rate of mitochondria isolated from Δ*nde1*Δ*nde2* yeast transformed with the p415TEF plasmid [(-)] or with p415TEF carrying the *L. infantum* NDH2 gene (Li *NDH2*) or the Li *NDH2* gene fused N-terminally to the MTS of the *N. crassa* ATP synthase subunit 9 (Li *Su9-NDH2*). Polarographic measurements were carried out in the presence of exogenously bolus of either NADH or NADPH. Data represent means and SDs of three independent experiments (*$P < 0.05$). (*D*) NDH2 is a functional, ETC-feeding enzyme of *Leishmania*. Rates of oxygen consumption by *L. infantum* promastigotes, either wt (Li) or transfected with the multicopy pTEX-*NEO*^{R}-*NDH2* plasmid (Li *NDH2*+). Polarographic measurements were performed in the absence [(-)] or presence of complex II and complex IV inhibitors (TTFA and KCN, respectively). Bars represent means and SDs of five independent experiments (**$P < 0.01$). Succinate-dependent oxygen consumption (violet vertical typing) can be calculated by subtracting from the basal oxygen consumption rates, the values measured in presence of TTFA. Likewise, NADH-dependent oxygen consumption (black vertical typing) can be estimated by subtracting from the succinate-independent oxygen consumption rates, the respiratory rates measured in presence of KCN. (*E*) Up-regulation of NDH2 accelerates both succinate- and NADH-dependent oxygen consumption. Graph shows the respiration rates of Li and Li *NDH2*+ promastigotes calculated as specified in *D*. ns, not significant.

(HR). This involved two successive rounds of transfection with *NDH2*-targeting constructs carrying *HYG*^{R} and *PHLEO*^{R} selectable markers (Fig. 3*A*). Following this approach, we managed to isolate viable *ndh2*$^{+/-}$ heterozygous parasites (Fig. 3*B*, *Left*) that showed no gross defects when compared to wild-type parasites (*SI Appendix*, Fig. S5) but not *ndh2*$^{-/-}$ homozygous knockouts. Disruption of both *NDH2* alleles was, nevertheless, possible in *L. infantum* parasites previously complemented with an *NEO*^{R}-*NDH2* episome (Fig. 3*A*; Li *NDH2*+) (Fig. 3*B*, *Right*). The finding that promastigotes do not tolerate full deletion of chromosomal *NDH2*, unless they have alternative (episomal) copies of this gene, is a strong indicator of *NDH2* essentiality.

Unequivocal proof that NDH2 is crucial for survival of Li promastigotes was obtained by subjecting *NDH2*+/*ndh2*$^{-/-}$

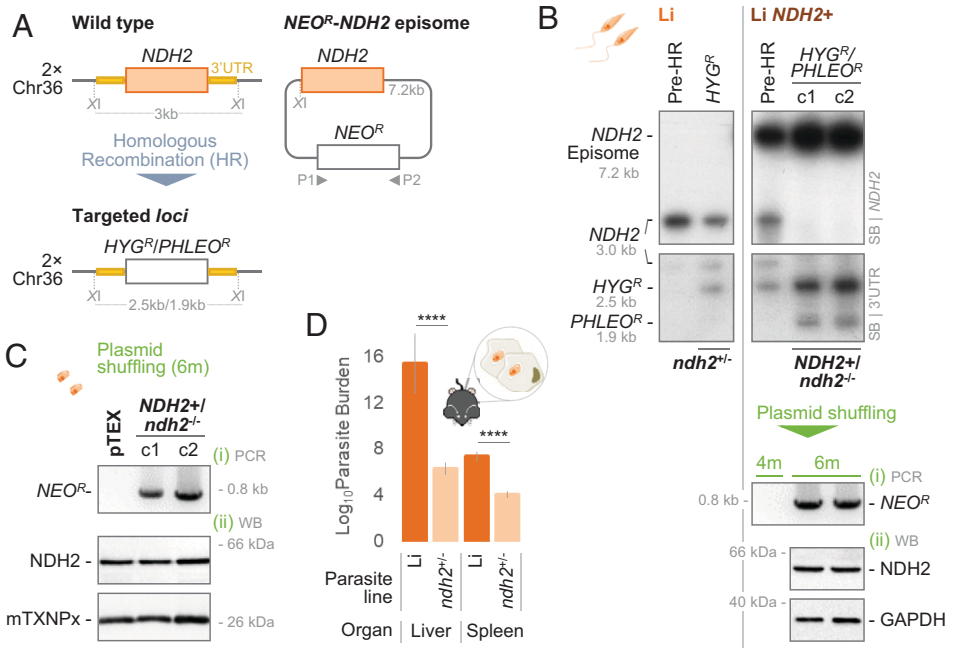

Fig. 3. NDH2 is essential for survival of *L. infantum* promastigotes and amastigotes. (*A*) HR strategy employed to target the diploid *NDH2* alleles in the *L. infantum* genome. The *NDH2* ORF (orange box) is shown in its wild-type *locus* at chromosome 36, flanked by 5' and 3' UTRs (yellow boxes). These were used as homology regions to target the *NDH2 locus* with disruption constructs carrying hygromycin- and phleomycin-resistance genes (HYG^R and $PHLEO^R$, respectively). To the right is the simplified scheme of the NEO^R-*NDH2* episome (pTEX-based), used as *NDH2*-complementing vector (NEO^R stands for neomycin-resistance). Also depicted are the sites of DNA restriction by *Xho*I (XI) and the size of the corresponding digestion fragments. Location of oligonucleotides (primers P1 and P2) used in PCR diagnosis are indicated by gray arrowheads. (*B*, *Upper*) Southern blot analysis of genomic DNA from *L. infantum* promastigotes: wild-type (Li) and NEO^R-*NDH2*-complemented (Li *NDH2*+) parasites before (pre-HR) and after (HYG^R, HYG^R/$PHLEO^R$) sequential transfections with HYG^R and $PHLEO^R$ disruption cassettes. DNAs were digested with *Xho*I and hybridized with the *NDH2* ORF and 3'UTR. After the first transfection round with HYG^R, Li parasites lose one *NDH2* allele, yielding $ndh2^{+/-}$ heterozygous knockouts. Elimination of the remaining *NDH2* allele after the second transfection round with $PHLEO^R$ (HYG^R/$PHLEO^R$) was only possible in Li *NDH2*+ parasites, the resulting transgenics being *NDH2*+/$ndh2^{-/-}$ (two independent clones, c1 and c2, are shown). (*Lower*) Plasmid-shuffling assay of *NDH2*+/$ndh2^{-/-}$ transgenics (c1 and c2). Parasites were tested for the loss of the NEO^R-*NDH2* plasmid after being kept in culture for 6 mo without neomycin drug pressure. The loss/retention of NEO^R-*NDH2* was tested by (i) PCR amplification of the NEO^R gene (P1/P2) and (ii) Western blot (WB) analysis of NDH2 expression with anti-NDH2 antiserum (loading control with anti-GAPDH). A total 4 mo were enough for control Li *pTEX* parasites to lose the NEO^R vector. (*C*) Plasmid-shuffling assay performed for 6 mo on axenic amastigotes of *L. infantum* pTEX and *NDH2*+/$ndh2^{-/-}$ (c1 and c2) lineages (differentiated from the corresponding promastigote lines, obtained according to *A* and *B*). The loss/retention of NEO^R-*NDH2* was tested by (i) PCR amplification of the NEO^R gene (P1/P2) and (ii WB analysis of NDH2 expression with anti-NDH2 antiserum (loading control with anti-mTXNPx). (*D*) Deletion of one *NDH2* allele impairs survival of *L. infantum* intracellular amastigotes residing in murines. Wild-type (Li) and heterozygous knockout ($ndh2^{+/-}$) parasites were inoculated intravenously in C57BL/6 mice, and 21 d later, their survival appraised by assessing the parasite burdens in infected livers and spleens. Data represent means and SDs of values obtained for five independent mice (****$P < 0.0001$). Uncropped membranes are shown in *SI Appendix*, Fig. S8.

transgenics to a plasmid-shuffling assay. This assay consisted in consecutively passaging *NDH2*+/$ndh2^{-/-}$ promastigotes in neomycin-free medium throughout 6 mo. In the absence of selective drugs, maintenance of the NEO^R gene in the NEO^R-*NDH2* plasmid should not confer any selective advantage to parasites; hence, it should be lost over time (32). The plasmid would, nevertheless, be retained if its *NDH2* ORF was critical for parasite survival. When assessed for the absence/presence of the NEO^R-*NDH2* vector by PCR, after 6 mo in culture without neomycin pressure, *NDH2*+/$ndh2^{-/-}$ promastigotes were found to keep the NEO^R-*NDH2* vector, unlike control parasites (wild-type carrying the empty vector), which lost the plasmid in just 4 mo (Fig. 3*B*, *Right*, i). These results show that the NEO^R-*NDH2* episome confers a selective advantage to parasites that have no other copies of *NDH2*, probably because it guarantees the sustained expression of the crucial NDH2 enzyme (Fig. 3*B*, *Right*, ii).

Next, we asked whether the essential character of *NDH2* also extended to the mammalian, disease-causing stage of *Leishmania*, the amastigote. We started by performing a plasmid-shuffling assay with *NDH2*+/$ndh2^{-/-}$ parasites differentiated to axenic amastigotes from the corresponding promastigote mutant lines. We found that *NDH2*+/$ndh2^{-/-}$ axenic amastigotes did not lose the NEO^R-*NDH2* episome after 6 mo of consecutive passages in medium without neomycin (unlike control parasites) (Fig. 3*C*), suggesting that *NDH2* is also crucial in this life cycle stage. Another piece of evidence supporting *NDH2* essentiality for *L. infantum* amastigotes came from an in vivo infection experiment. Here, we assessed the impact of decreased expression of NDH2 that resulted from elimination of one *NDH2* allele (in $ndh2^{+/-}$ heterozygous parasites) (*SI Appendix*, Fig. S4, *Right*) on parasite survival in the context of a murine infection. Its results, depicted in Fig. 3*D*, show that $ndh2^{+/-}$ parasites yielded significantly lower infection burdens in livers and spleens of infected C57BL/6 mice than control wild-type parasites, supporting the difficulty of *L. infantum* to survive as intracellular amastigotes when expression of NDH2 is limited by depletion of one gene allele.

Together, our data show that NDH2 is crucial for survival of *L. infantum* throughout its life cycle.

NDH2 Displays Unique and Crucial Functions Not Compensated by a Fully Active Complex I. Our finding that NDH2 is essential in *Leishmania* is intriguing, as these parasites have the potential to encode a fully operational complex I (reviewed in ref. 17),

which shares with NDH2 the capacity to transfer electrons from NADH into the respiratory chain. The inability of complex I to compensate for the lack of NDH2 in *ndh2*$^{-/-}$ knockouts can, nevertheless, be explained in a scenario in which 1) complex I is silent or 2) complex I is active but unable to meet the crucial function(s) of NDH2.

We started by investigating whether complex I is silent or active in *L. infantum*. First, by monitoring NADH dehydrogenase activity in intact promastigotes using resazurin as final electron acceptor, we found that reduction of this substrate was barely affected by the complex I inhibitors piericidin A and rotenone (Fig. 4*A*, Li). Second, we observed that the rate of oxygen consumption by promastigotes was only marginally impaired (~20% decrease) by piericidin A (Fig. 4*B*, Li). Third, mitochondrial extracts of promastigotes resolved in Blue Native polyacrylamide gel electrophoresis (BN-PAGE) failed to reveal in-gel NADH:NBT oxidoreductase activity (Fig. 4*C*, Li). Taken together, these results suggest that complex I activity is negligible in *L. infantum* promastigotes.

Next, we asked whether NDH2 would still be crucial in parasites expressing a fully active complex I. To accomplish this, we set out to genetically disrupt *NDH2* in complex I–expressing *Leishmania* parasites. Since generation of *L. infantum* parasites expressing complex I is technically unfeasible—it is constituted by a high number of subunits—we took advantage of our circumstantial finding that another *Leishmania* species (*L. major*) is endowed with an active complex I. This was shown by our observations that *L. major* promastigotes 1) decrease resazurin reduction in response to piericidin A and rotenone treatment (Fig. 4*A*, Lm), 2) show ~50% decrease oxygen consumption in the presence of piericidin A (Fig. 4*B*, Lm), and 3) yield a NADH:NBT–active

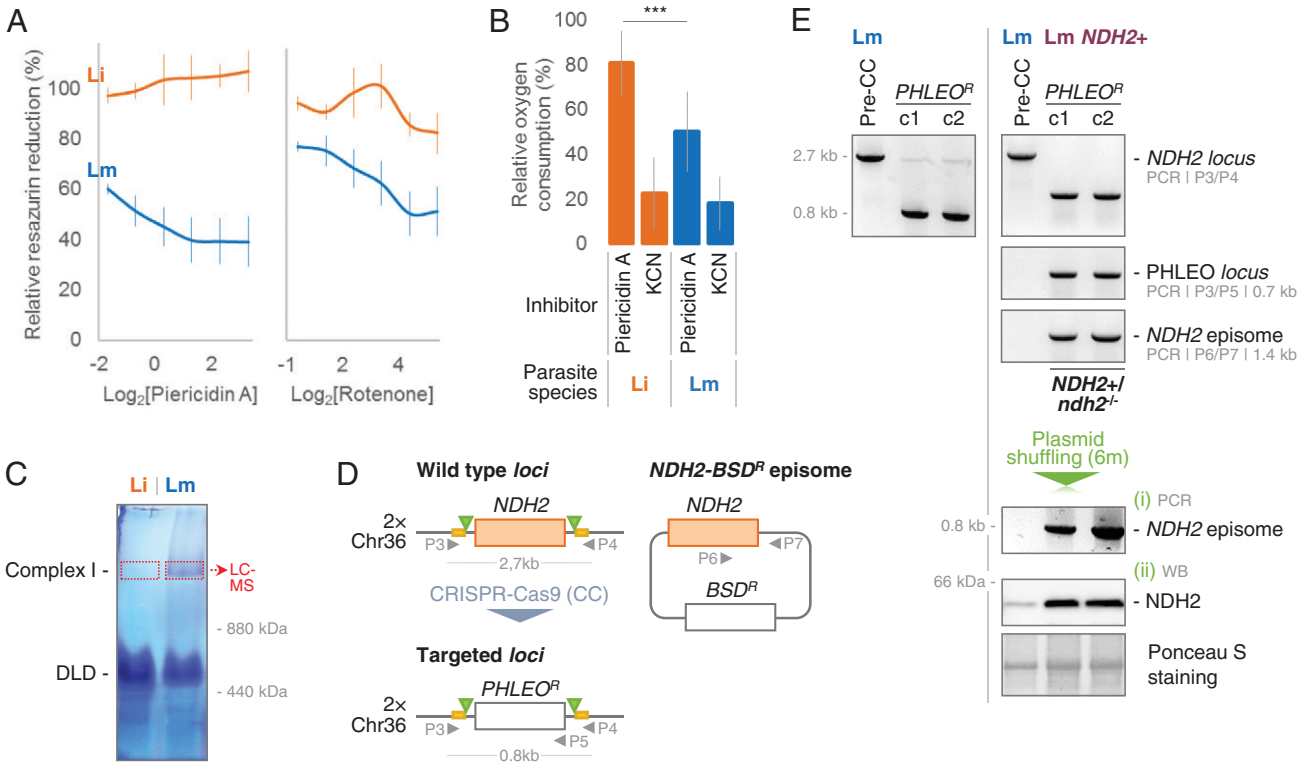

Fig. 4. Expression of a functional complex I does not rescue the lethal phenotype of *ndh2*$^{-/-}$ knockouts. The existence of an active complex I in promastigotes of *L. infantum* (Li) (and of *L. major* [Lm]) was investigated by different methodologies (*A*–*C*). (*A*) Li and Lm promastigotes were exposed to increasing concentrations of complex I inhibitors piericidin A (*Left*) and rotenone (*Right*), in the presence of resazurin, and subsequently monitored for the end-product of resazurin reduction by active NADH (and NADPH) dehydrogenases. Data represent means and SDs of three independent experiments (each performed in triplicate). (*B*) The rates of oxygen consumption by Li and Lm promastigotes were measured in the presence of piericidin A and KCN (inhibitors of complex I and complex IV, respectively). Data represent means and SDs of at least three independent experiments (***$P < 0.001$). (*C*) Crude mitochondrial extracts, obtained by digitonin solubilization of Li and Lm promastigotes, were resolved by Blue Native PAGE and subsequently subjected to an in-gel NADH:NBT oxidoreductase activity assay. Either one or two bands with NADH-NBT oxidoreductase activity were detected in Li or Lm, respectively, whose molecular weights comply with the size of dihydrolipoamide dehydrogenase (DLD)–containing complexes and complex I. The monomer and dimer of ferritin, used as molecular markers, are indicated. (*D*) CRISPR-Cas9 (CC) strategy to target the diploid *NDH2* alleles in the *L. major* genome. The *NDH2* ORF (orange box) is shown in its wild-type locus at chromosome 36, alongside with the sites for Cas9 binding and cleavage (green arrowheads). One donor DNA cassette, containing the phleomycin-resistance gene (*PHLEO*^{R}) and flanked by sequences of ~30 nucleotides identical to the target locus (yellow boxes), were used for homology-directed repair of the nicks produced by Cas9. To the right is the simplified scheme of the *BSD*^{R}*-NDH2* episome (pGL-based), used as *NDH2*-complementing vector (*BSD*^{R} stands for blasticidin-resistance). Also depicted are the location of oligonucleotides (primers P3 through P7; gray arrowheads) employed in PCR diagnosis of transfected parasites and the size of the PCR products obtained from P3/P4-driven amplification of *NDH2* and *PHLEO*^{R} loci. (*E*) PCR diagnosis of Lm promastigotes: wild-type (Lm) and *BSD*^{R}*-NDH2*-complemented (Lm *NDH2*+) parasites before (pre-CC) and after (*PHLEO*^{R}) CRISPR-Cas9–driven attempts to disrupt *NDH2* with the *PHLEO*^{R} cassette. PCR reactions were carried out with primers flanking the *NDH2*/*PLHEO*^{R} ORFs (P3/P4) as well as with primers amplifying *PHLEO*^{R} inserted into the *NDH2* locus (P3/P5) and the *BSD*^{R}*-NDH2* episome (P6/P7). Disruption of *NDH2* was not successful in Lm promastigotes (*Left*) but readily accomplished in Lm *NDH2*+ parasites, the resulting transgenics being *NDH2*+/*ndh2*$^{-/-}$ (two independent clones, c1 and c2, are shown). Plasmid-shuffling assay of *NDH2*+/*ndh2*$^{-/-}$ transgenics (c1 and c2). Parasites were tested for the loss of the *BSD*^{R}*-NDH2* plasmid after being kept in culture for 6 mo without blasticidin drug pressure. The loss/retention of *BSD*^{R}*-NDH2* was tested by (i) PCR amplification of the episomic copy of *NDH2* gene (P6/P7) and (ii) Western blot (WB) analysis of NDH2 expression with anti-NDH2 antiserum (Ponceau S staining of the membranes is shown as loading control). Uncropped membranes are shown in *SI Appendix*, Fig. S8.

band in native PAGE that falls within the molecular weight predicted for complex I (Fig. 4*C*, Lm) and (unlike the corresponding gel slice of *L. infantum*) tests positive for complex I peptide fragments by liquid chromatography-mass spectrometry (LC-MS) analysis (*SI Appendix*, Table S1). Starting from this complex I–expressing *L. major* species, we reattempted the genetic disruption of *NDH2*, this time, following a CRISPR-Cas9–based strategy (Fig. 4*D*). Strikingly, we found that the presence of complex I did not enable the isolation of viable *ndh2*$^{-/-}$ homozygous knockouts. Consistent with our observations in *L. infantum*, disruption of both *NDH2* alleles in *L. major* was only possible when parasites were pretransfected with a *BSD*^{R}*-NDH2*–complementing plasmid (Fig. 4*E*). Importantly, the resulting *NDH2+/ndh2*$^{-/-}$ *L. major* transgenics retained the *BSD*^{R}*-NDH2* plasmid in a plasmid-shuffling assay (Fig. 4*E*), providing definitive proof for *NDH2* essentiality in this complex I–expressing parasite species.

In conclusion, the essentiality of NDH2 indicates that it has unique and essential function(s) in *Leishmania* parasites, unmet by complex I.

Sustained Expression of NDH2 Renders Complex I Dispensable. In the previous section, we have shown that *Leishmania* cannot tolerate NDH2 elimination. However, type I mitochondrial NADH dehydrogenases might also display unique functions in these parasites. Complex I is well known for its ability to couple NADH oxidation to proton pumping into the IMS, an activity unparalleled by type II NADH dehydrogenases. To understand whether complex I functions are also crucial for parasite survival, we pursued the genetic inactivation of this enzymatic complex in both *L. infantum* and *L. major*, resorting to a CRISPR-Cas9–based strategy to target *NDUFS1* (*SI Appendix*, Fig. S6 and Fig. 5*A*)—a gene encoding a subunit of complex I essential for its assembly and activity (33, 34). Following this approach, we found that *L. infantum* promastigotes tolerate complex I disruption (Fig. 5*B* and *SI Appendix*, Fig. S7), which is consistent with the negligible expression of this enzyme in this parasite species (Fig. 4 *A*–*C*). Conversely, in the complex I–expressing *L. major*, we failed to isolate viable *ndufs1*$^{-/-}$ homozygous mutants. Interestingly, however, if *L. major* promastigotes were precomplemented with an episomal version of

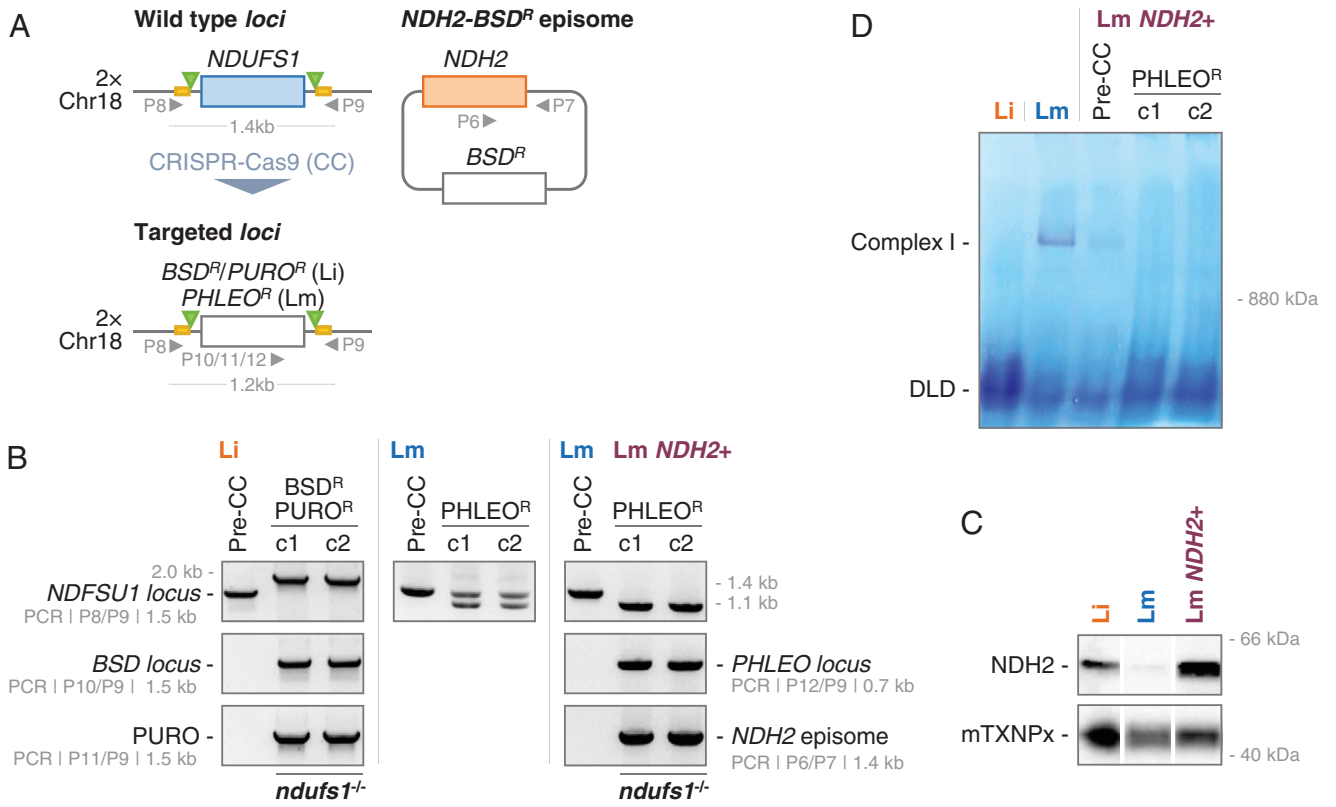

Fig. 5. Complex I is dispensable under sustained NDH2 expression. (*A*) CRISPR-Cas9 (CC) strategy employed to eliminate the *NDUFS1* genomic locus. The *NDUFS1* ORF (blue box) is shown in its wild-type locus at chromosome 18 alongside the sites for Cas9 binding and cleavage (green arrowheads). One donor DNA cassette for Lm, containing the phleomycin-resistance gene (*PHLEO*^{R}), and two donor DNA cassettes for Li, containing blasticidin and puromycin-resistance genes (*BSD*^{R} and *PURO*^{R}), both flanked by sequences of ~30 nucleotides identical to the target locus (yellow boxes) were used for homology-directed repair of the nicks produced by Cas9. To the right is the simplified scheme of the *BSD*^{R}*-NDH2* episome (pGL-based), used as *NDH2*-complementing vector. Also depicted are the location of oligonucleotides (primers P8 through P12; gray arrowheads) employed in PCR diagnosis of transfected parasites and the size of the PCR products obtained from P8/P9-driven amplification of *NDUFS1* loci. (*B*) PCR diagnosis of Li promastigotes: wild-type (Li), before (pre-CC) and after (*BSD*^{R}*-PURO*^{R}) CRISPR-Cas9–driven attempts to disrupt NDUFS1 with the *BSD*^{R}*-PURO*^{R} cassettes. PCR reactions were carried out with primers flanking the *NDUFS1*/*BSD*^{R}*-PURO*^{R} ORFs (P8/P9) as well as with primers amplifying *BSD*^{R} and *PURO*^{R} inserted into the *NDUFS1* locus (P10/P9 and P11/P9, respectively). Disruption of *NDUFS1* in Lm promastigotes: wild-type (Lm) parasites, before (pre-CC) and after (*PHLEO*^{R}) CRISPR-Cas9–driven attempts to disrupt *NDUFS1* with the *PHLEO*^{R} cassette. PCR reactions were carried out with primers flanking the *NDUFS1*/*PLHEO*^{R} ORFs (P8/P9) as well as with primers amplifying *PHLEO*^{R} inserted into the *NDUFS1 locus* (P12/P9) and the *BSD*^{R}*-NDH2* episome (P6/P7). Disruption of *NDUFS1* was not successful in Lm promastigotes (*Middle panel*) but readily accomplished in Lm *NDH2*+ parasites, the resulting transgenics being *NDH2+/ndufs1*$^{-/-}$ (two independent clones, c1 and c2, are shown). (*C*) Western blot analysis of NDH2 expression of the indicated *Leishmania* strains with anti-NDH2 antiserum (loading control with anti-mTXNPx). (*D*) Crude mitochondrial extracts from the indicated strains of *Leishmania* were analyzed by BN-PAGE upon digitonin solubilization. The native gel was stained for NADH:NBT activity identifying complex I. The dimeric form of ferritin is indicated. Uncropped membranes are shown in *SI Appendix*, Fig. S8.

NDH2 (Lm *NDH2*+) to drive NDH2 overexpression (Fig. 5*C*), *ndufs1*$^{-/-}$ mutants could be readily isolated, as confirmed by PCR (Fig. 5*B* and *SI Appendix*, Fig. S7) and NADH:NBT in-gel (Fig. 5*D*) analyses. We reckon that the low levels of NDH2 expression in wild-type *L. major* (Fig. 5*C*) do not suffice to sustain critical levels of NADH oxidation and/or feeding of the ETC, when complex I is abrogated.

In conclusion, NDH2 is per se capable of supporting the crucial function(s) of complex I, rendering this multisubunit enzyme dispensable for parasite survival.

Discussion

Type I and II NADH dehydrogenases are structurally very distinct enzymes yet display overlapping functions: they both maintain NADH/NAD+ balance, while fueling the mitochondria respiratory chain. When coexisting in the matrix face of the mitochondria, these systems are redundant. Here, we report on an internal type II NADH dehydrogenase of *Leishmania* that deviates from this trend. Our findings have distinct implications with regards to both *Leishmania* physiology and the treatment of infections caused by these parasites.

The work establishes that *Leishmania*, like many other organisms, makes use of type II NADH dehydrogenases to oxidize mitochondrial-derived NADH. This conclusion is supported by the following observations. First, the sequence of *Leishmania* NDH2 retains key residues conserved in all type II NADH dehydrogenases, including binding motives for the interaction with the two dinucleotide substrates, FAD and NAD(P)H. Second, analysis of yeast mutant strains devoid of homologous enzymes and expressing Li NDH2 directly demonstrated that this protein could act as a respiratory enzyme, preferentially oxidizing NADH. Finally, the findings that NDH2 faced the *Leishmania* mitochondrial matrix, that its overexpression increased the parasite respiratory rate, and that the enzyme could replace complex I activity provided evidence that NDH2 functions as an internal NADH dehydrogenase.

We show that NDH2 is crucial for *Leishmania* viability all along their life cycle. In support of this interpretation, NDH2 essentiality was found a trait not only of a species (Li) in which complex I is silent but also of an organism (Lm) in which this enzymatic complex is functional and provides most of the NADH dehydrogenase activity of the cell (Fig. 4 *A* and *B*). Such a pivotal role of NDH2 irrespective of the concomitant expression of type I NADH dehydrogenase suggests that the enzyme endows parasites with a function that complex I cannot supply.

Two hypotheses might account for NDH2 essentiality in *Leishmania*. NDH2 may confer parasites with a metabolic advantage relative to complex I. Because this enzyme does not export protons, growth at its expenses would bypass the negative effects that can accompany excessive mitochondrial membrane potential (35); this could benefit *Leishmania* by enabling them to take advantage of nutrient-rich environments and/or of specific nutrients for rapid growth. Such premises are in line with the observed upregulation of NDH2 in logarithmic-phase parasites comparative to stationary-phase cells (Fig. 1*A*). They are also backed by published reports. This is the case of a study showing that *N. crassa* resorts to its internal NDH2 (NDI1) for proliferating during log phase, when carbon sources are abundant, while relying on the energetically more favorable complex I during the stationary phase, when nutrients become limiting and growth is reduced (36). Similarly, the recent identification of NDH2 as a crucial enzyme for *Mycobacterium tuberculosis* growth in the presence of fatty acids (37) indicates that metabolization of specific substrates may require a type II NADH dehydrogenase. Finally, in what would be a different type of metabolic advantage, the *Leishmania* NDH2 could have a function in quinone synthesis as described for the plastid homologous enzyme that is essential for prenylquinone production (38).

NDH2 could be essential to *Leishmania* if it displayed dual subcellular location. A second NDH2-derived activity (located in another organelle or in the external face of the inner mitochondrial membrane) could not be substituted by complex I; hence, if indispensable to the cell, its loss would not be tolerated. Although our data show that NDH2 is predominantly a mitochondrial matrix protein in both *Leishmania* (Fig. 1 *B* and *C*) and yeast (*SI Appendix*, Fig. S2), it is interesting to note that in the latter organism, a small fraction of activity was detected in the IMS. The reasons for this apparent dual localization are unclear. It may be a genuine biological feature of *Leishmania* NDH2 or an artifact of expression in the yeast system. Nonetheless, it is noteworthy that dual localization of other NADH dehydrogenases has been reported (39–41) and suggested to be associated to a gain of function. For example, yeast Nde1, a classical NDH2 in the IMS, can also become cytosolic exposed, engaging, in this circumstance, in programmed cell death (41). Hence, even if no precedent for a matrix/IMS located NDH2 exists, this conjecture will be exploited in upcoming studies. In this context, it is interesting that *T. brucei* NDH2 has been reported to face the matrix (20) and the intermembrane space (21), a conflicting result that could easily be explained by NDH2 being located at both sides of the inner membrane.

Our results also offer important insights into the significance of complex I in *Leishmania*. Like other trypanosomatids, all species of *Leishmania* hold the genetic information needed to express this dehydrogenase (a multisubunit complex of up to 60 dissimilar subunits); nonetheless, its presence and importance to parasites has remained elusive. Data obtained here clearly establish that *Leishmania* is heterogeneous in what regards the presence of this dehydrogenase, some species expressing it (Lm) while others do not (Li). Importantly, even in species in which complex I activity is prominent, the function of this enzyme can be fulfilled by NDH2, provided this is expressed at sufficient levels. This conclusion is supported by the observation that a gene encoding a crucial complex I subunit could be successfully removed from both Lm and Li genomes (hence, blocking any possibility of formation of a functional enzyme) without impacting parasite survival (Fig. 5 *B* and *C*).

At least in promastigotes, complex I can be envisaged as a complement to NDH2 activity, the latter enzyme acting as the main mitochondrial NADH dehydrogenase in *Leishmania* mitochondria. In fact, when the levels of NDH2 are per se high enough, complex I becomes fully dispensable, as observed in Li. We anticipate that previous failures in revealing complex I activity in species as diverse as *Leishmania amazonensis* (14), *Leishmania donovani* (15), and the reptilian species *Leishmania tarentolae* (42) also reflect enzyme inessentiality. This assumption agrees with the observation that disruption of complex I in *L. tarentolae*, due to loss of minicircle-encoded guide RNAs editing several complex I subunit genes (43), did not affect parasite viability in culture.

Future studies will address whether the intracellular mammalian stage of *Leishmania* can also thrive without an active complex I. Interestingly, *Trypanosoma cruzi* field isolates harboring deletions in mitochondrial DNA rendering complex I inactive do not seem to affect the parasite biology, since these mutant strains are pathogenic to humans (44). Similarly, disruption of complex I in bloodstream *T. brucei* forms (45) did not yield any growth phenotypes in vivo. We also noticed that complex I was not able to rescue the growth defect of the Li *ndh2*$^{+/-}$ mutant in a mouse model of infection. Still, a definite statement regarding the role of *Leishmania* complex I in mammals requires evaluation of the ability of Li *ndufs1*$^{-/-}$ strain to infect mice. Certainly, assigning an important role for complex I in vivo would provide an answer for the maintenance of the different complex I subunit genes in species such as *L. infantum*.

Leishmaniasis therapy requires the development of novel treatments. Mitochondrial NADH oxidation as well as ATP generation through oxidative phosphorylation are essential metabolic functions for *Leishmania*, particularly in intracellular amastigotes (46, 47). As type II dehydrogenases are key for these functions, interference with the activity of NDH2 came up as an attractive strategy to fight Leishmaniasis. Nevertheless, the likelihood of redundancy between internal type II enzymes and complex I questioned whether inactivation of NDH2 would perturb parasite physiology to the point that elimination would ensue. Data presented here overturn this concern, positioning instead NDH2 as a very interesting target for future Leishmaniasis drug development. Indeed, in addition to being absent from mammalian hosts, this enzyme is essential to *Leishmania*, including to the mammalian disease-causing stage. The fact that several inhibitors have been developed for homologous enzymes of other pathogens (48–51), including a high-affinity inhibitor (52–54), sustains the idea that selective inhibition of NDH2 might not be a chimera.

This work identifies an organism in which type II NADH dehydrogenases are essential in the presence of active type I enzymes. This organism, *Leishmania*, emerges therefore as an unanticipated model system for future studies aiming at uncovering the still enigmatic advantage(s) provided by NDH2 that determined their presence in so many bacteria, yeast, protists, and plants along evolution.

Materials and Methods

Ethics Statement. Mice were raised at the i3S Animal Facility under specific pathogen–free conditions. Experimental animal procedures conformed to directives approved by the Local Animal Ethics Committee of i3S and licensed by DGAV (Direção Geral de Alimentação e Veterinária, Govt. of Portugal). The i3S animal house is certified by DGAV. Animals were handled in strict accordance with good animal practice as defined by national authorities (DGAV, directive 113/2013 from August 7, 2013) and European legislation (Directive 2010/63/EU).

Parasite Cultures. *L. infantum* promastigostes (strain MHOM MA67IT-MAP263) were cultured at 25 °C in Roswell Park Memorial Institute (RPMI) 1640 Glutamax supplemented with 10% (volume/volume) inactivated fetal bovine serum (iFBS), 50 U/mL^{-1} penicillin, 50 μg/mL^{-1} streptomycin (all from Gibco), and 25 mM Hepes sodium salt pH 7.4 (Sigma-Aldrich). *L. major* (strain MHOM/SA/85/JISH118) were grown at 25 °C in RPMI, as described for *L. infantum*. To obtain promastigotes at different growth phases, parasites were seeded at 1 × 10^6 cells/mL^{-1} (day 0) and harvested at the indicated time points. Axenic amastigotes of *L. infantum* were differentiated from parasites recently recovered from the spleen of infected mice, as described before (55), and maintained at 37 °C, 5% CO_2, in modified 199 medium supplemented with 0.5% (weight/volume) soybean tryptocasein, 15 mM D-glucose, 4 mM $NaHCO_3$, 25 mM Hepes pH 6.5, 20% (volume/volume) iFBS, 2 mM Glutamax (Gibco), and 0.023 mM hemin (Sigma).

Yeast Plasmids Expressing Li NDH2. Li *NDH2* ORF was obtained by digestion of pTEX-*NEO^R-NDH2* (see *Complementing Plasmids*) with *SpeI*/*Xho*I and cloned into the corresponding sites of p415TEF vector, creating plasmid p415TEF-*NDH2*, from which protein expression was under the control of a TEF promoter (56). For targeting to the mitochondrial matrix, Li *NDH2* ORF was genetically fused with the N-terminal MTS from subunit 9 of the F0-ATPase (Su9) from *N. crassa* (p415TEF-*Su9*). For that, pTEX-*NEO^R-NDH2* was double digested with *Bam*HI/*Xho*I, and the 900-bp fragment containing the 3' end of the gene was cloned into p415TEF-Su9. The resulting recombinant plasmid was digested with *Bam*HI and a 600-bp fragment isolated by digestion of pTEX-*NEO^R-NDH2* with *Bam*HI, containing the 5' end of Li *NDH2* ORF, inserted there, generating p415TEF-*Su9-NDH2*.

Mitochondrial Oxygen Consumption Assay. The oxygen consumption rate of mitochondria isolated from BY4742wt, Δ*nde1*::kanMX4 Δ*nde2*::HIS3, and Δ*ndi1*::hphNT2 strains transformed with the plasmids (p415TEF empty, p415TEF-*NDH2*, or p415TEF-*Su9-NDH2* was assessed using a Clark-type oxygen electrode) (Hansatech Instruments, Germany). For measurements, mitochondria were resuspended in 0.6 M Sorbitol, 20 mM Hepes pH 7.4 and 1 mM EDTA buffer supplemented with 1 mM $MgCl_2$ at a concentration of 250 μg/mL. Temperature was controlled at 25 °C. Signals were recorded for at least 1 min to establish baseline oxygen consumption. Subsequently, oxygen consumption was measured following the addition of either NADH or NADPH at a final concentration of 7.05 mM.

Basal Oxygen Consumption Assay. Parasite culture of logarithmic promastigotes were counted, washed, and suspended at 3 × 10^8 parasites/mL in phosphate-buffered saline. Oxygen consumption was constantly measured at room temperature using a Clark-type oxygen electrode (Hansatech) in a total volume of 1 mL. Assays contained 3 × 107 parasites, 0.3 M sucrose, 10 mM potassium phosphate pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA (ethyleneglycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 10 mM KCl, 4 μM CCCP (carbonyl cyanide m-chlorophenyl hydrazone), and 0.02% (weight/volume) bovine serum albumin. Respiratory chain inhibitors piericidin A, TTFA, antimycin A, and KCN were added to final concentrations of 5 μM, 1 mM, 0.37 μM, and 1 mM, respectively. Results were analyzed using the O2view software version 1.02 (Hansatech) (57).

Complementing Plasmids. Plasmid pTEX-*NEO^R-NDH2* was assembled by cloning the Li *NDH2* ORF (PCR-amplified with primers P13 and P14) into the *SpeI*/*Xho*I restriction sites of the expression vector pTEX-*NEO^R* (58).

The pGL-*BSD^R-NDH2* expression plasmid was generated as follows. First, the 3' untranslated region (3' *UTR*) of the tryparedoxin 1 (*TXN1*) gene was obtained by sequentially digesting the pGL-5'3'*UTR-TXN1* (55) plasmid with *Bgl*II and *Bam*HI. The 3' UTR region was cloned into the pGL-*BSD^R-SeqA* plasmid (59) previously digested with *Bgl*II and treated with alkaline phosphatase to prevent self-ligation. The pGL-*BSD^R-NDH2* plasmid was assembled by cloning the PCR-amplified (primers P15 and P16) Lm *NDH2* ORF upon digestion with *Sma*I and *Kpn*I into the equivalent sites of pGL-*BSD^R-SeqA*-3'UTR.

Replacement Vectors for Li *NDH2* Deletion by HR. The Li *NDH2* replacement vectors were assembled by cloning fragments of the 5' and 3' UTRs of the gene (PCR-amplified from the *L. infantum* genome using primer pairs P17/P18 and P19/P20) into the *Hind*III/*Spe*I and *Bam*HI/*Xba*I sites of plasmids pGL345 and pGL726, carrying the hygromycin B phosphotransferase (*HYG^R*) or the phleomycin hydrolase (*PHLEO^R*) coding sequences, respectively (60). Before transfection of *L. infantum*, the replacement cassettes were excised out by digestion with *Hind*III/*Xba*I and purified from agarose gels.

Generation of sgRNA Expression Vectors and Donor Fragments for CRISPR-Cas9–Driven *L. major NDH2* and *NDUFS1* Deletion. Promastigotes of *L. major* were engineered to express *Streptococcus pyogenes* Cas9 nuclease gene from the pLP-*HYG^R-Cas9* plasmid (61). These parasites were transfected with pSPneogRNA5'H-HHgRNA3'H plasmid and then, upon selection, transfected with the donor DNA.

The pSPneogRNA5'H-HHgRNA3'H plasmid expressing the 5' and 3' guide RNAs was generated according to the protocol described by ref. 61. Briefly, the guide sequences were designed for *NDH2* (P21-P24) and for *NDUFS1* (P25-P28), the double-stranded 5' and 3' guide RNAs (gRNAs) were cloned into the *Bbs*I-restricted pSPneogRNAH and pSPneoHHgRNAH plasmids, respectively. The rRNAPgRNA5'H fragment (360 bp) was then excised from pSPneogRNA5'H by sequentially digesting the plasmid with *Bam*HI and *Hind*III and introduced into the *Bgl*II/HindIII restriction sites of pSPneoHHgRNA3'H.

The *PHLEO^R* cassette donors were obtained from pGL-*SSA-PHLEO^R* plasmid (59) by PCR amplification with primers P29/P30 for *NDH2* and primers P31/P32 for *NDUFS1*.

Production of sgRNA Templates and Targeting Fragments for CRISPR-Cas9–Driven *L. infantum NDUFS1* Deletion. Promastigotes of *L. infantum* were transfected with pT007_Cas9_T7_Tub plasmid to express Cas9 nuclease and T7 RNA polymerase genes (62). These parasites were transfected with a mixture of sgRNAs and donors (6 μg of each) to target *NDUFS1* alleles. Both sgRNAs and donors were PCR amplified in a reaction mixture containing 0.2 mM deoxyribonucleotides triphosphate (dNTPs) , 2 μM of each primer, and 1 Unit Q5-fidelity DNA polymerase (New England Biolabs) in 1× Q5 reaction buffer. For amplification of sgRNAs, the sgRNA scaffold primer P33 and the target-specific forward primer P34 or P35 were used. For amplification of *BSD^R* and *PURO^R* donor fragments, 30 ng of pTBlast or pTPuro plasmid were used (62) with primers P36 and P37 specific for *NDUFS1*.

In Vivo Virulence. Parasites were passaged through mice once prior to infection experiments. Inoculation of C57BL/6 mice (6 to 8 wk-old) was performed intravenously with 2 × 10^7 stationary-phase promastigotes of each strain. Twenty-one days postinfection, mice were euthanized and their livers and spleens excised, weighed, and homogenized in Schneider's medium (Sigma-Aldrich) supplemented with 10% (volume/volume) iFBS, 100 U/mL^{-1} penicillin, 100 μg/mL^{-1} streptomycin, 5 mM Hepes pH 7.4, and 5 μg/mL^{-1} phenol-red

(Sigma-Aldrich). The parasite load (parasites per gram of organ) was estimated using a limiting dilution assay and calculated as described before (63).

Data Availability. All study data are included in the article and/or *SI Appendix*.

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