



Deposited via The University of York.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/id/eprint/244813/>

Version: Published Version

Article:

Kropp, Ashleigh, Asadollahi, Kazem, Stapleton, Jamie A et al. (2026) Quinone-transporting filaments expand bioenergetic capacity in Gram-positive Bacillota. Nature Microbiology. ISSN: 2058-5276

<https://doi.org/10.1038/s41564-026-02450-z>

Reuse

This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here:

<https://creativecommons.org/licenses/>

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

Quinone-transporting filaments expand bioenergetic capacity in Gram-positive Bacillota

Received: 27 September 2025

Accepted: 13 July 2026

Published online: 17 August 2026

 Check for updates

Ashleigh Kropp^{1,2,3}, Kazem Asadollahi^{1,10}, Jamie A. Stapleton^{4,10}, Luka Simsi^{5,10}, Pok Man Leung^{3,10}, Rachel L. Darnell^{6,10}, Christopher K. Barlow⁷, Benjamin G. Hartmann⁸, Nicholas D. J. Yates⁴, Tasha Lumbantobing⁷, Daniel R. Fox^{1,2,3}, Chris Greening^{1,3}, Oleksii Zdorevskyi^{1,5}, Vivek Sharma^{1,5,9}, James N. Blaza^{1,8}, Alison Parkin^{1,4} & Rhys Grinter^{1,2,3} 

Cellular respiration depends on transferring electrons to hydrophobic quinones in membrane bilayers, meaning bioenergetic capacity is constrained by available membrane surface area. While Gram-negative bacteria expand this capacity through internal membrane invaginations and eukaryotes use membrane-bound organelles, whether Gram-positive bacteria have alternative capacity-generating mechanisms is unknown. Here we show that *Bacillus subtilis* forms a quinone-transporting pseudomembrane composed of filaments of the NADH dehydrogenase Ndh and the quinone-transporting protein Ncp. Cryo-EM, lipidomics and molecular dynamics reveal that Ndh and Ncp co-assemble with lipids into a 4:4 stoichiometric complex with a solvent-excluded hydrophobic lumen containing phospholipids, which sequesters quinones. These complexes further assemble into filaments, linking chambers into a continuous conduit that amplifies quinone reduction. Phylogenetic analysis suggests that this capacity is widespread in Bacillota. Quinone-transporting filaments thus reveal a strategy to expand the quinone pool and bioenergetic capacity while occupying minimal membrane space.

Cellular respiration is fundamentally constrained by the surface area available to house electron-transport machinery^{1,2}. Overcoming this limitation has been proposed as a central driver of eukaryogenesis, with mitochondria and chloroplasts providing massively expanded internal membranes to support energy conversion^{3–5}. Certain Gram-negative bacteria also employ organelles for energy conversion, notably thylakoids, chlorosomes and anammoxosomes⁶. Others use extensive membrane invaginations to boost electron input capacity^{3–5,7–10}. Gram-positive bacteria appear to lack internal membranes or organelles that expand their respiratory capacity¹¹. In addition, they only have a single membrane, which must accommodate other essential

processes such as cell wall synthesis, nutrient import and secretion^{11,12}. Because respiratory ATP synthesis depends on electron transfer within this limited membrane surface, mechanisms that expand this capacity would confer an advantage under rapid growth or metabolic stress¹³. Central to this process are respiratory quinones, highly hydrophobic molecules that shuttle electrons between respiratory dehydrogenases and terminal oxidases¹⁴. For respiratory enzymes, accessing membrane-embedded quinones poses a fundamental challenge. Many enzymes solve this problem by using integral membrane subunits, often containing haem or iron–sulfur clusters, that reduce quinones directly within the bilayer, and require permanent membrane real estate^{15,16}.

Others peripherally associate with the membrane to reach the quinone pool without dedicated transmembrane subunits^{17,18}. Another recently discovered strategy involves extracting quinones from the membrane and reducing them in the cytosol using either membrane-associated quinone transport channels or a soluble quinone-binding shuttle protein^{19–21}.

Nicotinamide adenine dinucleotide (NADH) is a key intermediate electron carrier in cellular metabolism, transporting low-potential electrons primarily from organic carbon oxidation to the electron-transport chain via the reduction of respiratory quinone^{22,23}. While this reaction is classically associated with the multisubunit complex I (type-1 NADH dehydrogenase), many bacteria instead rely on type-2 NADH dehydrogenase (NDH-2). Unlike complex I, NDH-2 is a compact, ~50 kDa flavoprotein that does not span the membrane or pump protons^{24–28}. It is often the dominant or sole NADH-oxidizing enzyme in bacteria, underscoring its physiological importance^{17,29}.

The soil-dwelling facultatively anaerobic Gram-positive bacterium *Bacillus subtilis* encodes three putative NDH-2 enzymes, *ndh*, *yumB* and *yutJ*, to transfer electrons from NAD(P)H to the respiratory chain^{29,30}. Among these, Ndh is the primary NADH dehydrogenase during vegetative growth²⁹. NDH-2 enzymes often peripherally associate with the membrane to transfer electrons to quinone within the bilayer^{17,26,31–34}. However, *B. subtilis* Ndh lacks key residues in this region for membrane interaction, raising the question of how it accesses and reduces quinones. The *ndh* gene shares an operon with *yjIC*, which encodes a protein with a C-terminal DUF1641 domain²⁹. Recently discovered DUF1641-domain proteins form quinone-transporting complexes with both H₂- and formate-oxidizing enzymes (Huc and ForCE)^{19,20}.

Here we identify that the *yjIC* gene product, which we designate the Ndh-coupling protein (Ncp), forms a stable NADH-oxidizing complex with Ndh. Unlike the Huc and ForCE complexes, the Ndh–Ncp complex assembles into tube-like filaments with internally connected hydrophobic chambers lined by many embedded phospholipids^{19,20}. These Ndh–Ncp ‘pseudomembrane’ tubes enable the sequestration and long-range transport of respiratory quinones, hundreds of angstroms outside of the membrane, for reduction by electrons from NADH. By doing this, Ndh–Ncp filaments extend the surface area for quinone reduction, providing a solution to the limitation of membrane real estate available for energy generation, functionally convergent with mitochondrial cristae and respiratory supercomplexes^{3,4,35,36}. Genomic analysis shows that Ndh–Ncp complexes are widely distributed across Bacillota, indicating that respiratory chain expansion via protein–lipid pseudomembrane filaments is a conserved and fundamental mechanism in bacterial bioenergetics.

Results

Ndh from *B. subtilis* forms a functional complex with Ncp

The *ndh* gene from *B. subtilis* is important for optimal growth and forms an operon with *nep* (formerly *yjIC*)²⁹. To test whether *ndh* and *nep* are functionally associated, we generated *B. subtilis* knockout strains lacking either gene ($\Delta ndh::kan$ or $\Delta nep::kan$) and assessed

their growth in liquid culture. Both $\Delta ndh::kan$ and $\Delta nep::kan$ strains exhibited a slower growth rate, with defects of similar magnitude at 37 °C, suggesting that loss of *nep* phenocopies loss of *ndh* (Fig. 1a and Extended Data Fig. 1a). In addition, when grown at 30 °C (which reduces sporulation of *B. subtilis*), the growth yield of both strains was substantially reduced (Extended Data Fig. 1b,c).

Given the genetic linkage between *ndh* and *nep*, and the homology of Ncp to HucM, we hypothesized that Ndh and Ncp form a complex that couples Ndh activity to the membrane. To investigate this, we constructed an *Escherichia coli* expression construct encoding both *nep* and *ndh* genes in tandem, with *nep* N-terminally twin-strep tagged. Expression and purification from clarified cell lysate resulted in the isolation of a complex containing both Ndh and Ncp, which had an elongated peak on size exclusion chromatography (SEC), with mass photometry indicating a non-uniform size distribution (Extended Data Fig. 1d and Supplementary Fig. 1). UV–visible spectra demonstrated that the purified Ndh–Ncp complex contains flavin in an oxidized state with absorbance peaks at ~390 nm and 450 nm. The addition of NADH led to the formation of a charge-transfer complex (CTC) between NADH and FAD⁺, indicated by an absorbance peak at 660 nm (Fig. 1b and Extended Data Fig. 1e)²⁶. This CTC wavelength is comparable to that of NDH-2 from *Caldalkalibacillus thermarum* and *Staphylococcus aureus* but is shorter than the 740 nm of the distantly related eukaryotic NDH-2 from *Saccharomyces cerevisiae*^{26,33,37}.

We next measured the catalytic kinetics of Ndh–Ncp using the quinone analogues menadione (MDA) or decylubiquinone (DQ) as electron acceptors. Ndh–Ncp oxidizes NADH with a Michaelis constant (K_M) of 50 μ M (confidence interval (CI) 36–69 μ M) and a maximum velocity (V_{max}) of 110 s^{–1}, with MDA as the electron acceptor (Fig. 1c and Extended Data Fig. 1f). The complex has lower affinity and is ~10-fold slower than NDH-2 from *C. thermarum* with MDA as an electron acceptor ($K_M = 29 \mu$ M, $V_{max} = 1,190$ s^{–1})²⁶. Using DQ as an electron acceptor for Ndh–Ncp resulted in a lower V_{max} , suggesting that Ndh–Ncp prefers menaquinone over ubiquinone (Extended Data Fig. 1g,h). The slower kinetics of *B. subtilis* Ndh–Ncp compared with *C. thermarum* NDH-2 suggest that this complex may restrict the access of soluble quinones to the Ndh electron acceptor sites compared to characterized NDH-2 enzymes.

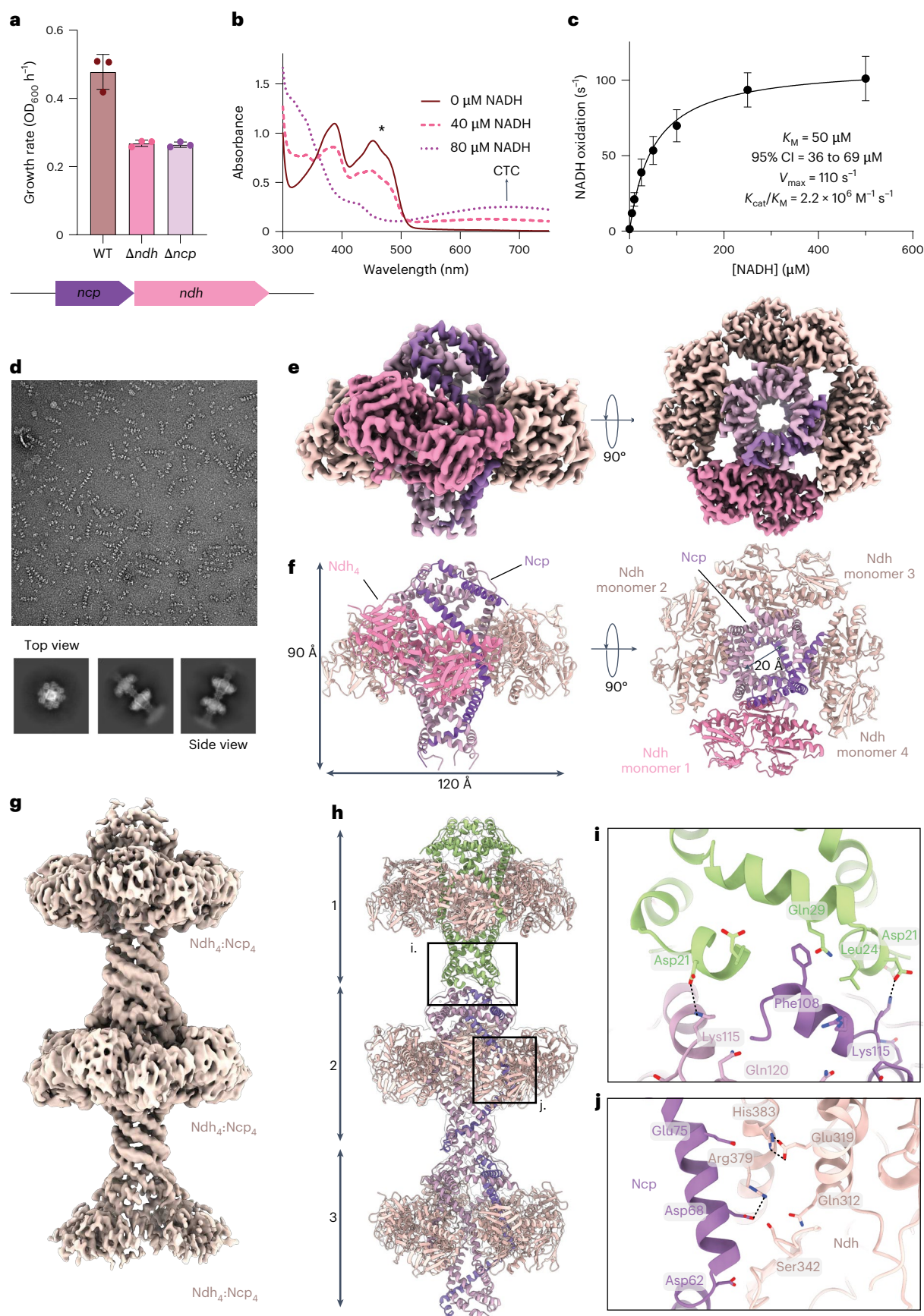
Ndh and Ncp form a complex that associates into filaments

To gain insight into the structure of the Ndh–Ncp complex, we performed negative-stain electron microscopy. Strikingly, these micrographs revealed that purified Ndh–Ncp forms filaments with as many as 13 repeating units (Fig. 1d). To determine the high-resolution structure of this complex, we performed cryo-electron microscopy (cryo-EM) on Ndh–Ncp, followed by single-particle and helical reconstruction (Fig. 1d). We resolved the structure of a single Ndh–Ncp complex at a global resolution of 2.11 Å, revealing a 4:4 stoichiometry of Ndh and Ncp subunits (Supplementary Fig. 2 and Table 1). Ncp forms a hollow tetrameric tube at the centre of the complex, which scaffolds four Ndh subunits. The complex has C4 symmetry, spans ~90 Å in length and

Fig. 1 | *B. subtilis* Ncp complexes with Ndh and is essential for its activity.

a, Growth rates of WT, Δndh or Δnep *B. subtilis* 168 strains during exponential phase. Data presented as mean $\pm$ s.d. of $n = 3$ biological replicates. **b**, Representative UV–visible spectra of Ndh–Ncp complex with increasing concentrations of NADH. The asterisk (*) shows the typical flavin peak at 450 nm, and the arrow represents the charge-transfer complex (CTC) at ~660 nm. **c**, Michaelis–Menten kinetics of Ndh–Ncp incubated with 500 μ M MDA and varying concentrations of NADH. Data presented as mean $\pm$ s.d. of $n = 3$ independent replicates. **d**, Representative negative-stain EM micrograph showing filamentous Ndh–Ncp (top, representative micrograph from $n = 10$ replicates) and cryo-EM 2D class averages of filaments (bottom). **e**, Cryo-EM map of the Ndh–Ncp complex showing the Ndh subunits in pale pink and one

representative in bright pink, and the Ncp in purple. The images show the side (left) and top (right) views of the complex. **f**, Cartoon representation of the cryo-EM structure of the Ndh–Ncp complex. Four Ndh monomers (light pink) couple to Ncp (purple) with C4 symmetry. Left: side view. Right: top view of the complex. **g**, Cryo-EM map of the Ndh–Ncp helical refinement showing three subunits in the filament. **h**, Ndh–Ncp filament structure showing three units in the filament. A cartoon representation is shown, with the top Ncp tube in green, the following two tubes in purple and the interacting Ndh enzymes in pale pink. **i**, Interaction between the ‘tail’ (green) of Ncp from one unit and the ‘head’ (purple) Ncp from another unit in the filament. Key residues that promote filament formation are highlighted. **j**, Interface between one Ndh enzyme and Ncp monomer, highlighting key residues that drive the interaction.



~120 Å in width, and has openings in the Ncp tube of ~20 Å in diameter at its head and tail (Fig. 1e,f). The interface between the Ndh monomers and the Ncp scaffold is primarily driven by electrostatic interactions mediated by a positively charged Ndh C-terminal helix, which is complemented by the negative charge of the Ncp scaffold in this region (Fig. 1j and Extended Data Fig. 1i). To reconstruct the Ndh–Ncp filament, we performed a helical refinement, generating a map with three connected Ndh–Ncp complexes (Fig. 1g,h, Extended Data Fig. 1j, and Supplementary Fig. 2, Video 1 and Table 1). These Ndh–Ncp subunits stack head-to-tail to form the filament, with the connecting interface composed entirely of the Ncp subunit and driven by a phenylalanine (Phe108) on the Ncp head embedding into the tail complex, as well as a salt bridge between Lys115 (head) and Asp21 (tail) (Fig. 1h,i). Sequence analysis of Ncp homologues from diverse Gram-positive bacterial species indicates that key interacting residues on both sides of the filament interface are highly conserved (Extended Data Fig. 2a,b), indicating that natural selection maintains the integrity of this interface, suggesting that it is physiologically relevant. Consistent with this, mutagenesis of key interacting residues of the interface diminished filament formation (Extended Data Fig. 2c–g).

To determine whether Ndh–Ncp filament formation also occurs under native expression conditions in *B. subtilis*, we extracted the complex directly from cells and analysed it using negative-stain EM. To identify NADH-oxidase activity corresponding to Ndh–Ncp, we performed activity staining on lysates from wild-type, *Andh* and *Δncp* strains. High molecular weight staining was observed exclusively in the wild-type lysate, consistent with the apparent molecular weight of recombinant Ndh–Ncp on native polyacrylamide gel electrophoresis (PAGE) (Extended Data Figs. 1d and 3a). Guided by this activity, we fractionated *B. subtilis* lysates using anion exchange followed by size exclusion chromatography, tracking Ndh–Ncp by native PAGE activity staining (Extended Data Fig. 3b–e). SEC fractions containing NADH-oxidase activity in the expected size range were analysed by negative-stain EM. These fractions contained stacked filaments closely resembling those formed by recombinant Ndh–Ncp, with dimensions consistent with the cryo-EM structure (Extended Data Fig. 3f,g). We additionally confirmed that Ndh–Ncp is strongly enriched in this fraction by mass spectrometry (Supplementary Data 1). This indicates that Ndh–Ncp filaments form under physiological conditions in *B. subtilis*. Notably, this fractionation strategy did not substantially concentrate the lysate, suggesting that filament formation is not an artefact of overexpression or protein concentration.

The width of the Ncp tube varies, reaching ~45 Å in the region where Ndh attaches (Fig. 2a). The C-terminal ‘head’ of the Ncp tetramer contains the DUF1641 domain. This region is structurally similar to HucM²⁰, with each Ncp subunit travelling up and over the monomer immediately left of it, then under the monomer immediately right of it, stabilizing the complex and creating the opening at the head of the tube (Fig. 2b,c). The equivalent region in Huc mediates membrane interaction via positively charged residues and a bilayer-interacting tryptophan²⁰. Consistent with this, the Ncp head is positively charged, with Phe108 positioned to embed in the bilayer (Fig. 2c,d). Interestingly, this region is also responsible for filament formation, suggesting a dual role in lipid and protein interactions, reminiscent of the recently characterized CODH–CoxG complex²¹. Considering that Ndh–Ncp forms filaments and that the complex is purified from the soluble fraction when expressed recombinantly, interaction between the Ncp and the membrane is most probably dynamic in nature or specific to native host membranes.

The Ndh–Ncp filament consists of a tube formed by the head and tail region of the Ncp tetramer, which connects a broader chamber where Ndh associates with the complex. Both the tube and chamber are lined entirely with hydrophobic amino acids, forming a continuous conduit for hydrophobic quinones from the point of membrane attachment to the electron acceptor sites of Ndh (Fig. 2d). Strikingly,

additional density is present in the cryo-EM maps in the region of Ndh attachment, filling gaps between the Ndh and Ncp polypeptide chains (Fig. 2e and Supplementary Video 2). The shape and chemical environment of this density are consistent with it belonging to phospholipids. The solvent-facing portion of these densities, which probably corresponds to negatively charged phosphate groups of phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) lipids, is coordinated by complementary positively charged residues (lysine residues 51, 66, 81, 85, 95 and 121 from Ncp and Arg213 and Lys351 from Ndh), and density probably corresponding to aliphatic tails extends into the hydrophobic interior of the Ndh–Ncp complex (Fig. 2e–g). The presence of this density suggests that the Ndh–Ncp complex incorporates a substantial quantity of phospholipids as an additional structural and functional element. Overall, the structure of the Ndh–Ncp complex reveals similarities to Huc and ForCE, indicating a conserved role for the DUF1641 superfamily in enzyme scaffolding, membrane attachment and long-range quinone transport^{19,20}. However, filament formation by Ndh–Ncp and the incorporation of phospholipids into the structure indicate considerable structural and mechanistic differences and demonstrate pronounced structural diversity within the DUF1641 superfamily.

Ndh–Ncp binds NADH and menaquinone in conserved binding sites

Our initial cryo-EM data were collected on Ndh–Ncp in the absence of NADH. This structure had no NADH or quinone bound at their respective binding sites. To obtain the substrate-bound structure, we repeated the cryo-EM, supplementing Ndh–Ncp with 5× the molar concentration of NADH immediately before freezing. We resolved this complex at a global resolution of 2.52 Å, showing clear density for the FAD group, NADH and quinone, at their structurally conserved binding sites (Fig. 3a–e, and Supplementary Fig. 3, Video 3 and Table 1). FAD coordination by Ndh is comparable to that by other structurally characterized NDH-2 enzymes, with isalloxazine stabilization from Lys127, Glu168 and Lys373 side-chain residues and Ala311 and Gln312 main-chain nitrogens^{26,28,31–34}. The pyrophosphate groups engage in hydrogen bonding with the Tyr12, Gly13 and Asp295 main-chain nitrogens, and the adenine ring interacts with the Val80, Gln261 and Lys37 main-chain nitrogens (Fig. 3b,c)^{26,31–34}. Ndh binds NADH in the conserved region, interacting with the key WxxG motif (Trp254 and Gly257), as well as with the highly conserved residues Ile116, Gly163, Val196–Gly199, Val231, Pro309 and Thr310 (Fig. 3d)^{26,31–34}.

In our NADH-bound structure, we observed density at the conserved quinone-binding electron acceptor site^{31,32,34}. No exogenous quinone was added to the Ndh–Ncp complex, meaning that the observed quinone was probably extracted during *E. coli* expression and retained through protein purification. The absence of this density in the NADH-free structure may indicate that quinone binding is preceded by NADH binding. *E. coli*, the expression host for the Ndh–Ncp complex, produces ubiquinone-8 (UQ-8) and menaquinone-8 (MK-8) as its major quinones during aerobic and anaerobic conditions, respectively¹⁴. The shape and coordination environment of the quinone headgroup density in our structure is more consistent with MK-8, so we modelled this in our structure (Fig. 3e and Supplementary Fig. 4a,b). This is also supported by the superior kinetics for Ndh–Ncp with MDA versus DQ as the electron acceptor (Extended Data Fig. 1f,h)³⁸. The Ndh quinone binding site is conserved in other NDH-2 homologues, specifically the motif (AQxAXQ; Ala311, Gln312, Ala314, Gln316)^{26,31,34}. The MK-8 binding site faces the interior of the Ndh–Ncp tube, indicating that quinone enters the Ndh electron acceptor site from the internal hydrophobic chamber of the complex (Fig. 3e and Supplementary Fig. 4c,d). Considering this, the slower kinetics of Ndh–Ncp may result from restricted access to the electron acceptor site, due to the tube slowing the movement of the soluble quinones used in this assay (Fig. 1c).

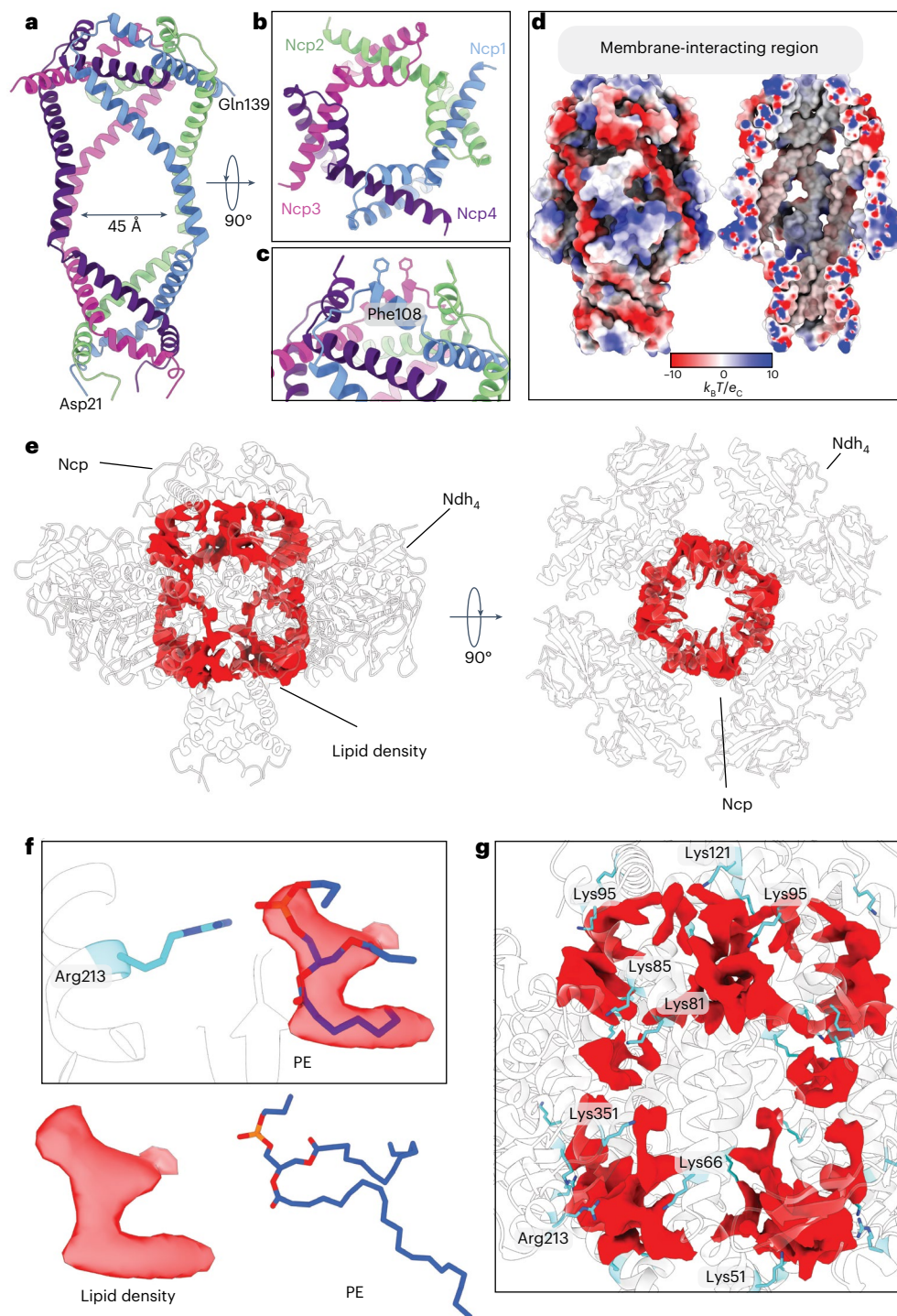


Fig. 2 | Ncp facilitates membrane interaction and Ndh-Ncp associates with lipids. **a**, Cartoon representation of the structure of Ncp tetramer alone, with each Ncp monomer in a different color. The Ncp tube broadens where Ndh binds, reaching a width of 45 Å. **b**, The DUF1641 domain forms the opening of the tube and **c**, Phe108 from each monomer facilitates membrane interaction. **d**, The membrane-interacting region of Ncp is characterized by positively charged residues (blue) flanking the hydrophobic Phe108 (white) that inserts into the hydrophobic bilayer. Internally, the Ndh-Ncp complex is hydrophobic

(white). Scale of electrostatic surface potential ($k_B T/e_c$) shown on the bar below. **e**, Cartoon representation of the Ndh-Ncp cryo-EM structure in white with grey outline, with density corresponding to phospholipids in red. **f**, Example of a phosphatidylethanolamine (PE) molecule modelled into lipid density from the cryo-EM maps of the Ndh-Ncp complex, with the phosphate head group coordinated by Arg213 from Ndh. **g**, Positively charged residues in blue from the Ndh and Ncp subunits coordinate lipid density (red) throughout the complex.

The Ndh-Ncp complex binds phospholipids and extracts menaquinone

Our cryo-EM structure suggests that phospholipids form an important component of the Ndh-Ncp complex and that the hydrophobic

chamber accommodates respiratory quinones. To identify lipids associated with the complex, we performed lipidomics on the purified complex. This analysis revealed a substantial lipid content with a considerable quantity of PE and PG lipid species (Fig. 3f, and Supplementary

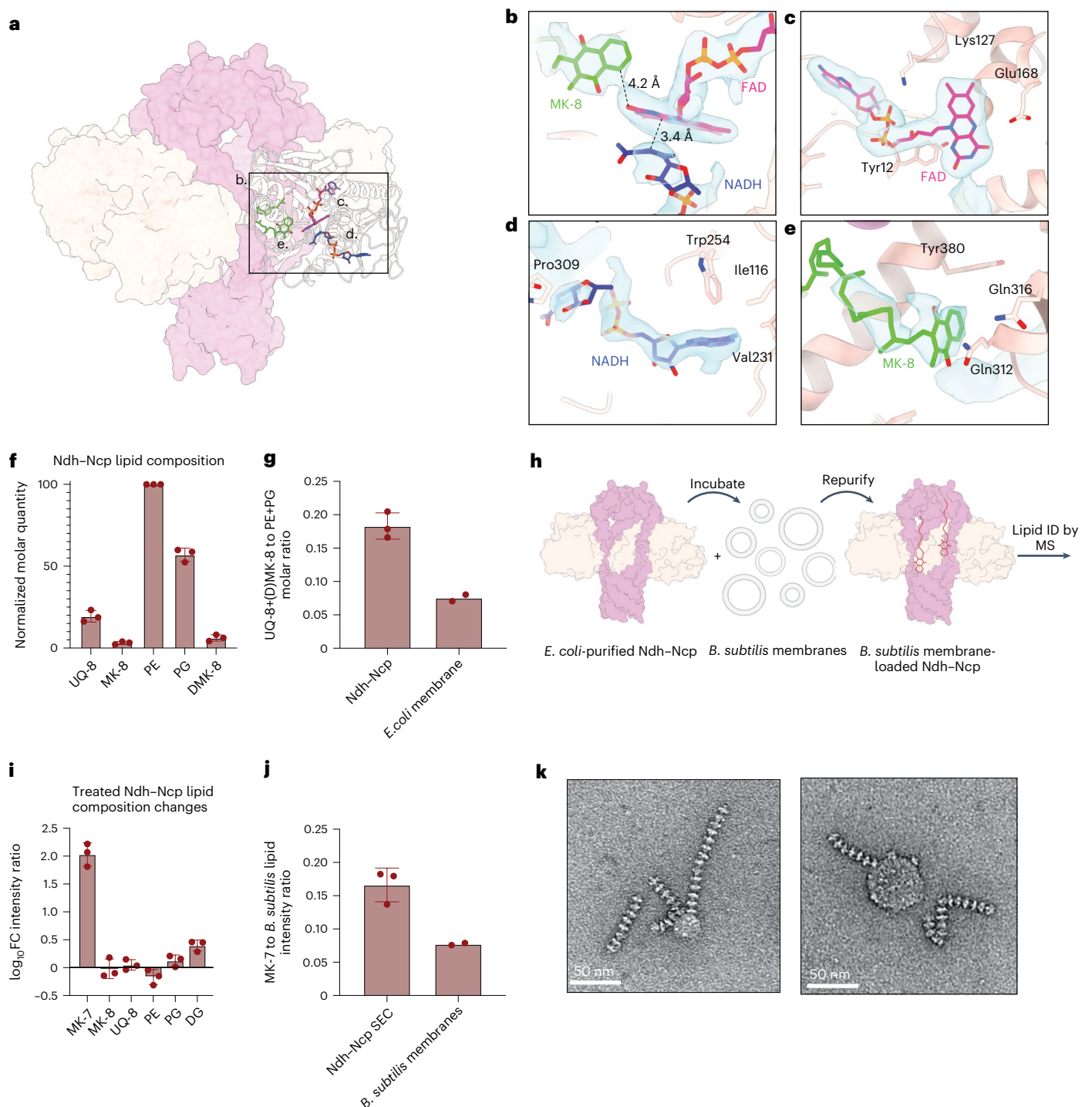


Fig. 3 | Ndh-Ncp binds NADH and MK-8. **a**, Cartoon representation of the cryo-EM structure of Ndh-Ncp with NADH and quinone bound, showing their location in one Ndh enzyme. **b**, Interatomic distances between each of the ligands; NADH in blue, FAD in pink and MK-8 in green. **c**, Coordination of FAD is driven by conserved Lys127, Glu168, Lys373 and Tyr12. **d**, NADH has a conserved binding mode. **e**, MK-8 is coordinated by Tyr380, Ala311, Gln312 and Gln316. Density for all three cofactors is shown overlaid on the structure, contoured at 5 σ . **f**, Graph showing the molar quantity of lipids in the Ndh-Ncp purified sample, normalized to the most abundant lipid, PE. Data were from positive ion mode MS, and each type of lipid was summed into its associated group (that is, PE lipids of differing lengths were summed into the PE group). **g**, Molar ratio of UQ-8, MK-8 and DMK-8 to PE and PG in purified Ndh-Ncp complex compared to membranes isolated from the *E. coli* expression strain. **h**, Schematic of the experimental protocol for the incubation of Ndh-Ncp with *B. subtilis* membranes. **i**, Graph showing

the fold change (FC) in lipids and quinones after treatment. **j**, Relative MS peak intensity ratio of MK-7 to lipids unique to *B. subtilis* membranes in Ndh-Ncp complex reisolated after incubation with *B. subtilis* membranes compared to the membranes used for the incubation. **k**, Negative-stain EM of streptactin-isolated Ndh-Ncp after incubation with *B. subtilis* membranes, showing attachment to membrane vesicles (representative micrograph from $n = 20$). PE, phosphatidylethanolamine; PG, phosphatidylglycerol; UQ-8, ubiquinone-8; MK-8, menaquinone-8; MK-7, menaquinone-7; DMK-8, demethylmenaquinone-8; DG, diglyceride; PC, phosphatidylcholine. Lipidomics data (**f**, **g**, **j**) were derived from 3 independent experiments ($n = 3$) for Ndh-Ncp complexes and two independent experiments ($n = 2$) for *E. coli* and *B. subtilis* membranes, with all samples analysed concurrently. Data represent mean $\pm$ s.d. Panel **h** created in BioRender: Kropp, A. <https://biorender.com/udvq7pg> (2026).

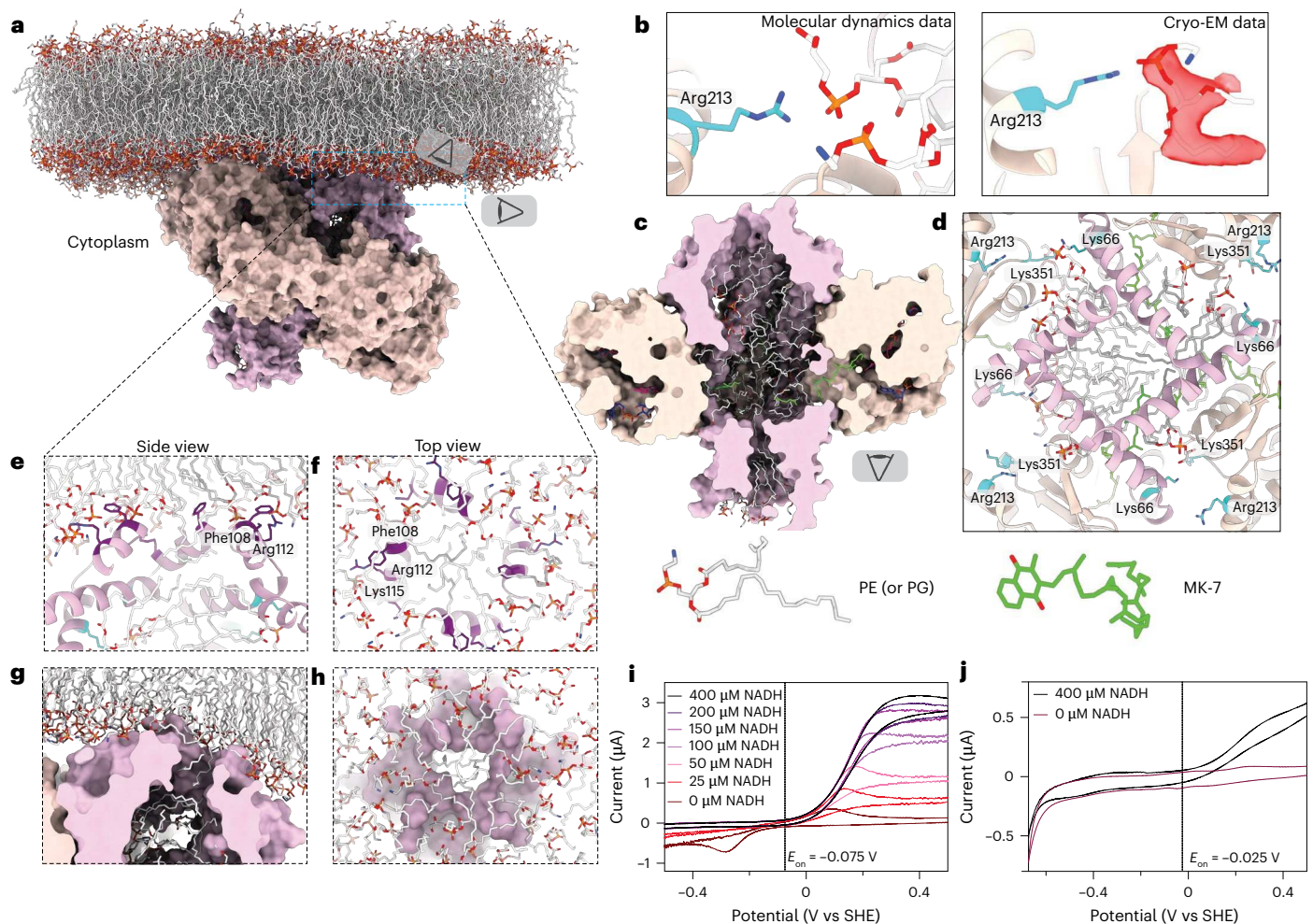


Fig. 4 | Molecular dynamics simulations demonstrate the Ndh-Ncp protein-lipid architecture. **a**, Ndh-Ncp simulation was set up with a bilayer consisting of PE and PG lipids. Lipids and MK-7 were docked inside the Ncp on the basis of structural and lipidomics data. **b**, A PE and PG molecule from an MD snapshot showing the PG coordinated by Arg213 (left) and cryo-EM data showing a PE molecule in the cryo-EM density coordinated by Arg213 (right). **c**, Snapshot of the last frame of the simulation with PE/PG present within Ndh-Ncp. Cutaway shows the occupation of these molecules within the Ncp and quinone binding site. MK-7 is shown in green and PE/PG in white. **d**, Snapshot of the last frame of the simulation showing the position of phospholipid headgroups in proximity to positively charged residues predicted to coordinate the lipid headgroups (lipids in white, coordinating residues in cyan). The complex is viewed from the bottom. **e**, Side view and **f**, top view snapshot from the last frame of the MD simulation of the Ncp interacting with the bilayer. Key residues Phe108, Arg112 and Lys115 are shown in dark purple. **g**, Side view and **h**, top view of the last frame from MD data of the Ncp tube interacting with the bilayer. Lipids from the bilayer enter the Ncp with the hydrophobic tail directed into the Ncp tube. **i**, Representative cyclic voltammograms of Ndh-Ncp stabilized on a phospholipid membrane recorded at varying NADH concentrations from -0.675 to $+0.625$ V vs SHE at 10 mV s^{-1} in the presence of MK-7. **j**, Representative cyclic voltammograms of Ndh-Ncp recorded at 0 μM NADH (pink) or 400 μM NADH (black) from -0.675 to $+0.625$ V vs SHE at 10 mV s^{-1} in the absence of exogenous MK-7. The onset potential (E_{on}) for each experiment is noted on the voltammograms in **i** and **j**.

Data 2 and 3). These data support our interpretation of the cryo-EM density as PE and PG phospholipids. These lipids are probably derived from the *E. coli* plasma membrane and associate with the complex during or after assembly. The high lipid content of the tubular assembly suggests a protein-lipid hybrid architecture capable of extending membrane-like environments beyond the bilayer.

In addition to phospholipids, abundant features with masses and elution profiles corresponding to UQ-8, MK-8 and demethylmenaquinone-8 (DMK-8) were associated with the complex (Fig. 3f, and Supplementary Data 2 and 3). The presence of MK-8 supports its assignment at the electron-acceptor site in the NADH-bound structure. UQ-8 was the most abundant quinone associated with the complex (Fig. 3f); however, as *B. subtilis* primarily utilizes MK-7, this probably reflects the *E. coli* expression system rather than substrate preference. The presence of both ubiquinone and menaquinone in the complex suggests that association is driven primarily by interactions

with their hydrophobic isoprenyl tails, which can be accommodated within the central cavity. To quantify quinone enrichment, we compared standardized lipidomics profiles of the Ndh-Ncp complex with those of *E. coli* membranes, calculating the molar ratio of total quinones (UQ and MK species) to total PE and PG lipids. This ratio was higher in the Ndh-Ncp complex, indicating that the complex concentrates quinones relative to the membrane (Fig. 3g). Given this enrichment, quinones can probably occupy both the canonical Ndh binding sites and the central cavity, although additional quinones could not be resolved structurally due to the dynamic nature of this hydrophobic environment.

To assess whether the Ndh-Ncp complex can extract quinones in a native context, we incubated the purified complex with isolated *B. subtilis* membranes, then repurified it and analysed its lipid content (Fig. 3h, Supplementary Note 1 and Extended Data Fig. 4a). *B. subtilis* produces MK-7 as its sole respiratory quinone, and accordingly, MK-7

was the only quinone species identified with increased abundance³⁸, with levels increased by ~90-fold relative to pre-incubation (Fig. 3i, and Supplementary Fig. 4 and Data 3)³⁸.

To assess enrichment of MK-7 in membrane-incubated Ndh–Ncp relative to the membrane, we compared the within-sample peak intensity ratio of MK-7 to a set of *B. subtilis*-specific lipid species in the post-incubation complex and *B. subtilis* membranes (Supplementary Data 3). The ratio was higher in the Ndh–Ncp sample, indicating increased relative abundance of MK-7 in the complex (Fig. 3j and Extended Data Fig. 4c–f). Similarly, the ratio of total quinone signal to *B. subtilis*-specific lipids was elevated in the complex, suggesting that MK-7 accumulation is not simply due to exchange with pre-bound quinones but reflects net enrichment (Extended Data Fig. 4d,e). Although these ratios were derived from raw peak intensities and may be affected by matrix effects, consistent enrichment across multiple lipid species supports a qualitative conclusion of MK-7 enrichment following membrane incubation.

The architecture of the Ndh–Ncp–phospholipid complex

Our structural and lipidomic analysis indicates that Ndh–Ncp forms a hybrid protein–phospholipid complex. However, the density in our cryo-EM maps is insufficiently resolved to model the location of phospholipids in this complex. To resolve this, we performed molecular dynamics (MD) simulations of Ndh–Ncp in complex with PE and PG and associated with a PE/PG lipid bilayer. Due to the size and complexity of the system, we performed extensive optimization simulations to determine optimal lipid numbers and starting positions (Supplementary Note 2, Extended Data Fig. 5a and Supplementary Table 3). The Ndh–Ncp chamber is considerably larger than the Huc or ForCE complexes^{19,20}, allowing it to accommodate 39 phospholipids (Supplementary Table 3). In this model, the charged phosphate-containing head groups of PE and PG occupy gaps in the complex between the Ndh and Ncp subunits, facing towards the external solvated environment (Fig. 4a,c,d). The phospholipid headgroup positioning and coordination correlate well with our cryo-EM data, indicating that the MD simulations provide a realistic model of the complex (Fig. 4b). The aliphatic lipid tails extend into and occupy the internal chamber of the complex (Fig. 4c,d). The lipid tails are dynamic throughout the simulations, which is consistent with the poor density in our cryo-EM maps and the role of the complex in long-range quinone transport, which requires lipid mobility within the chamber.

As indicated by our structural analysis, the Ncp tube interacts with the membrane through four Phe108 residues that stably embed in the bilayer and four Arg112 and Lys115 residues that coordinate the charged phospholipid headgroups (Fig. 4e,f). Over the course of the simulations, the Ndh–Ncp complex tilts so that one of the Ndh subunits contacts the membrane, with the opening of the Ncp tube penetrating the hydrophobic region of the lipid bilayer (Fig. 4a,g and Extended Data Fig. 6b). To accommodate the Ncp tube in the membrane, the phospholipid headgroups of the bilayer move away from the opening of the Ncp tube, and tails of bilayer phospholipids occupy the opening of the Ncp tube (Fig. 4g,h). This arrangement connects the hydrophobic lumen of the bilayer with the interior of the Ndh–Ncp

complex, allowing for diffusion of quinone between the two structures, a process critical for long-range quinone transport. This arrangement contrasts with the recently published ForCE structure, in which phospholipid headgroups are modelled within the hydrophobic ForE tube with tails directed outward, an assignment weakly supported by the density that would occlude the proposed quinone-access pathway¹⁹. Our MD model offers a more chemically realistic view of DUF1641–membrane interaction.

Quinone diffusion drives electron transfer from Ndh–Ncp to the membrane

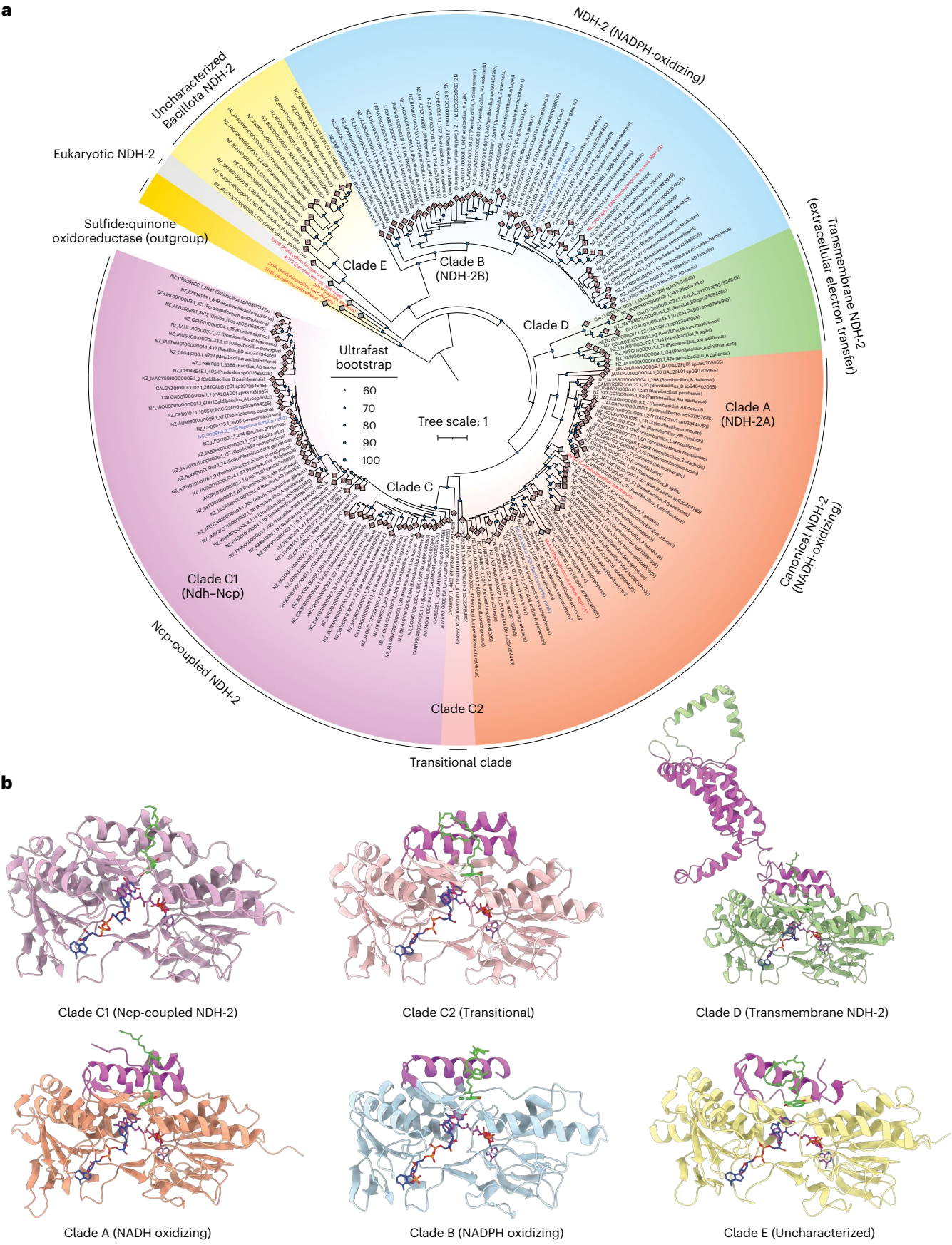
To directly assess the ability of the Ndh–Ncp complex to utilize quinone to transfer electrons from NADH oxidation to a lipid bilayer, we performed cyclic voltammetry on the complex immobilized on an electrode-bound, MK-7-supplemented phospholipid membrane, stabilized by a mixed self-assembled monolayer consisting of EO₃-cholesterol and 6-mercaptohexanol. In the absence of NADH, MK-7 oxidation and reduction peaks were observed in this system at +0.09 V and –0.28 V vs standard hydrogen electrode (SHE), respectively (Fig. 4i). The addition of NADH yielded a catalytic oxidative current with an onset potential of –0.075 V, close to the midpoint potential of MK-7 (ref. 39), indicating that MK-7 is responsible for mediating electron transfer between the electrode and the Ndh. The reductive MK-7 peak disappeared upon NADH addition, indicating that MK-7 is reduced at the Ndh active site rather than at the electrode. The electrochemical K_M for NADH, determined from the magnitude of the catalytic current at +0.3 V vs SHE at different NADH concentrations, was 91.6 μ M, in agreement with our previous kinetic assays (Extended Data Fig. 6a). Control experiments lacking the enzyme showed no NADH oxidation (Extended Data Fig. 6b). These observations are consistent with the behaviour of other NDH-2 enzymes, including homodimeric *C. thermarum* NDH-2 (ref. 40).

We next examined the electrochemical behaviour of the complex in the absence of MK-7 in the electrode-associated bilayer. Cyclic voltammetry revealed that under these conditions, Ndh–Ncp produced a catalytic oxidative current upon the addition of 400 μ M NADH, but at a reduced magnitude. We attribute this activity to the presence of MK-8 in Ndh–Ncp sequestered from *E. coli*, which decayed over repeated scans, suggesting that quinones present in the Ndh–Ncp complex are responsible for electron transfer from NADH to the electrode, and the magnitude of this transfer reduces over time as they diffuse into the lipid bilayer (Fig. 4j and Extended Data Fig. 6c). We calculated a quinone diffusion constant of $8.2 \times 10^{-4} \text{ s}^{-1}$ from the decay rate (Extended Data Fig. 6c), indicating the rate of diffusion between Ndh–Ncp and the membrane. Together with our lipidomics analysis showing quinone enrichment by Ndh–Ncp, these electrochemical data demonstrate that the complex not only sequesters quinones from the membrane but also retains them in quantities capable of sustaining Ndh catalysis away from the bilayer, providing a mechanism for extending respiratory activity beyond the membrane.

Cyclic voltammetry experiments of Ndh–Ncp immobilized on a bilayer containing UQ-8 demonstrated that Ndh is selective for MK-7, with a much greater onset potential (>0.5 V) observed for UQ-8 (Fig. 4j and Extended Data Fig. 6c,e). This is consistent with the kinetic

Fig. 5 | Ndh–Ncp is a recently evolved NDH-2 clade widespread among Bacillota. **a**, Maximum-likelihood phylogenetic tree of NDH-2 from Ndh–Ncp-encoding Bacillota genomes. Sequences are from a representative subset of 60 phylogenetically divergent Bacillota species (see Extended Data Fig. 7a and Supplementary Data 4 for all Ndh–Ncp-encoding species), highlighting the common presence of multiple divergent NDH-2 genes within the same organism, which probably mediate different physiological functions. The tree was constructed on the basis of 195 amino acid sequences using the LG + I + R10 model and rooted using sulfide:quinone oxidoreductases (Sqr) as an outgroup. Taxonomy of the genomes follows GTDB R09-RS220. Coloured tip labels show reference NDH-2/Sqr sequences (red) and the three *Bacillus subtilis* NDH-2 (blue).

Branch circles show ultrafast bootstrap support values with 1,000 replicates. Diamond labels at the tip denote identifiers of the 60 genomes. The scale bar corresponds to the expected number of substitutions per site. **b**, Chai1-predicted structural models for representatives of each NDH-2 clade, coloured to match the tree. Membrane interaction regions are coloured in purple. Clade C1: *B. subtilis* Ndh (NC_000964.3_1275) (experimental data), Clade C2: *MYW30-H2 sp022818495* (CP089291.1_2664), Clade D: *B. daliensis* (NZ_JAJSB010000001.1_475), Clade A: *B. subtilis* YumB (NC_000964.3_3321), Clade B: *B. subtilis* YutJ (NC_000964.3_3331) and Clade E: *B. daliensis* (NZ_JAJSB010000001.1_165). Model validation is provided in Supplementary Fig. 5.



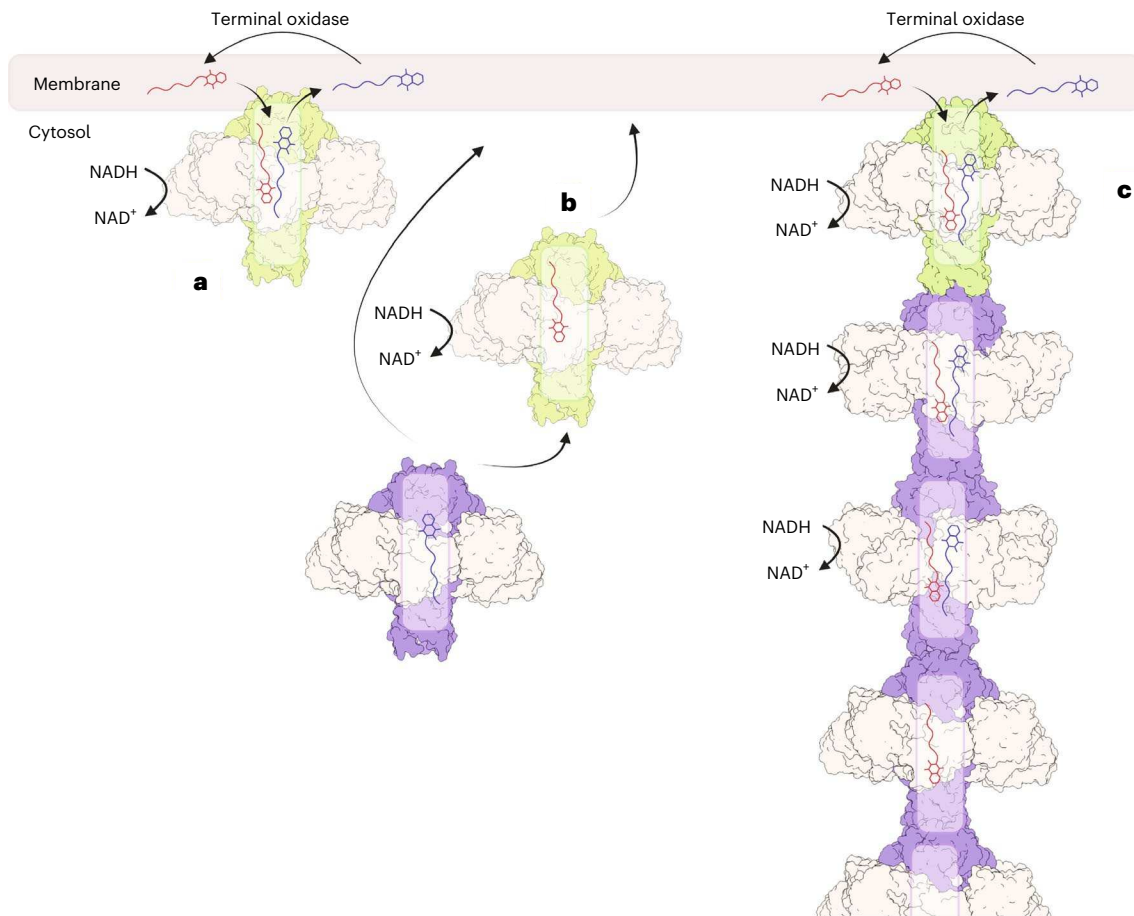


Fig. 6 | A model for quinone transfer and reduction by the Ndh–Ncp complex. **a**, Ndh can interact with the respiratory membrane through the C-terminal head region of the Ncp. The Ncp sequesters MK-7 (menaquinone in red) from the membrane for delivery to the electron acceptor sites in Ndh, where MK-7 is reduced (menaquinol in blue) by electrons from NADH oxidation. **b**, Ndh–Ncp can either remain at the membrane or dissociate using the MK-7 stored in the

tube. Ndh–Ncp associates with other Ndh–Ncp complexes or directly with the membrane using the same interface. **c**, Ndh–Ncp forms a filament allowing for MK-7 to diffuse from the membrane through several Ncp tubes to the associated Ndh–Ncp chambers for storage and reduction. Created in BioRender: Kropp, A. <https://biorender.com/I14erwr> (2026).

experiments and further indicates that the MK-8 associated with the Ndh–Ncp complex is responsible for facilitating electron transfer in the electrochemical experiments in the absence of quinone supplementation in the bilayer.

The Ndh–Ncp complex is widespread throughout Bacillota

To understand the distribution and evolution of Ndh–Ncp in Gram-positive bacteria, we performed a comprehensive survey of Ndh and Ncp homologues in all 113,104 representative archaeal and bacterial species genomes from the Genome Taxonomy Database (GTDB) (Supplementary Data 4)⁴¹. The Ndh–Ncp complex is exclusively found in the phylum Bacillota, where 8.5% of genes encoding Ndh homologues occur directly adjacent to an *ncp* gene. The 684 Ndh–Ncp-encoding species span 30 different families, indicating that this complex is widely encoded by taxonomically diverse members of this phylum (Extended Data Fig. 7a). Genome-wide metabolic analysis suggested that the Ndh–Ncp complex occurs in aerobic or facultatively anaerobic lineages but is absent in obligately anaerobic lineages such as Clostridia and Negativicutes (Supplementary Data 4). All Ndh–Ncp-encoding species co-encode at least one additional, non-Ncp-associated NDH-2 enzyme (Extended Data Fig. 7b), suggesting distinct physiological roles of these enzymes, as exemplified by *yumB* and *yutJ* encoded by *B. subtilis*²⁹.

Phylogenetic analysis of Ndh homologues across Bacillota revealed three major monophyletic NDH-2 clades (A, B, C) and three smaller

clades (D, E, F) (Fig. 5a,b, Extended Data Fig. 8a and Supplementary Data 4). All Ncp-associated NDH-2 enzymes are contained within clade C where Ncp-associated Ndh enzymes form a monophyletic group, which we refer to as Clade C1, defined by *ndh–ncp* synteny and the evolution of an Ndh C-terminal domain that mediates the stable interaction interface with Ncp (Fig. 5a, and Extended Data Figs. 8 and 9e). In addition, we identify a transitional lineage that we term clade C2. Enzymes in clade C2 retain a C terminus widely conserved in the NDH-2 family that mediates direct membrane interaction (Fig. 5a,b and Extended Data Fig. 8). Phylogenetic analysis resolves two C2 sub-branches distinguished by the presence or absence of a DUF1641-containing gene within their genome (Fig. 5a and Extended Data Fig. 8b). The DUF1641-encoding genes of this C2 branch lack the strict *ndh–ncp* synteny that defines clade C1, suggesting an intermediate stage in which the *ncp* progenitor was acquired by the ancestral bacterium, possibly forming a complex with a different enzyme, but the NDH-2 enzyme continued to interact with the membrane directly. From this transitional state, we hypothesize that the Ncp-associated Ndh enzymes in clade C1 emerged via spontaneous complex formation with the DUF1641-containing protein, followed by co-evolution to form the Ndh–Ncp. The short branch length of clade C1 relative to other clades suggests that the complex formation with Ncp is a relatively recent evolutionary innovation, but has become widely disseminated in Bacillota (Fig. 5a and Extended Data Fig. 8a).

For comparison, clades A and B represent widespread NDH-2 subtypes that frequently co-occur and genomically co-localize (separated

by 4–20 genes) across divergent Bacillota species (Fig. 5a,b, Extended Data Figs. 8a and 7b, and Supplementary Data 4). Clade A represents the canonical monotopic NADH-oxidizing NDH-2, containing *yumB* of *B. subtilis* and the structurally resolved NDH-2 enzymes from *S. aureus* and *C. thermarum* (Extended Data Fig. 9a–c). These structures feature C-terminal helices that anchor NDH-2 at the membrane surface, facilitating quinone access (Extended Data Fig. 9a–c)^{33,34,42}. Clade B is represented by the biochemically characterized monotopic NADPH-oxidizing NDH-2 from *S. aureus* (NDH-2B)⁴², in addition to *yutJ* of *B. subtilis* (Fig. 5a,b, and Extended Data Figs. 8a and 9d). Their genomic co-localization suggests ancestral relationships and complementary functions in NADH and NADPH oxidation. Interestingly, clade D represents NDH-2 enzymes that associate with the membrane through transmembrane helices, with previous work indicating that this clade forms a component of an extracellular electron-transport system (Extended Data Fig. 9f)⁴³.

Because genomes containing *ndh-ncp* also typically encode clade A and clade B NDH-2 enzymes, we next asked why multiple NDH-2 subtypes coexist. Transcriptomic data from *B. subtilis* show that *ndh* (Ndh–Ncp) is expressed primarily during exponential growth, when respiratory activity is highest (Extended Data Fig. 7c)⁴⁴. By contrast, *yumB* (clade A) is expressed during stationary phase and sporulation^{29,44}, when energy demand is reduced, and fewer respiratory complexes occupy the membrane. *yutJ* (clade B) shows lower expression throughout, consistent with a specialized role in NADPH oxidation (Extended Data Fig. 7c)^{29,44}. These complementary modes of expression and membrane interaction underscore the distinct physiological roles of Ndh–Ncp and clade A NDH-2, a division of labour that appears conserved across Bacillota.

Discussion

Our findings show that *B. subtilis* Ndh, the primary NADH dehydrogenase in this organism, forms a stable complex with the DUF1641 protein Ncp, which enables filament formation and quinone-mediated electron transfer to the respiratory chain (Fig. 6). A defining feature of this complex is its large internal chamber, which accommodates numerous phospholipids, creating a solvent-excluded hydrophobic lumen that mimics the membrane bilayer. We observe Ndh–Ncp filaments of up to 13 units, with chambers connected into a continuous hydrophobic conduit. This arrangement could allow 52 Ndh subunits to access quinones with a membrane footprint similar to a single NDH-2 dimer embedded in the bilayer. These pseudomembrane filaments would extend NADH-driven quinone reduction into the cytosol, expanding metabolic capacity by increasing the surface area available for respiration. This structure provides an elegant solution to the constraint of limited membrane surface area^{1,2}. In addition, the ability of Ndh–Ncp to sequester quinone may also enable these filaments to function as a ‘quinone battery’, concentrating quinone for reduction by Ndh, which can be exchanged during interaction with the membrane. These features distinguish Ndh–Ncp from other DUF1641-containing enzyme complexes such as Huc and ForCE, which incorporate few or no observable phospholipids and do not form filaments^{19,20}.

These properties place Ndh–Ncp alongside other strategies to expand bioenergetic capacity. Thylakoids, mitochondrial cristae and the intracytoplasmic membranes of nitrifying and anaerobic bacteria all expand membrane area to support electron transfer^{3–5,7–9}. Ndh–Ncp represents a conceptually distinct solution to this problem, a protein–lipid filament that substitutes for additional membrane surface area, by creating an internal hydrophobic tunnel for quinone transfer. This mechanism is structurally similar to electron-transferring redox enzyme filaments such as hydrogen-dependent CO₂ reductase (HDCR), yet functionally more analogous to classical membrane elaborations⁴⁵.

Consistent with a role in expanding respiratory capacity, Ndh–Ncp predominates during exponential growth, when respiratory demand is highest and the membrane most crowded, whereas the

membrane-anchored YumB is produced under the lower respiratory load of stationary phase and sporulation^{29,44}. The distribution of Ndh–Ncp across diverse Bacillota further supports the utility of this complex; although recent in evolutionary terms, it has been selected as an efficient strategy to enhance respiratory flexibility. Beyond its biological role, the Ndh–Ncp complex provides design principles for synthetic bioenergetic systems. The ability to sequester and transport hydrophobic electron carriers using soluble protein-based filaments expands possibilities for building synthetic respiratory systems with greater spatial control of electron transfer than classical bilayers.

In summary, the Ndh–Ncp complex expands the conceptual and mechanistic framework of respiratory biology. By demonstrating that respiratory enzymes can operate through protein-based pseudomembrane filaments, this work reveals a mechanism for spatially separating respiratory redox chemistry from the membrane. This strategy alleviates the challenge of limited membrane surface area while preserving efficient quinone reduction. Future work should determine the quinone capacity of these complexes, how their filaments behave in vivo and how they integrate with other respiratory systems. Together, our results establish Ndh–Ncp as a striking example of how evolution has reimagined membrane function, and provide a foundation for engineering synthetic energy-transducing systems.

Methods

Data analysis and visualization

In addition to programs described elsewhere in the manuscript, Microsoft Excel 2016, GraphPad Prism 10 and Origin were used for data analysis and visualization.

Cloning and mutant construction

The *Ndh* and *ncp* genes were amplified from *B. subtilis* 168 genomic DNA (gDNA) as one continuous fragment and subcloned into a pET22b plasmid, between BamHI and NheI sites, modified to encode an N-terminal twin strep II tag, yielding the expression vector p22b–Ndh–Ncp. To generate Ndh–Ncp mutants for recombinant purification, we used the Q5 mutagenesis kit (NEB) to add 4 mutations to generate Mutant 1 (Q14A, Q16A, Q17A, D21A) and Mutant 2 (D21A, Q22A, Q29A, E30A) in the p22b–Ndh–Ncp vector. Mutants were confirmed by whole plasmid sequencing (Plasmidosaurus).

To generate isogenic deletion mutants, gDNA was isolated from the *B. subtilis* kanamycin gene deletion library strains $\Delta yj1C::kan$ (now $\Delta ncp::kan$) and $\Delta ndh::kan$ as previously described^{46,47}. Subsequently, our *B. subtilis* 168ca (trp+) wild-type strain was transformed with the isolated gDNA and mutants were selected on kanamycin-containing nutrient agar plates⁴⁶. Colonies were clean streaked on fresh nutrient agar–kanamycin plates and stored at –80 °C. Deletions were confirmed by PCR and Sanger sequencing. All primers, strains and plasmids are listed in Supplementary Table 2.

B. subtilis growth assay

B. subtilis 168ca (trp+) wild-type⁴⁸ and isogenic mutants $\Delta ncp::kan$ and $\Delta ndh::kan$ were routinely grown overnight in lysogeny broth (LB: 10 g l^{–1} tryptone, 5 g l^{–1} yeast extract, 10 g l^{–1} sodium chloride) at 37 °C with aeration, with the addition of kanamycin at a final concentration of 5 µg ml^{–1} for the deletion mutants. The following day, cultures were normalized to a starting optical density at 600 nm (OD₆₀₀) of 0.05 in fresh LB and transferred in 200 µl aliquots, in technical triplicate and biological duplicate, to a 96-well plate at either 37 °C or 30 °C. Growth was subsequently monitored at OD₆₀₀ every 15 min for 12 h in a BMG Spectrostar Nano plate reader. Growth rates were calculated using OD₆₀₀ values between 0.1 and 1.6, equating to bacterial growth during exponential phase. The rate of growth was calculated for each replicate and plotted as three individual data points. Growth yield experiments were carried out in LB + 0.5% glucose to reduce sporulation and yield calculated as OD₆₀₀ reading at 24 h after inoculation.

Expression of Ndh–Ncp

p22b–Ndh–Ncp and mutants were transformed into *E. coli* C41 cells and grown as a starter culture in LB media until the culture reached the stationary phase, ~12–16 h. Large cultures of 500 ml terrific broth media (TB: 12 g l⁻¹ tryptone, 24 g l⁻¹ yeast extract, 12.26 g l⁻¹ K₂HPO₄, 2.31 g l⁻¹ KH₂PO₄, 4 g l⁻¹ glycerol) in a 2.5 l flask were inoculated with 5 ml of the starter culture and grown with shaking at 180 r.p.m. and 37 °C until they reached ~1.0 OD₆₀₀. The cultures were then cooled at 4 °C for 30 min before being returned to an incubator at 20 °C and induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were incubated for a further 16–18 h before collection. Cells were collected by centrifugation at 3,500 × g for 20 min at 4 °C using a Sorvall high-speed centrifuge. Once pelleted, cells were frozen and stored at –80 °C.

Recombinant purification of Ndh–Ncp

Purification occurred in a two-step process, beginning with affinity purification and followed by size exclusion chromatography. Initially, cells were thawed and resuspended with lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0, 1× cOmplete EDTA-free protease inhibitor (Roche, 11836145001), 1 mg ml⁻¹ DNase and lysozyme) at room temperature. Cells were then lysed by two passages through a cell disruptor (Emulsiflex C-5), and the lysate was clarified by centrifugation at 30,000 × g for 20 min at 4 °C. Lysate was then incubated for 5 min with BioLock biotin blocking buffer (IBA Lifesciences IBA, 2-0205-050) and loaded onto 1 ml StrepTrap XT (Cytiva). Once loaded, the column was washed with lysis buffer for ~30 column volumes (CV). The protein was eluted with elution buffer (50 mM Tris, 150 mM NaCl and 50 mM biotin, pH 8.0) in ten 1-ml fractions. Resulting fractions were analysed using SDS–PAGE, then pooled and concentrated to 500 µl for SEC using a 4-ml centrifugal concentrator with a 100-kDa molecular weight cut-off (MWCO) (Amicon, UFC803008). Concentrated protein was then loaded onto a Superose 6 Increase 10/300 GL column (Cytiva, 17517201) in SEC buffer (50 mM Tris, 150 mM NaCl, pH 8.0) and eluted in 0.5-ml fractions. Fractions were analysed by SDS–PAGE for peak identification and purity. Peaks corresponding to the correct complex were either pooled and concentrated for biochemical assays, or individual fractions were concentrated to separate each region of the peak for cryo-EM.

Native PAGE and coupled activity stain

Native PAGE was run following manufacturer instructions (ThermoFisher Native PAGE Bis-tris). Briefly, either protein or lysate was prepared and the concentration determined, then samples were mixed with 4× loading dye to a final volume of 20 µl. Samples were loaded onto 3–12% 10-well gels, along with Native Mark molecular weight marker (ThermoFisher), and run in native running buffer (ThermoFisher) at 150 V for 90 min. Following this, gels were either stained with Coomassie (Bulldog Acqua Stain) or incubated in buffer (50 mM Tris, 150 mM NaCl, pH 8.0) containing 500 µM nitroterazolum blue (NBT) and 500 µM NADH. The activity-stained gels were imaged as the colour developed upon NBT reduction (to purple) indicating NDH-2 activity.

Fractionation of Ndh–Ncp from *B. subtilis*

B. subtilis cells were grown overnight at 37 °C in LB media, then the following day, 1 l of LB media in 5 l flasks was inoculated to 0.01 OD₆₀₀. Cells were grown for 6 h post-inoculation and then collected by centrifugation and frozen. Cell pellet (3 l) was thawed and resuspended in 50 mM Tris pH 8.0 until homogeneous, and 2 mg ml⁻¹ of lysozyme was added to the resuspended cell solution. The cells were incubated with gentle stirring for 30 min at 37 °C. Cells were then further lysed by two passages through a cell disruptor (Emulsiflex C-5), and the lysate was clarified by centrifugation at 30,000 × g for 20 min at 4 °C. Lysate was applied to a QFF HP anion exchange column (Cytiva) and eluted over 10 CV with a linearly increasing gradient of 1 M NaCl. Eluted fractions were tested for activity with a plate-based activity assay (200 µl 500 µM

NADH and 500 µM NBT in the plate and 2–30 µl protein added to the cell, NDH-2 activity confirmed with a purple colour change). Active fractions were pooled and subjected to SEC on a Superose 6 column. Fractions that were active were subjected to SDS–PAGE, native PAGE and negative-stain EM to visualize Ndh–Ncp.

Mass photometry

These measurements were carried out using a Refeyn 2MP mass photometer. Protein samples and standards were diluted in phosphate buffered saline (PBS) for measurements. Initially, standards of apoferitin and BSA were measured to generate a standard curve for molecular weight calculations. Then each protein sample was measured at different concentrations (10–100 nM) to determine the optimal concentration for accurate reads. At the sample-dependent optimal concentration, each sample was measured three times and one representative from each sample was used in the figure. Data were analysed using Refeyn DiscoverMP software.

Visible-wavelength spectroscopic measurements

These measurements used a SpectraMax ABS Plus (Molecular Devices) 96-well plate reader fully housed in an anaerobic glovebox (O₂ < 3 ppm; Belle Technology). A concentration of 85 µM of enzyme was carefully deoxygenated, diluted with anaerobic buffer (50 mM Tris–Cl pH 8, 150 mM NaCl), and spectra were recorded in a quartz cuvette following the addition of NADH (0–100 µM). Data were plotted using GraphPad Prism 10.6.

Steady-state oxidation of NADH measurements

The oxidation of NADH was measured at 340–380 nm ($\epsilon = 4.81 \text{ mM}^{-1} \text{ cm}^{-1}$) in a SpectraMax ABS Plus (Molecular Devices) 96-well plate reader. The buffer (50 mM Tris–Cl pH 8, 150 mM NaCl) contained 0.15% asolectin–CHAPS to provide a hydrophobic phase for the short-chain quinones. Menadione reaction kinetics were measured with the addition 4 nM Ndh–Ncp at a concentration range of 0–120 µM menadione or 0–500 µM NADH. Decylubiquinone was measured with the addition of 8 nM Ndh–Ncp over a concentration range of 0–120 µM decylubiquinone or 0–500 µM NADH. The data were fit to the Michaelis–Menten equation ($v = V_{\text{max}} \times [S]/(K_M + [S])$), with $[S]$ corresponding to substrate concentration, using Solver in Microsoft Excel. Data were plotted in GraphPad Prism 10.6 for figures.

Negative-stain and cryo-EM imaging and data collection of Ndh–Ncp

Negative-stain grids (carbon film 300 mesh, EMS) were prepared using first glow discharge for 30 s at 15 mA, and then the protein was applied (0.01 mg ml⁻¹) for 1 min, followed by blotting of the excess protein. Three rounds of uranyl acetate (1–2%) application followed (two at 30 s, the final for 1 min, each followed by blotting). Grids were then imaged in a Talos L120C transmission electron microscope (ThermoFisher) at ×72,000 magnification.

Cryo-EM grids were prepared using a Vitrobot Mark IV vitrification device (ThermoFisher) pre-cooled to 4 °C and equilibrated to 100% humidity. UltrAuFoil 1.2/1.3 300 mesh grids (Quantifoil) were glow discharged for 180 s at 15 mA. Protein (4 µl) was applied at 6 mg ml⁻¹ concentration to the grid after glow discharge. For the NADH-bound structure, Ndh–Ncp (at 6 mg ml⁻¹) was incubated with 5× molar concentration of NADH (~130 µM) 10 min before freezing. The grid was then plunge frozen into liquid ethane using the Vitrobot. Grids were immediately clipped for the autoloader and stored long term in liquid nitrogen.

Samples were initially screened on a Talos Arctica 200 kV transmission electron microscope, collecting a small dataset (200–300 movies) to generate two-dimensional (2D) class averages with cryoSPARC live⁴⁹. Large datasets were collected on a Titan Krios G4 transmission electron microscope fitted with a Gatan K3 direct electron detector with energy filter (Ndh–Ncp) or a Falcon4i direct electron detector (Ndh–Ncp

NADH/quinone bound). Data were collected at $\times 130,000$ magnification ($0.655 \text{ \AA pixel}^{-1}$) with a total dose of $39 \text{ e}^- \text{ \AA}^{-2}$, fractionated into 40 frames for Ndh–Ncp, and $\times 96,000$ magnification ($0.808 \text{ \AA pixel}^{-1}$) with an exposure time of 3 s and a total dose of $50 \text{ e}^- \text{ \AA}^{-2}$, fractionated into 50 frames for Ndh–Ncp NADH/quinone bound. A C2 aperture of $42 \text{ \mu m}$ was used with the objective aperture inserted and a defocus range between $-1.2 \text{ \mu m}$ and $-0.6 \text{ \mu m}$. Data collections were automated using the EPU v.3.6 software (ThermoFisher).

Cryo-EM data processing and analysis

Ndh–Ncp. Micrographs (7,876) were grouped into their respective optics groups using the Python script AFIS.py, and the grouped micrographs were then motion corrected using UCSF MotionCor3 and contrast transfer function (CTF) estimation (CTFFIND-4.1) in Relion (5.0)⁵⁰. The particle coordinates were then determined with crYOLO (1.7.6) using a general model and extracted in Relion with a box size of 512 px (refs. 50,51). Initial 2D classification was carried out in Relion 5.0, then extracted and imported into cryoSPARC v.4.6.0 for ab initio reconstruction, with one class with C4 symmetry⁴⁹. Homogeneous refinement of 483,556 particles was followed by non-uniform refinement with C4 symmetry. 3D classification followed with 6 classes. Four classes were selected containing 389,955 particles and subjected to another round of non-uniform refinement with C4 symmetry. This generated a map of $2.14 \text{ \AA}$ global resolution which was improved to $2.12 \text{ \AA}$ resolution with local refinement, masking only one unit of the filament. From this, helical refinement was carried out with a helical rise of $99.22 \text{ \AA}$, twist of 23.95° and final resolution of $3.09 \text{ \AA}$. Alternatively, the particles from the local refinement were reclassified with heterogeneous refinement, masking one class with ‘bad particles’. The resulting ‘good particles’ were reclassified in 2D space, and 356,549 particles were selected for local refinement, resulting in maps reaching a global resolution of $2.11 \text{ \AA}$ (Supplementary Fig. 2).

Ndh–Ncp NADH/quinone bound. Micrographs (11,418) were grouped into their respective optics groups using the Python script AFIS.py, and the grouped micrographs were then motion corrected using UCSF MotionCor3 in Relion 5.0. The motion-corrected particles.star files were transferred to cryoSPARC v.4.6.0 for CTF estimation^{49,50}. Particle coordinates were determined initially using Blob Picker to generate 2D averages to train Topaz Picking⁵². Topaz Picking was trained only on the 2D class of the top view, in an effort to find more of this underrepresented view. Topaz Picking resulted in 5,095,952 particles, which were extracted at a 420-pixel box size, binned to 120 px , and 2D classification resulted in 2,590,595 particles selected. Ab initio reconstruction was carried out with two classes and C4 symmetry, with the largest class containing 1,301,383 particles used for homogeneous refinement (C4). Homogeneous refinement was followed by heterogeneous refinement with two classes, one masked with the ‘junk’ ab initio class, the other masked from homogeneous refinement. A total of 797,409 particles were used for non-uniform refinement to generate a new model for Topaz Picking. A total of 1,894,345 particles resulted and were extracted at a 420-pixel box size, binned to 120 px . A total of 1,267,220 particles were selected from 2D classification and re-extracted without binning. 2D classification further resulted in 1,217,300 particles selected and ab initio reconstruction was carried out with two classes and C4 symmetry. Homogeneous refinement (C4) with 632,082 particles resulted in maps to a global resolution of $2.58 \text{ \AA}$. Further non-uniform refinement and 3D classification using heterogeneous refinement (two rounds) resulted in a non-uniform refinement with 341,297 particles to a global resolution of $2.52 \text{ \AA}$ (Supplementary Fig. 3).

Of note, processing of both datasets was also carried out using C1 symmetry for 3D refinement; however, maps from C1 processing were of lower resolution, displayed anisotropy due to orientation bias, and did not reveal additional structural features. As such, subsequent processing focused on C4 symmetry.

Model building and visualization

An initial model of the Ndh–Ncp complex was generated with AlphaFold3 (ref. 53), and Ncp and Ndh were then separated into individual molecules using Pymol (3.1.8)⁵⁴. The Ncp tetramer and Ndh enzymes were docked separately into the maps generated, using ChimeraX 1.8 fit map⁵⁵. The models were built and refined using Coot 0.9.8.1 and Phenix (1.2)^{56,57}. FAD, MK-8 and NADH were modelled into the complexes using Coot⁵⁷. Model quality was validated using MolProbity⁵⁸. All figures were made using ChimeraX (1.8)⁵⁵.

Mass spectrometric lipid detection and analysis

Ndh–Ncp purified in *E. coli* was incubated with *B. subtilis* 168 membranes extracted from 4 l of *B. subtilis* cultures or left untreated. To prepare the membranes, *B. subtilis* was grown in LB at 30°C for 24 h, then collected by centrifugation at $3,500 \times g$ and frozen at -20°C . Cells were lysed in buffer (50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, pH 7.5) and incubated with 1 mg ml^{-1} lysozyme at 37°C for 30 min. Cells were then incubated with $1 \times$ cComplete EDTA-free protease inhibitor (Roche, 11836145001) and 1 mg ml^{-1} DNase for 10 min at room temperature, followed by lysis with two passages through a cell disrupter (Emulsiflex C-5). The lysate was clarified by centrifugation at $27,000 \times g$ for 20 min at 4°C , and the membranes were then collected by ultracentrifugation at $100,000 \times g$ for 1 h at 4°C . Membranes were resuspended in 50 mM Tris, 150 mM NaCl, pH 7.5 and incubated for 6 h at 4°C with 5 mg of Ndh–Ncp, rotating slowly. The membranes were then pelleted by ultracentrifugation, and the resulting protein was purified initially by affinity chromatography with a StrepXT column (Cytiva), then the flow through of unbound samples was purified by SEC on a Superose 6 Increase 10/300 GL column (Cytiva, 17517201) in SEC buffer (50 mM Tris, 150 mM NaCl, pH 8.0). Resulting eluted fractions were pooled, concentrated and frozen for mass spectrometry. Samples from each fraction were analysed by negative-stain TEM before concentration.

Samples corresponding to $48.6 \text{ \mu g}$ of purified protein (determined using absorbance at 280 nM) were prepared for liquid chromatography–mass spectrometry (LC–MS) analysis using a modified Folch extraction. Briefly, a $100 \text{ \mu l}$ solution of purified protein ($\sim 48.6 \text{ \mu g}$) or $1 \text{ \mu l}$ of isolated membrane diluted to $100 \text{ \mu l}$ with LC–MS-grade water was treated with $2,000 \text{ \mu l}$ of 2:1 chloroform:methanol v/v containing 50 ng ml^{-1} of menaquinone-7 (d7) (90%, LGC Standards, TRC-KIT6138) and EquiSPLASH (Avanti, 330731-1EA) lipid mixture with each standard at 50 ng ml^{-1} , after which the mixture was shaken for 10 min and allowed to stand for a further 50 min. Water ($400 \text{ \mu l}$) was added, and the mixture was shaken for 10 min, after which the sample was allowed to stand until the two phases had completely separated. The lower chloroform-rich phase was then transferred to a 1.4 ml total recovery glass microvial (ThermoFisher, 6PSV9-CHV1) and the solvent was removed under a stream of nitrogen gas. Samples were then reconstituted in $60 \text{ \mu l}$ of 7:3 LC Solvent A:LC Solvent B (described below). Samples were analysed using a Vanquish Horizon ultra-high-performance liquid chromatography platform (ThermoFisher) coupled to an Orbitrap Exploris 480 MS (ThermoFisher) using a C18 column (Zorbax Eclipse Plus C18 Rapid Resolution HD, $2.1 \times 100 \text{ mm}$, $1.8 \text{ \mu m}$, Agilent, 959758-902) with a binary solvent system: solvent A = 40% isopropanol/60% water and solvent B = 98% isopropanol/2% water, both containing 2 mM formic acid and 8 mM ammonium formate. Linear gradient time–%B were as follows: 0 min–0%, 8 min–35%, 16 min–50%, 19 min–80%, 23 min–100%, 28 min–100%, 30 min–0%, 32 min–0%. The flow rate was $250 \text{ \mu l min}^{-1}$, the column temperature 50°C , and the sample injection volume was $10 \text{ \mu l}$. Each sample was analysed three times, first at an MS1 level using fast polarity switching, then by data-dependent acquisition (DDA) in a single polarity, that is, one analysis in positive mode and one analysis in negative mode. For the polarity switching analysis, the mass spectrometer operated at a resolution of 120,000 from $m/z = 140$ –2,000 and DDA was performed with an MS1 survey scan $m/z = 450$ –2,000 at a resolution of 60,000. The resulting data-dependent MS/MS was conducted at a

resolution of 15,000 using an isolation window of 1.5 m/z and a stepped normalized collision energy of 20, 40 and 60. The method cycled with a loop count of 7 and dynamic exclusion of 8.0 s. The following source conditions were used in all experiments: spray voltage 3.5 kV (positive), 2.5 kV (negative); capillary temperature 325 °C; sheath gas 60; aux gas 15; sweep gas 2 and probe temp 320 °C.

Lipidomic data analysis was performed using untargeted feature extraction on the MS1 polarity switching data in mzMine (4.3)⁵⁹. Briefly, this involved untargeted feature extraction from each sample, alignment of features, gap filling and identification against those features previously identified. Features that did not exceed the signal found in the extraction blanks by at least 5-fold were excluded. Lipids were then putatively identified by comparison against the lipid identification, which was achieved by comparing the MS/MS spectra against the Lipid-Blast database⁶⁰ and/or the MSIAL-TandemMassSpectralAtlas-VS69 (ref. 61), followed by manual review of the putative annotations. Unassigned features were then annotated on the basis of their accurate mass against the Lipid Maps structural database^{62,63} (MSIID) and/or the MycoMass database⁶⁴. Raw and processed data can be found at <https://zenodo.org/records/21271664>. In addition, the molar amount of PE, PG, MK, DMK and CoQ was determined by comparison of each lipids' peak area against that of the isotopically labelled standard of the same class for PE and PG, or against the menaquinone-7 (d7) standard for MK, DMK and CoQ lipids. Peak integration was performed using TraceFinder 5.2 SP3 (ThermoFisher).

To assess enrichment of MK-7 and related quinone species in the Ndh–Ncp complex following incubation with *B. subtilis* membranes, we performed a semi-quantitative analysis based on within-sample lipid intensity ratios. This analysis was designed to provide a qualitative indication of quinone enrichment across compositionally distinct samples rather than absolute quantification. Lipidomic features ($n = 10,387$; unprocessed output) were filtered to retain a high-abundance, reproducibly detected subset suitable for ratio-based comparisons. Features lacking a putative annotation and internal standards were removed. Remaining features were ranked by summed intensity across all samples, and features with a summed intensity below 1×10^8 across all conditions were excluded, retaining approximately the top 6% of detected features for downstream analysis. To ensure reliable detection, features were required to exceed 3-fold the maximum blank signal in at least two replicates within at least one condition. Features were retained if they met a coefficient of variation threshold of $\leq 30\%$ in at least one sample group, reflecting the substantial compositional differences between sample types (*E. coli* membranes, *B. subtilis* membranes and purified complexes). Filtered features were consolidated at the lipid species level by summing intensities across adducts, yielding 278 unique lipid species. To define a *B. subtilis*-specific reference lipid set, species were selected that were present in *B. subtilis* membranes but absent or present at $<10\%$ relative intensity in the Ndh–Ncp complex before membrane incubation, yielding 113 species (excluding MK-7 itself, which served as the ratio numerator).

Because class-matched internal standards were not available for all lipid classes in the reference pool, intensity ratios were calculated from unnormalized peak intensities to avoid asymmetric normalization between numerator and denominator. The deuterated MK-7 internal standard was used to monitor quinone signal stability across samples; however, as no equivalent deuterated standard was available for the denominator lipid classes, it was not applied to ratio calculations. Quinone enrichment was quantified as the ratio of endogenous MK-7 intensity, or the summed intensity of all detected quinone species, to the summed intensity of the *B. subtilis*-specific reference lipid set within each sample. To assess robustness to denominator selection and mitigate potential biases arising from class-specific ionization or chromatographic effects, analogous ratios were calculated using independent reference sets, including a subset putative of dihexosyl-diacylglycerol (Hex₂DG) species ($n = 5$) and a single abundant putative

ether-linked diacylglycerol species (DG O-32:2; DG P-14:0/18:1). Consistent enrichment trends across these independent analyses indicate that the observed quinone enrichment is not driven by the behaviour of a specific lipid class or feature subset^{65–67}.

Molecular dynamics simulations

We performed all-atom classical molecular dynamics (MD) simulations on the high-resolution cryo-EM structures of *B. subtilis* Ndh–Ncp complex with and without MK-7/NADH. Simulations were performed with GROMACS (2022.3/4)⁶⁸ using the CHARMM36m force field for proteins and CHARMM36 for lipids, ions, water and redox-active components (FAD, MK-7, NADH)^{69–72}. The membrane bilayer was constructed with the CHARMM-GUI⁷³ tool, and comprised 950 POPE (~79%) and 259 POPG (~21%) lipids. The total system size was ~664,000–666,000 atoms depending on lipid and substrate arrangements (see more below). In total, six simulation setups were constructed (Supplementary Table 3).

Two strategies were applied for lipid placement in the Ndh–Ncp complex. In the first (setup 1), lipids were manually embedded into Ndh–Ncp interface cavities with VMD's graphical interface⁷⁴. The lipid head groups were oriented towards the solvent and tails directed inward, resulting in 33 POPE and 7 POPG molecules in a tightly packed chamber. In the second approach (setup 2), a pre-equilibrated bilayer patch was aligned using the 'psfgen' plugin⁷⁴ with Ncp, and only lipids inside the chamber were retained, resulting in a total of 20 POPE and 7 POPG molecules (setups 2, 3, 4, 6, Supplementary Table 3). Lipids near the N-terminal region were added similarly.

All membrane-protein model systems were solvated with TIP3P water and 150 mM Na⁺/Cl[−], using VMD's 'autoionize' and 'solvate' plugins^{74–76}. Protonation states were predicted using PROPKA at pH 7.0 (ref. 77). In all Ndh monomers, His383 was modelled protonated in non-substrate-bound setups, whereas Glu168 was protonated only in the MK-7/NADH-bound setups. Simulations were performed in the isothermal-isobaric (NPT) ensemble (310 K, 1 atm) with semi-isotropic pressure coupling. MD equilibration used the V-rescale thermostat and C-rescale barostat, followed by production simulations with Nosé–Hoover thermostat and Parrinello–Rahman barostat^{78–82}. A 2-fs timestep was used with the leapfrog integrator, and all covalent bonds involving hydrogen atoms were constrained using the LINCS algorithm^{83,84}. Non-bonded interactions were applied with a 12 Å cut-off and particle-mesh Ewald (PME)^{85,86} for long-range electrostatics.

The membrane patch was pre-equilibrated for 10 ns by applying harmonic position restraints (20,000 kJ mol^{−1} nm^{−2}) to the phosphate groups of all lipids, followed by a 30-ns production run to generate a fully equilibrated lipid bilayer. Simulations of membrane-protein systems (Supplementary Table 3) began with a 1,000-step energy minimization using NAMD (2.14)^{87,88}, with harmonic position restraints (force constant of 41.4×10^3 kJ mol^{−1} nm^{−2}) applied to all heavy atoms of the protein and its substrates. This was followed by a steepest-descent minimization in GROMACS 2022.3 with an energy tolerance of 250 kJ mol^{−1} nm^{−1}, using restraints (20,000 kJ mol^{−1} nm^{−2}) on the same atoms. Subsequently, equilibrations were performed in the NPT ensemble with identical position restraints for 100 ns to relax the lipids within the Ncp tube, followed by a 100-ns equilibration by keeping the same harmonic restraints now only on the protein backbone and FAD atoms. Three 1-μs production simulation replicates were run per setup, where replicates 2 and 3 were generated by extending the last equilibration step by 1 and 2 ns, respectively. In addition, in substrate-bound systems, weaker restraints (2,000 kJ mol^{−1} nm^{−2}) were applied to NADH and MK-7 headgroups during equilibration setups. It is noteworthy that MK-7 molecules (setup 5), after binding to FAD for ~300 ns, diffused from their structural locations. Therefore, in additional simulations (setup 6, Supplementary Table 3), harmonic distance restraints were applied between FAD (N5) and NADH (C5N), and between FAD (N3) and the MK-7 (OR8) atoms, using the GROMACS pull code with a force constant of 5,000 kJ mol^{−1} nm^{−2} (ref. 68). Volume calculations of solvent accessible

surface (SAS) were performed with the MSMS program⁸⁹ combined with the Openstructure software package⁹⁰.

Synthesis and characterisation of S-cholesterol (for electrochemistry membrane bilayer)

Synthesis of ethyl-trioxyethanol-cholesteryl 2. 2-[2-(2-Aminoethoxy)ethoxy]ethanol **1** (1.08 g, 1.060 mmol, 7.22 mmol) was dissolved in anhydrous dichloromethane (DCM) (25 ml) and triethylamine (0.82 g, 1.12 ml, 8.04 mmol) was then added. Separately, cholesteryl chloroformate (3.62 g, 8.04 mmol) was dissolved in anhydrous DCM (50 ml). The solution of cholesteryl chloroformate was then added dropwise to the solution of 2-[2-(2-aminoethoxy)ethoxy]ethanol, and the resultant solution was stirred for 48 h at room temperature under N₂. After this time, the reaction mixture was diluted with additional DCM (100 ml) and washed with 1 M HCl (2 × 40 ml), then brine (40 ml). The organic layer was dried over MgSO₄ and concentrated in vacuo to yield a crude residue, which was purified via silica column chromatography (hexane → ethyl acetate) to yield **2** as a colourless oily foam (3.95 g, 97%). Note, when performing TLC, that the location of **2** can be visualized with permanganate stain (Supplementary Fig. 6a).

Preparation of ethyl-trioxythioacetate-cholesteryl 3. **2** (1.95 g, 3.47 mmol) was dissolved in anhydrous DCM (50 ml). Triethylamine (630 µl, 4.51 mmol) was then added, followed by *p*-toluenesulfonyl chloride (0.860 g, 4.51 mmol). The reaction solution was then stirred at room temperature under N₂ overnight. After this time, the reaction solution was diluted with additional DCM (50 ml) and washed with 1 M HCl (2 × 20 ml), saturated NaHCO₃ (20 ml) and then brine (20 ml). The organic layer was dried over MgSO₄ and concentrated in vacuo to yield the crude tosylate intermediate. The resulting residue was then redissolved in anhydrous dimethylformamide (DMF) (20 ml) and potassium thioacetate (1.19 g, 10.4 mmol) was then added. The resultant solution was then placed under a nitrogen atmosphere and stirred at 90 °C for 3 h, during which time a strong brown colour developed. The reaction solution was then cooled to room temperature and the majority of the DMF was removed in vacuo. DCM was then added (100 ml) and the resultant solution was transferred to a separating funnel. The solution was washed with water (2 × 50 ml) and then brine (20 ml), and subsequently dried over MgSO₄. The product, **3**, was then isolated via silica column chromatography (10% ethyl acetate (EtOAc) in petroleum ether), yielding a deep-brown oil (0.910 g, 42%) (Supplementary Fig. 6b).

Synthesis of ethyl-trioxythiol-cholesteryl 4. **3** (0.882 g, 1.42 mmol) was dissolved in ethanol (10 ml). Separately, potassium hydroxide (169 mg, 3.01 mmol) was dissolved in ethanol (10 ml) and delivered to the solution of **3**. The reaction mixture was placed under a nitrogen atmosphere and stirred at room temperature for 3 h before being diluted in DCM (50 ml). The reaction mixture was then transferred to a separating funnel. The solution was washed with 1 M HCl (10 ml), followed by brine (20 ml), and then dried over MgSO₄. Concentration in vacuo yielded a crude brown residue, which was further purified via silica column chromatography (hexane → ethyl acetate). **4** was isolated in resolved fractions as both its free-thiol monomeric form (0.419 g, 51%) and its corresponding disulfide dimer (0.209 g, 26%) in a combined yield of 77% (both the free-thiol and disulfide forms of the product can be used identically for self-assembled monolayer (SAM) formation). Both forms of **4** were brown in colour (Supplementary Fig. 6c).

Electrode modification

Liposomes were formed from a solution of 5 mg ml⁻¹ soy phospholipids (Sigma Aldrich) in aqueous buffer (20 mM MOPS, 30 mM Na₂SO₄, pH 7.40). This suspension was extruded through an Avanti mini-extruder containing a Cytiva 0.1 µm membrane filter 31× to generate liposomes. When quinone supplementation was required,

0.44 mg ml⁻¹ menaquinone-7 ('MK-7'; Cambridge Bioscience) or 0.44 mg ml⁻¹ ubiquinone-8 ('UQ-8'; Merck) was added to the lipid suspension before extrusion.

BASi gold disc electrodes (3 mm diameter) were first mechanically polished in air using alumina slurries of decreasing particle size (1, 0.3, then 0.05 µm, Metprep) applied to polishing pads (Beuhler), with 2-min polishes used per alumina size followed by sonication for 3 min in water, then ethanol. The electrodes were then taken into a glovebox (in-house construction, Belle Technology purification columns, [O₂] < 30 ppm) and an electrochemical polish of 50 cyclic voltammograms from -1.3 to 0.7 V vs Ag/AgCl at 100 mV s⁻¹ in 0.5 M NaOH were followed by 50 cyclic voltammograms from -0.25 to 1.55 V vs Ag/AgCl at 100 mV s⁻¹ in 0.5 M H₂SO₄. Remaining in the glovebox, the cleaned electrodes were left to dry and then immersed in 1 ml of 2-propanol solution containing 8.9 mM 6-mercaptohexanol and 1.1 mM EO₃-cholesterol (S-cholesterol) for 16 h to form a mixed SAM, following previously published procedure⁴⁰. Following this SAM formation, the electrodes were removed from the glovebox and rinsed with ethanol and dichloromethane in the laboratory atmosphere and dried under N₂. The electrodes were then returned to the glovebox, and to form the liposome functionalization, 5 µl liposome was pipetted onto the tip of the electrode, and 1 µl 120 mM CaCl₂ in the same buffer solution was added with gentle mixing using the pipette tip. This was left to dry (~30 min), then the same liposome and CaCl₂ dropcast plus drying process was repeated. Enzyme modification of the electrode was then achieved by applying 5 µl 7 mg ml⁻¹ *Bacillus subtilis* Ndh-Ncp in 50 mM Tris, 150 mM NaCl, pH 7.4 to the electrode, and leaving this to dry for ~30 min.

Electrochemical set up

All experiments were carried out in a 3-electrode cell containing 10 ml buffer solution (20 mM MOPS, 30 mM Na₂SO₄, pH 7.40), which was stirred continuously throughout. The cell used a modified gold disc working electrode (see above), a platinum wire counter electrode and an Ag/AgCl reference electrode, which was determined to be offset from SHE by +0.225 V based on calibration experiments using ferricyanide (Extended Data Fig. 6f)⁹¹. All experiments were carried out in a glovebox under a nitrogen atmosphere ([O₂] < 30 ppm) at 19 °C. During a NADH titration experiment, the potential was continuously cycled at 10 mV s⁻¹, with aliquots of a concentrated stock solution of 100 mM NADH added in increments at approximately -0.4 V vs SHE during every even scan. Every odd scan is displayed.

Genomic survey, synteny characterization and phylogenetic analysis

To profile the distribution and diversity of the Ndh-Ncp complex across the prokaryotic tree of life, we retrieved all 113,104 representative archaeal and bacterial species genomes from the Genome Taxonomy Database (GTDB) release 09-RS220 (ref. 41). Genes were predicted using Prodigal (v.2.6.3)⁹² and functionally annotated against KEGG⁹³ (accessed 22 November 2021) and Pfam (v.34.0)⁹⁴ protein databases through DRAM (v.1.2.4)⁹⁵. DUF1641-domain family proteins (including Ncp) were identified by searching against the Conserved Domain Database (CDD)⁹⁶ profiles pfam07849 (DUF1641-domain) and COG2427 (YjgD/ForE, DUF1641-domain protein) using rpsblast (-evalue 1 × 10⁻⁵ -max_hsps 1 -max_target_seqs 5) in BLAST+ (v.2.16.0)⁹⁷, as well as against all entries of DUF1641-domain proteins from UniProt⁹⁸ using DIAMOND (v.2.1.10)⁹⁹. Hits were cross-compared and manually inspected to filter false positives. For further curation and the analysis of genetic context, five genes upstream and downstream of DUF1641-domain hits and their annotations by DRAM were retrieved. Type II NADH dehydrogenases (NDH-2) in the neighbourhood and across genomes were identified by matches to KEGG Orthology KO K03885. In total, 43,976 and 5,479 genomes were found to encode NDH-2 and DUF1641-domain proteins, respectively; 684 genomes from exclusively the phylum Bacillota

encode the Ndh–Ncp complex wherein *ndh* and *ncp* are always genomically co-located next to each other (Supplementary Data 4).

Genomes and NDH-2 sequences of Bacillota were further analysed to investigate the potential physiological relevance and evolution of the Ndh–Ncp complex. The presence of major aerobic and anaerobic respiratory enzymes (including complexes I–V and terminal oxidases) in Bacillota genomes was identified on the basis of DRAM and KEGG annotation. A phylogenomic tree of the Ndh–Ncp encoding Bacillota was constructed by aligning the concatenated 120 GTDB bacterial marker genes using GTDB-Tk (v.2.4.0)¹⁰⁰, followed by a maximum-likelihood phylogenetic reconstruction using IQ-TREE (v.2.4.0)¹⁰¹ (model: WAG + G20; 1,000 ultrafast bootstrap replicates; mid-point rooted). For phylogenetic analysis of Bacillota NDH-2, Ncp-associated and non-Ncp-associated sequences with lengths over 300 amino acids were respectively clustered at identity thresholds of 80% and 60% using MMseqs2 (ref. 102) (–c 0.7–cov-mode 1 –cluster-mode 2–s 7.5). A multiple sequence alignment of NDH-2 cluster representatives, structurally resolved NDH-2 (PDB 5NA4, 4NWZ, 4G73, 5JWB) and sulfide:quinone oxidoreductases (PDB 3H8L, 3HYV, 3KPK) was built using the align function of Muscle (v.5.3)¹⁰³. Using ModelFinder^{103,104} implemented in IQ-TREE v.2.4.0, the best-fit model according to Bayesian information criterion (BIC) was determined to be LG + I + R10. A maximum-likelihood phylogenetic tree was then constructed using IQ-TREE v.2.4.0 (–m LG + I + R10 –B 1000 –bnni) and rooted using sulfide:quinone oxidoreductases as outgroups (Extended Data Fig. 9). To visualize the common presence of multiple copies of NDH-2 of different clades within the same microorganism, a maximum-likelihood phylogenetic tree using all NDH-2 sequences from a subset of 60 representative and phylogenetically divergent Ndh–Ncp encoding Bacillota species was also constructed with the same method (Fig. 5 and Supplementary Data 4). All phylogenetic trees were visualized using iTOL (v.7)¹⁰⁵. Major clades of NDH-2 were defined in this study on the basis of phylogeny, structural features (for example, transmembrane domain, interaction with Ncp) and reported physiological roles (for example, NADPH/NADH preference, extracellular electron transfer). NDH-2 and Ncp sequences, synteny of Ndh–Ncp, NDH-2 classification, genome statistics and metabolic annotations of the Bacillota genomes are summarized in Supplementary Data 4.

Structural modelling

Modelling was performed on the Chai1 or AlphaFold3 online server^{53,106}. Entire sequences were provided along with NADH (or NADPH)/FAD/MK-7/MK-8/UQ-8 smiles string.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The cryo-EM structures are available in the PDB and EMDb under the codes PDB 9PXX, EMD-71972 (Ndh–Ncp with MK-8/NADH), PDB 9PXL, EMD-71973 (Ndh–Ncp) and PDB 9PXM, EMD-71974 (Ndh–Ncp filament). All phylogenetics sequences and accession numbers are found in Supplementary Data File 4. Raw and processed lipidomics data have been deposited in Zenodo at <https://doi.org/10.5281/zenodo.21271664> (ref. 107). Molecular dynamics simulation data have been deposited in Zenodo at <https://doi.org/10.5281/zenodo.21226551> (ref. 108). Source data containing raw and uncropped gels and numerical values for all graphs are provided with this paper. All other data are provided with the manuscript.

References

- Szenk, M., Dill, K. A. & de Graff, A. M. Why do fast-growing bacteria enter overflow metabolism? Testing the membrane real estate hypothesis. *Cell Syst.* **5**, 95–104 (2017).
- Zhuang, K., Vemuri, G. N. & Mahadevan, R. Economics of membrane occupancy and respiro-fermentation. *Mol. Syst. Biol.* **7**, 500 (2011).
- Mannella, C. A. Structure and dynamics of the mitochondrial inner membrane cristae. *Biochim. Biophys. Acta Mol. Cell Res.* **1763**, 542–548 (2006).
- Mustárdy, L. & Garab, G. Granum revisited. A three-dimensional model—where things fall into place. *Trends Plant Sci.* **8**, 117–122 (2003).
- Mühleip, A. et al. Structural basis of mitochondrial membrane bending by the I–II–III2–IV2 supercomplex. *Nature* **615**, 934–938 (2023).
- Greening, C. & Lithgow, T. Formation and function of bacterial organelles. *Nat. Rev. Microbiol.* **18**, 677–689 (2020).
- van Niftrik, L. A. et al. The anammoxosome: an intracytoplasmic compartment in anammox bacteria. *FEMS Microbiol. Lett.* **233**, 7–13 (2004).
- Hanson, R. S. & Hanson, T. E. Methanotrophic bacteria. *Microbiol. Rev.* **60**, 439–471 (1996).
- Zhu, Y. et al. Structure and activity of particulate methane monooxygenase arrays in methanotrophs. *Nat. Commun.* <https://doi.org/10.1038/s41467-022-32752-9> (2022).
- Roger, A. J., Muñoz-Gómez, S. A. & Kamikawa, R. The origin and diversification of mitochondria. *Curr. Biol.* **27**, R1177–R1192 (2017).
- Rajagopal, M. & Walker, S. in *Protein and Sugar Export and Assembly in Gram-positive Bacteria* (eds Bagnoli, F. & Rappuoli, R.) 1–44 (Springer, 2017).
- Rohde, M. The Gram-positive bacterial cell wall. *Microbiol. Spectr.* <https://doi.org/10.1128/microbiolspec.gpp3-0044-2018> (2019).
- Kaila, V. R. & Wikström, M. Architecture of bacterial respiratory chains. *Nat. Rev. Microbiol.* **19**, 319–330 (2021).
- Franza, T. & Gaudu, P. Quinones: more than electron shuttles. *Res. Microbiol.* **173**, 103953 (2022).
- Yankovskaya, V. et al. Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science* **299**, 700–704 (2003).
- Volbeda, A. et al. Crystal structure of the O₂-tolerant membrane-bound hydrogenase 1 from *Escherichia coli* in complex with its cognate cytochrome *b*. *Structure* **21**, 184–190 (2013).
- Liang, Y., Bueler, S. A., Cook, G. M. & Rubinstein, J. L. Structure of mycobacterial NDH-2 bound to a 2-mercapto-quinazolinone inhibitor. *J. Med. Chem.* <https://doi.org/10.1021/acs.jmedchem.5c00049> (2025).
- Singh, H., Arentson, B. W., Becker, D. F. & Tanner, J. J. Structures of the PutA peripheral membrane flavoenzyme reveal a dynamic substrate-channeling tunnel and the quinone-binding site. *Proc. Natl Acad. Sci. USA* **111**, 3389–3394 (2014).
- Broc, M. et al. A scaffold for quinone channeling between membrane and soluble bacterial oxidoreductases. *Nat. Struct. Mol. Biol.* <https://doi.org/10.1038/s41594-025-01607-4> (2025).
- Grinter, R. et al. Structural basis for bacterial energy extraction from atmospheric hydrogen. *Nature* **615**, 541–547 (2023).
- Kropp, A. et al. Quinone extraction drives atmospheric carbon monoxide oxidation in bacteria. *Nat. Chem. Biol.* <https://doi.org/10.1038/s41589-025-01836-0> (2025).
- Bouranis, J. A. & Tfaily, M. M. Inside the microbial black box: a redox-centric framework for deciphering microbial metabolism. *Trends Microbiol.* **32**, 1170–1178 (2024).
- Covarrubias, A. J., Perrone, R., Grozio, A. & Verdin, E. NAD⁺ metabolism and its roles in cellular processes during ageing. *Nat. Rev. Mol. Cell Biol.* **22**, 119–141 (2021).
- Efremov, R. G., Baradaran, R. & Sazanov, L. A. The architecture of respiratory complex I. *Nature* **465**, 441–445 (2010).
- Kersch, S., Dröse, S., Zickermann, V. & Brandt, U. The three families of respiratory NADH dehydrogenases. *Results Probl. Cell Differ.* **45**, 185–222 (2008).

26. Blaza, J. N. et al. The mechanism of catalysis by type-II NADH:quinone oxidoreductases. *Sci. Rep.* **7**, 40165 (2017).
27. Marreiros, B. C., Sena, F. V., Sousa, F. M., Batista, A. P. & Pereira, M. M. Type II NADH:quinone oxidoreductase family: phylogenetic distribution, structural diversity and evolutionary divergences. *Environ. Microbiol.* **18**, 4697–4709 (2016).
28. Marreiros, B. C. et al. Structural and functional insights into the catalytic mechanism of the Type II NADH:quinone oxidoreductase family. *Sci. Rep.* **7**, 42303 (2017).
29. Gyan, S., Shiohira, Y., Sato, I., Takeuchi, M. & Sato, T. Regulatory loop between redox sensing of the NADH/NAD⁺ ratio by Rex (YdiH) and oxidation of NADH by NADH dehydrogenase Ndh in *Bacillus subtilis*. *J. Bacteriol.* **188**, 7062–7071 (2006).
30. Vamshi Krishna, K. & Venkata Mohan, S. Purification and characterization of NDH-2 protein and elucidating its role in extracellular electron transport and bioelectrogenic activity. *Front. Microbiol.* <https://doi.org/10.3389/fmicb.2019.00880> (2019).
31. Feng, Y. et al. Structural insight into the type-II mitochondrial NADH dehydrogenases. *Nature* **491**, 478–482 (2012).
32. Iwata, M. et al. The structure of the yeast NADH dehydrogenase (Ndi1) reveals overlapping binding sites for water- and lipid-soluble substrates. *Proc. Natl Acad. Sci. USA* **109**, 15247–15252 (2012).
33. Sena, F. V. et al. quinone oxidoreductase from *Staphylococcus aureus* has two distinct binding sites and is rate limited by quinone reduction. *Mol. Microbiol.* **98**, 272–288 (2015).
34. Heikal, A. et al. Structure of the bacterial type II NADH dehydrogenase: a monotopic membrane protein with an essential role in energy generation. *Mol. Microbiol.* **91**, 950–964 (2014).
35. Schagger, H. & Pfeiffer, K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J* **19**, 1777–1783 (2000).
36. Gong, H. et al. An electron transfer path connects subunits of a mycobacterial respiratory supercomplex. *Science* **362**, eaat8923 (2018).
37. Yang, Y. et al. Reaction mechanism of single subunit NADH-ubiquinone oxidoreductase (Ndi1) from *Saccharomyces cerevisiae*: evidence for a ternary complex mechanism. *J. Biol. Chem.* **286**, 9287–9297 (2011).
38. Taber, H. W., Dellers, E. A. & Lombardo, L. R. Menaquinone biosynthesis in *Bacillus subtilis*: isolation of men mutants and evidence for clustering of men genes. *J. Bacteriol.* **145**, 321–327 (1981).
39. Dharmaraj, K., Román Silva, J. I., Kahlert, H., Lendeckel, U. & Scholz, F. The acid-base and redox properties of menaquinone MK-4, MK-7, and MK-9 (vitamin K2) in DMPC monolayers on mercury. *Eur. Biophys. J.* **49**, 279–288 (2020).
40. Nakatani, Y. et al. Unprecedented properties of phenothiazines unraveled by a NDH-2 bioelectrochemical assay platform. *J. Am. Chem. Soc.* **142**, 1311–1320 (2020).
41. Parks, D. H. et al. GTDB: an ongoing census of bacterial and archaeal diversity through a phylogenetically consistent, rank normalized and complete genome-based taxonomy. *Nucleic Acids Res.* **50**, D785–D794 (2021).
42. Sena Filipa, V. et al. The two alternative NADH:quinone oxidoreductases from *Staphylococcus aureus*: two players with different molecular and cellular roles. *Microbiol. Spectr.* **12**, e0415223 (2024).
43. Gu, L., Zhao, S., Tadesse, B. T., Zhao, G. & Solem, C. Scrutinizing a *Lactococcus lactis* mutant with enhanced capacity for extracellular electron transfer reveals a unique role for a novel type-II NADH dehydrogenase. *Appl. Environ. Microbiol.* **90**, e0041424 (2024).
44. Jun, J.-S., Jeong, H.-E., Moon, S.-Y., Shin, S.-H. & Hong, K.-W. Time-course transcriptome analysis of *Bacillus subtilis* DB104 during growth. *Microorganisms* **11**, 1928 (2023).
45. Dietrich, H. M. et al. Membrane-anchored HDCR nanowires drive hydrogen-powered CO₂ fixation. *Nature* **607**, 823–830 (2022).
46. Harwood, C. R. & Cutting, S. M. *Molecular Biological Methods for Bacillus* (Wiley, 1991).
47. Koo, B.-M. et al. Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell Syst.* **4**, 291–305.e7 (2017).
48. Stavarakakis, M. D., Humphrey, M., van Rij, T., Harwood, C. R. & Strahl, H. Monitoring single cell bioenergetic status and cell lysis in dense and differentiating *Bacillus subtilis* cultures. Preprint at *bioRxiv* <https://doi.org/10.1101/2025.08.21.671461> (2025).
49. Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat. Methods* **14**, 290–296 (2017).
50. Zivanov, J. et al. New tools for automated high-resolution cryo-EM structure determination in RELION-3. *eLife* **7**, e42166 (2018).
51. Wagner, T. et al. SPHIRE-crYOLO is a fast and accurate fully automated particle picker for cryo-EM. *Commun. Biol.* **2**, 218 (2019).
52. Bepler, T. et al. Positive-unlabeled convolutional neural networks for particle picking in cryo-electron micrographs. *Nat. Methods* **16**, 1153–1160 (2019).
53. Abramson, J. et al. Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**, 493–500 (2024).
54. DeLano, W. L. *PyMOL: An Open-source Molecular Graphics Tool* (ScienceOpen, 2002).
55. Pettersen, E. F. et al. UCSF ChimeraX: structure visualization for researchers, educators, and developers. *Protein Sci.* **30**, 70–82 (2021).
56. Afonine, P. V. et al. Real-space refinement in PHENIX for cryo-EM and crystallography. *Acta Crystallogr. D Struct. Biol.* **74**, 531–544 (2018).
57. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132 (2004).
58. Chen, V. B. et al. Molprobity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D* **66**, 12–21 (2010).
59. Schmid, R. et al. Integrative analysis of multimodal mass spectrometry data in MZmine 3. *Nat. Biotechnol.* **41**, 447–449 (2023).
60. Kind, T. et al. LipidBlast in silico tandem mass spectrometry database for lipid identification. *Nat. Methods* **10**, 755–758 (2013).
61. Tsugawa, H. et al. A lipidome atlas in MS-DIAL 4. *Nat. Biotechnol.* **38**, 1159–1163 (2020).
62. Conroy, M. J. et al. LIPID MAPS: update to databases and tools for the lipidomics community. *Nucleic Acids Res.* **52**, D1677–d1682 (2024).
63. Sud, M. et al. LMSD: LIPID MAPS structure database. *Nucleic Acids Res.* **35**, D527–D532 (2007).
64. Layre, E. et al. A comparative lipidomics platform for chemotaxonomic analysis of *Mycobacterium tuberculosis*. *Chem. Biol.* **18**, 1537–1549 (2011).
65. Nitzschke, A. & Bettenbrock, K. All three quinone species play distinct roles in ensuring optimal growth under aerobic and fermentative conditions in *E. coli* K12. *PLoS ONE* **13**, e0194699 (2018).
66. Yasuhiro, K., Yuzuru, A. & Shoshichi, N. Composition and turnover of the phospholipids in *Escherichia coli*. *Biochim. Biophys. Acta* **144**, 382–390 (1967).
67. Neidhardt, F., Ingraham, J. & Schaechter, M. *Physiology of the Bacterial Cell: A Molecular Approach* (Sinauer Associates, 1990).
68. Abraham, M. J. et al. GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1–2**, 19–25 (2015).

69. Huang, J. et al. CHARMM36m: an improved force field for folded and intrinsically disordered proteins. *Nat. Methods* **14**, 71–73 (2017).
70. Huang, J. & MacKerell, A. D. Jr. CHARMM36 all-atom additive protein force field: validation based on comparison to NMR data. *J. Comput. Chem.* **34**, 2135–2145 (2013).
71. Klauda, J. B. et al. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. *J. Phys. Chem. B* **114**, 7830–7843 (2010).
72. Feng, S., Wang, R., Pastor, R. W., Klauda, J. B. & Im, W. Location and conformational ensemble of menaquinone and menaquinol, and protein–lipid modulations in archaeal membranes. *J. Phys. Chem. B* **125**, 4714–4725 (2021).
73. Jo, S., Kim, T., Iyer, V. G. & Im, W. CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comput. Chem.* **29**, 1859–1865 (2008).
74. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38 (1996). 27–38.
75. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W. & Klein, M. L. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926–935 (1983).
76. Beglov, D. & Roux, B. Finite representation of an infinite bulk system: solvent boundary potential for computer simulations. *J. Chem. Phys.* **100**, 9050–9063 (1994).
77. Olsson, M. H., Søndergaard, C. R., Rostkowski, M. & Jensen, J. H. PROPKA3: consistent treatment of internal and surface residues in empirical pK_a predictions. *J. Chem. Theory Comput.* **7**, 525–537 (2011).
78. Bussi, G., Donadio, D. & Parrinello, M. Canonical sampling through velocity rescaling. *J. Chem. Phys.* **126**, 014101 (2007).
79. Bernetti, M. & Bussi, G. Pressure control using stochastic cell rescaling. *J. Chem. Phys.* **153**, 114107 (2020).
80. Nosé, S. A molecular dynamics method for simulations in the canonical ensemble. *Mol. Phys.* **52**, 255–268 (1984).
81. Hoover, W. G. Canonical dynamics: equilibrium phase–space distributions. *Phys. Rev. A Gen. Phys.* **31**, 1695–1697 (1985).
82. Parrinello, M. & Rahman, A. Polymorphic transitions in single crystals: a new molecular dynamics method. *J. Appl. Phys.* **52**, 7182–7190 (1981).
83. Allen, M. P., Tildesley, D. J. & Banavar, J. R. Computer simulation of liquids. *Phys. Today* **42**, 105–106 (1989).
84. Hess, B., Bekker, H., Berendsen, H. J. C. & Fraaije, J. G. E. M. LINCS: a linear constraint solver for molecular simulations. *J. Comput. Chem.* **18**, 1463–1472 (1997).
85. Verlet, L. Computer “experiments” on classical fluids. I. Thermodynamical properties of Lennard–Jones molecules. *Phys. Rev.* **159**, 98–103 (1967).
86. Darden, T., York, D. & Pedersen, L. Particle mesh Ewald: an N-log(N) method for Ewald sums in large systems. *J. Chem. Phys.* **98**, 10089–10092 (1993).
87. Phillips, J. C. et al. Scalable molecular dynamics on CPU and GPU architectures with NAMD. *J. Chem. Phys.* **153**, 044130 (2020).
88. Hestenes, M. R. & Stiefel, E. Methods of conjugate gradients for solving linear systems. *J. Res. Nat. Bur. Stand.* **49**, 409–436 (1952).
89. McIntosh, T. J. & Simon, S. A. Area per molecule and distribution of water in fully hydrated dilauroylphosphatidylethanolamine bilayers. *Biochemistry* **25**, 4948–4952 (1986).
90. Sanner, M. F., Olson, A. J. & Spehner, J. C. Reduced surface: an efficient way to compute molecular surfaces. *Biopolymers* **38**, 305–320 (1996).
91. O’Reilly, J. E. Oxidation–reduction potential of the ferro-ferricyanide system in buffer solutions. *Biochim. Biophys. Acta* **292**, 509–515 (1973).
92. Hyatt, D. et al. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **11**, 119 (2010).
93. Kanehisa, M. & Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
94. Mistry, J. et al. Pfam: the protein families database in 2021. *Nucleic Acids Res.* **49**, D412–D419 (2020).
95. Shaffer, M. et al. DRAM for distilling microbial metabolism to automate the curation of microbiome function. *Nucleic Acids Res.* **48**, 8883–8900 (2020).
96. Lu, S. et al. CDD/SPARCLE: the conserved domain database in 2020. *Nucleic Acids Res.* **48**, D265–D268 (2019).
97. Camacho, C. et al. BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421 (2009).
98. The UniProt Consortium UniProt: the Universal Protein Knowledgebase in 2025. *Nucleic Acids Res.* **53**, D609–D617 (2024).
99. Buchfink, B., Xie, C. & Huson, D. H. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **12**, 59–60 (2015).
100. Chaumeil, P.-A., Mussig, A. J., Hugenholtz, P. & Parks, D. H. GTDB-Tk v2: memory friendly classification with the genome taxonomy database. *Bioinformatics* **38**, 5315–5316 (2022).
101. Minh, B. Q. et al. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Mol. Biol. Evol.* **37**, 1530–1534 (2020).
102. Steinegger, M. & Söding, J. MMseqs2 enables sensitive protein sequence searching for the analysis of massive data sets. *Nat. Biotechnol.* **35**, 1026–1028 (2017).
103. Edgar, R. C. Muscle5: high-accuracy alignment ensembles enable unbiased assessments of sequence homology and phylogeny. *Nat. Commun.* **13**, 6968 (2022).
104. Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A. & Jermini, L. S. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods* **14**, 587–589 (2017).
105. Letunic, I. & Bork, P. Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. *Nucleic Acids Res.* **52**, W78–W82 (2024).
106. Boitreaud, J. et al. Chai-1: decoding the molecular interactions of life. Preprint at *bioRxiv* <https://doi.org/10.1101/2024.10.10.615955> (2024).
107. Lumbantobing, T., Barlow, C., Kropp, A. & Grinter, R. Quinone-transporting filaments extend the respiratory chain of Gram-positive bacteria [Dataset]. *Zenodo* <https://doi.org/10.5281/zenodo.21271664> (2026).
108. Simsive, L., Sharma, V. & Zdorevskyi, O. Quinone-transporting filaments extend the respiratory chain of Gram-positive bacteria [Dataset]. *Zenodo* <https://doi.org/10.5281/zenodo.2126551> (2026).

Acknowledgements

We thank S. Valimehr and H. Brown (Ian Holmes Imaging Centre, Faculty of Science, University of Melbourne) for cryo-EM training and advice. We acknowledge the use of the facilities at the Melbourne Protein Characterisation Facility, of the Bio21 Molecular Science and Biotechnology Institute, the University of Melbourne and thank B. Michell for training and assistance. We thank S. Revitt-Mills (Department of Microbiology, Monash University) for providing the *B. subtilis* strain for lipid exchange experiments. We acknowledge the use of instruments at the Ian Holmes Imaging Centre. We thank D. Ghosal, D. Shepard and M. Johnson (Department of Biochemistry and Pharmacology, Bio21 Institute, University of Melbourne) for advice regarding *B. subtilis* tomography experiments. We thank H. Strahl (Centre for Bacterial Cell Biology, Newcastle University Biosciences Institute) for advice regarding *B. subtilis* genetics and physiology. We acknowledge extensive HPC resources from the Center for Scientific Computing (CSC), Finland and thank the IT support of the Faculty of Science, University of Helsinki.

Author contributions

R.G., A.K. and K.A. conceived and designed the project. R.G. and C.G. supervised the project. A.K. and K.A. expressed and purified proteins. A.K. performed biochemical characterization of Ndh-Ncp, *B. subtilis* fractionation and negative-stain TEM. A.K., K.A. and D.R.F. prepared cryo-EM grids and collected data. A.K., R.G. and K.A. processed cryo-EM data. A.K. and R.G. modelled and analysed the structures. R.L.D. generated *B. subtilis* mutants, performed growth assays and carried out native PAGE NADH-oxidase activity assays. J.A.S. and A.P. designed, performed and analysed cyclic voltammetry experiments. N.D.J.Y. and J.A.S. generated S-cholesterol for bilayer construction. J.N.B. and B.G.H. devised, performed and analysed UV-Vis and kinetics experiments. A.K. designed and carried out lipid exchange experiments, and C.K.B. and T.L. designed, performed and analysed mass spectrometry experiments. L.S., O.Z. and V.S. devised, performed and analysed molecular dynamics simulations. P.M.L. performed genomic surveys and phylogenetic analysis. A.K. and R.G. wrote and edited the manuscript with input from all authors.

Funding

A.K., R.G. and D.R.F. are members of the Australian Research Council Industrial Transformation Training Centre for Cryo-Electron Microscopy of Membrane Proteins for Drug Discovery (IC200100052). A.K. and D.R.F. were supported by an RTP scholarship. This work was supported by an Australian Research Council Discovery Project (DP260100290), UKRI Future Leader Fellowship to J.N.B. (MR/Z000084/1 & MR/T040742/1), a BBSRC sLoLa award to J.N.B. and R.L.D. (BB/X003035/1), an Australian Research Council DECRA fellowship to P.M.L. (DE250101210), and an EPSRC PhD studentship to B.G.H. (2885393). Funding for J.A.S., N.Y. and A.P. was provided by UKRI, EP/X027724/1. This study used BPA-enabled (Bioplatforms Australia) / NCRIS-enabled (National Collaborative Research Infrastructure Strategy) infrastructure located at the Monash Proteomics and Metabolomics Platform. This work was further supported by Monash eResearch capabilities, including Research Data Storage, M3 Massive HPC, and Nectar Research Cloud. V.S. acknowledges research funding from the Research Council of Finland (338176), the Sigrid Jusélius Foundation, the University of Helsinki, the Jane and Aatos Erkko Foundation and the Finnish Society of Sciences and Letters. O.Z. acknowledges research funding from the Research Council of Finland (1362411). L.S. acknowledges support from the SciDoc (Chems) doctoral programme of the University of Helsinki.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at <https://doi.org/10.1038/s41564-026-02450-z>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41564-026-02450-z>.

Correspondence and requests for materials should be addressed to Rhys Grinter.

Peer review information *Nature Microbiology* thanks Thomas Boesen, David Young and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permissions information is available at www.nature.com/reprints.

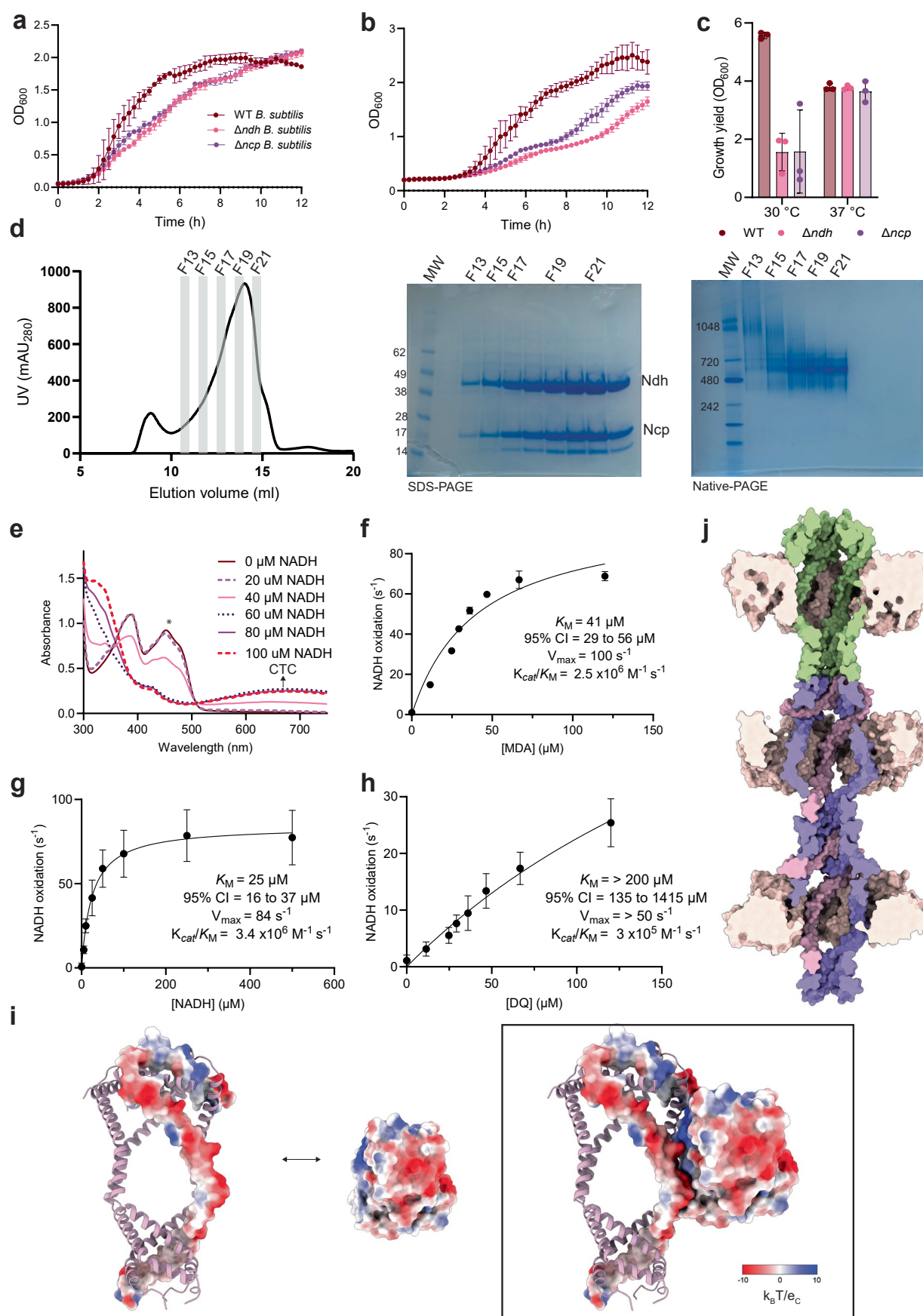
Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2026

¹Department of Biochemistry and Pharmacology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria, Australia. ²Centre for Electron Microscopy of Membrane Proteins, Monash Institute of Pharmaceutical Sciences, Parkville, Victoria, Australia.

³Department of Microbiology, Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia. ⁴Department of Chemistry, University of York, York, UK. ⁵Department of Physics, University of Helsinki, Helsinki, Finland. ⁶Centre for Bacterial Cell Biology, Newcastle University Biosciences Institute, Newcastle University, Newcastle-upon-Tyne, UK. ⁷Monash Proteomics and Metabolomics Platform, Department of Biochemistry, Monash Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia. ⁸York Structural Biology Laboratory, Department of Chemistry, University of York, York, UK. ⁹HiLIFE Institute of Biotechnology, University of Helsinki, Helsinki, Finland. ¹⁰These authors contributed equally: Kazem Asadollahi, Jamie A. Stapleton, Luka Simsi, Pok Man Leung, Rachel L. Darnell. ✉ e-mail: rhys.grinter@monash.edu

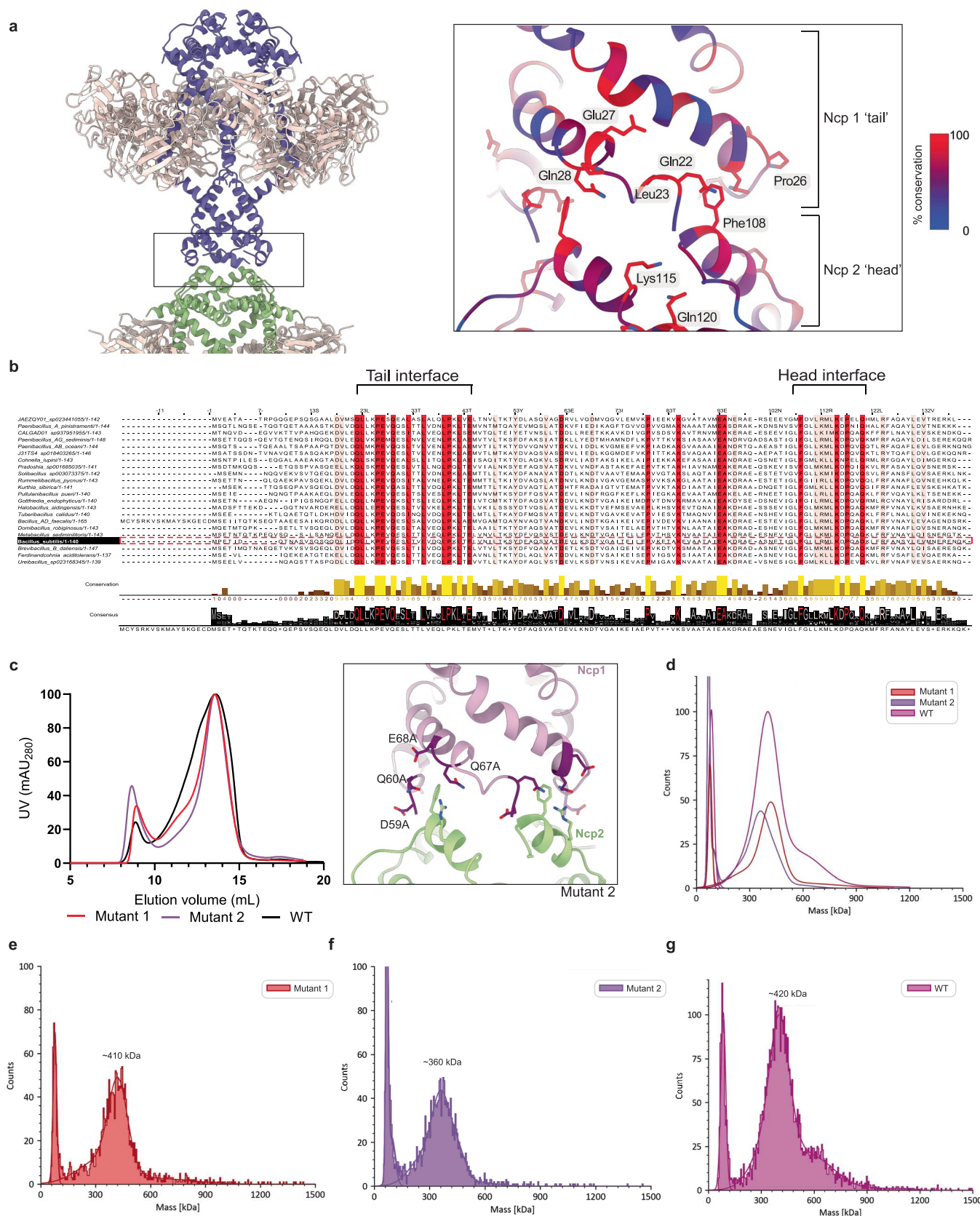


Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | *B. subtilis* Ndh-Ncp purification and characterisation.

a, A growth curve of WT, Δndh and Δncp *B. subtilis* 168 strain, grown at 37 °C, showing OD₆₀₀ vs. time (h). **b**, A growth curve of WT, Δndh and Δncp *B. subtilis* 168 strain, grown at 30 °C, showing OD₆₀₀ vs. time (h). **c**, The growth yield of WT, Δndh and Δncp *B. subtilis* 168 strain after 24 hours of growth at 30 or 37 °C, measured in OD₆₀₀. Data in panels a, b and c are from three or six biological replicates (n = 3, n = 6) with mean ± standard deviation displayed. **d**, The SEC profile (Superose 6 10/300) from the purification of Ndh-Ncp with the corresponding SDS-PAGE and Native-PAGE gels. Ndh has an expected size of 42 kDa and Ncp 19.4 kDa (with strep tag). Raw gel images are shown in the Source Data. **e**, Representative UV-Vis spectra of Ndh-Ncp complex with increasing concentrations of NADH (all concentrations shown). **f**, Michaelis-Menten kinetics of Ndh-Ncp incubated with

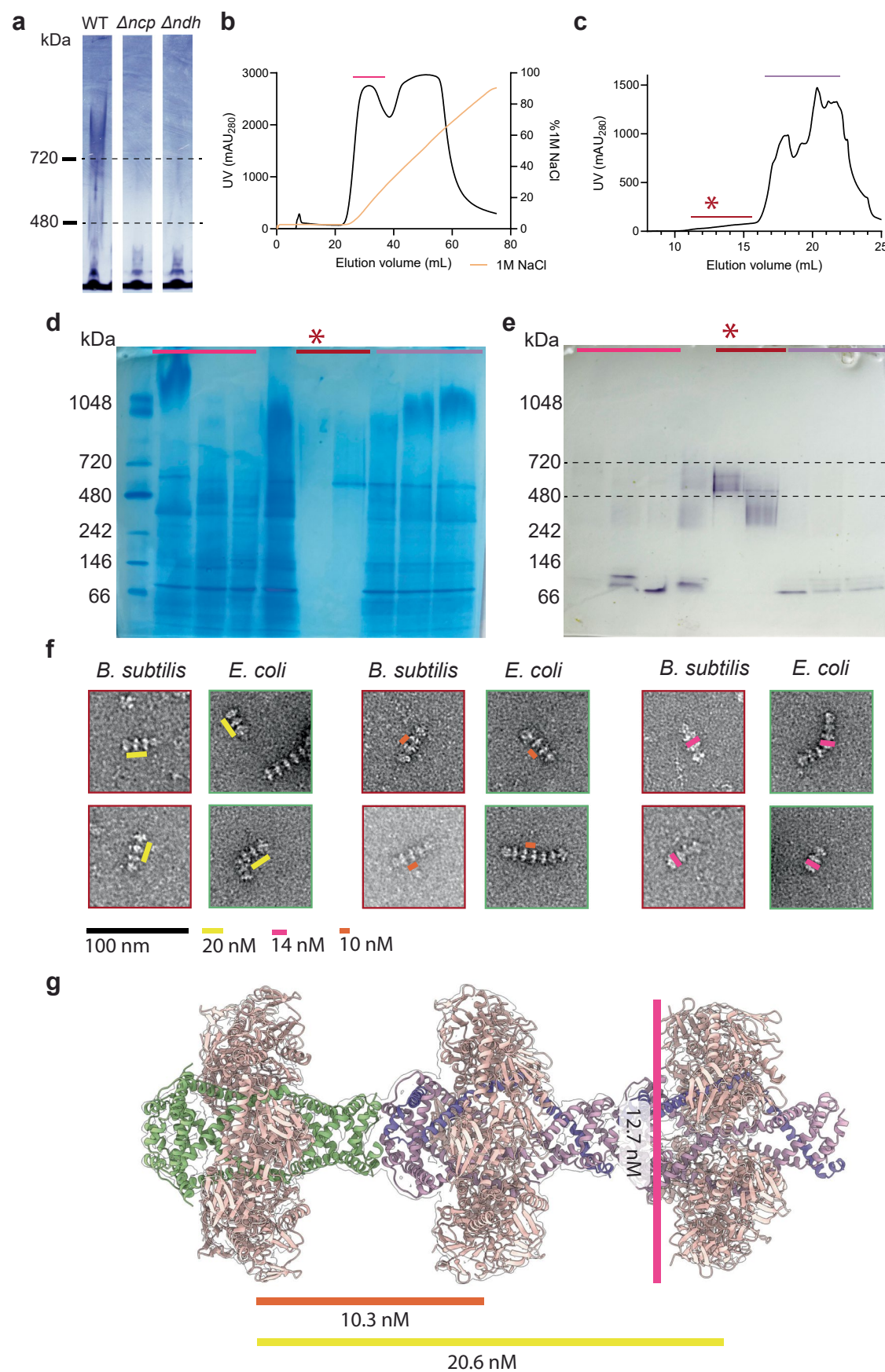
NADH and varying concentrations of MDA. **g**, Michaelis-Menten kinetics of Ndh-Ncp incubated with decylubiquinone (DQ) and varying concentrations of NADH. **h**, Michaelis-Menten kinetics of Ndh-Ncp incubated with NADH and varying concentrations of DQ. **i**, An electrostatic surface representation of the cryo-EM structure of one Ncp and one Ndh overlaid on the cartoon representation of NCP tube showing the interaction interface between one Ncp and one Ndh. Red colouring corresponds to negatively charged residues, white neutral, and blue positively charged residues. **j**, Surface representation of the Ndh-Ncp cryo-EM structure cut down the centre to show the continuous Ncp tube between complexes. Data in panels f, g and h are from three independent replicates (n = 3) with mean ± standard deviation displayed.



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Analysis of the Ndh-Ncp filament interface. **a**, A cartoon view of the Ndh-Ncp complex showing the filament interface (left), with a zoomed view of this region, showing the conservation of amino acids forming key interface contacts (right). **b**, A multiple sequence alignment of selected diverse Ncp homologues showing highly conserved amino acids at the head and tail regions of the filament interface. **c**, SEC chromatograms of purified WT

Ndh-Ncp, and Ndh-Ncp with mutations which disrupt the filament interface (mutations are detailed in the methods). **d**, Processed mass photometry data on WT and filament interface Ndh-Ncp mutants, showing mutations cause a loss of higher order oligomerisation. Data were normalised to WT counts. **e-g**, Raw mass photometry counts for curves depicted in panel **d**.

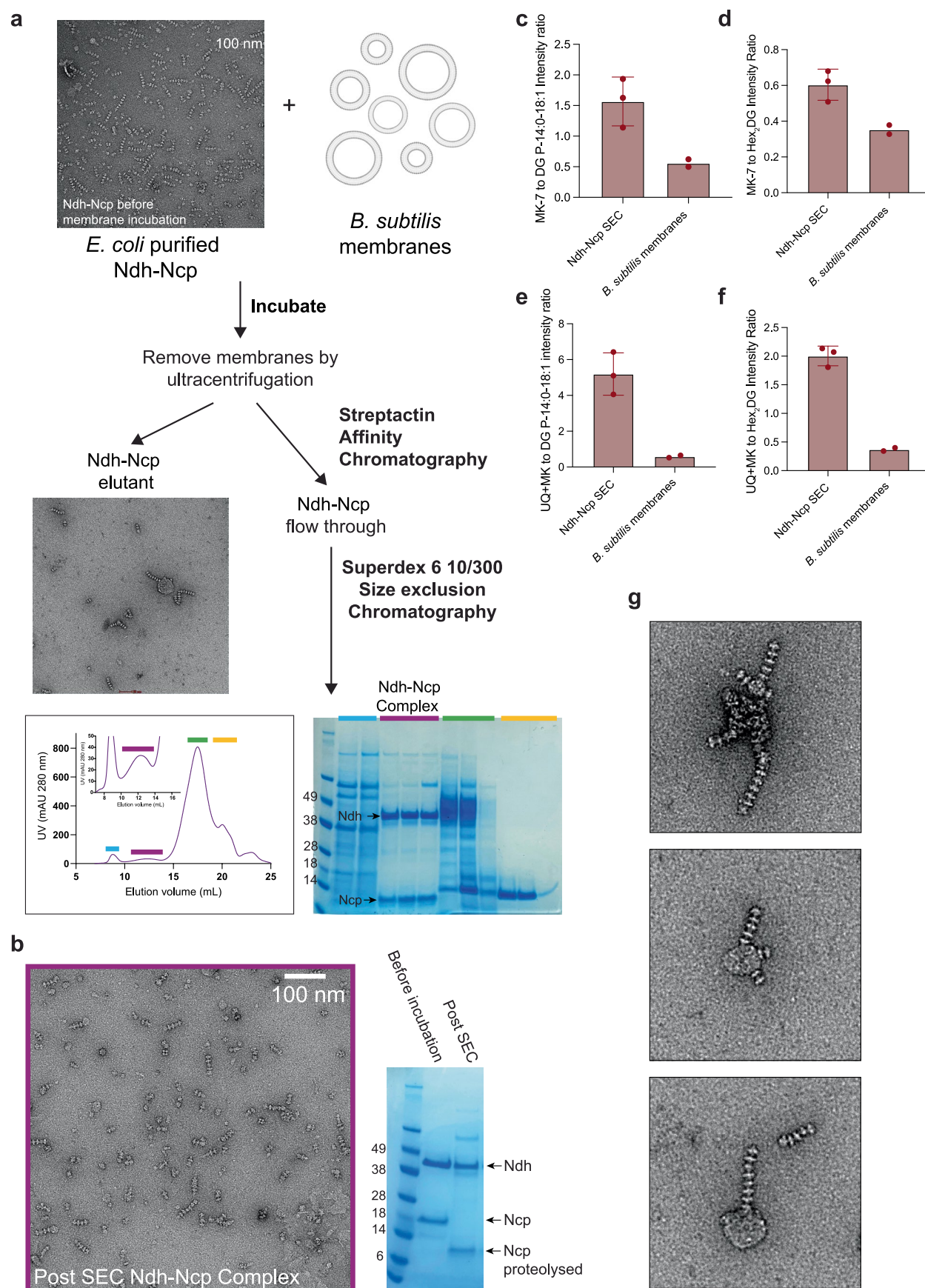


Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | Extraction of Ndh-Ncp filaments from *B. subtilis*.

a, Native-PAGE of WT, Δncp and Δndh *B. subtilis* cell lysates stained for NADH oxidase activity, showing activity attributable to the intact Ndh-Ncp complex ranging between the 480 and 720 kDa size markers. One representative gel shown from $n = 3$ replicates. **b**, An anion exchange chromatography trace of *B. subtilis* cell lysate eluting over an NaCl gradient. The peak containing strong NADH oxidase activity is marked by the pink line. **c**, A SEC chromatogram of the pooled NADH oxidase activity fractions from panel b. The fraction containing NADH oxidase activity in the size range corresponding to Ndh-Ncp is indicated with a star. **d**, A Coomassie stained Native-PAGE gel of fractions from

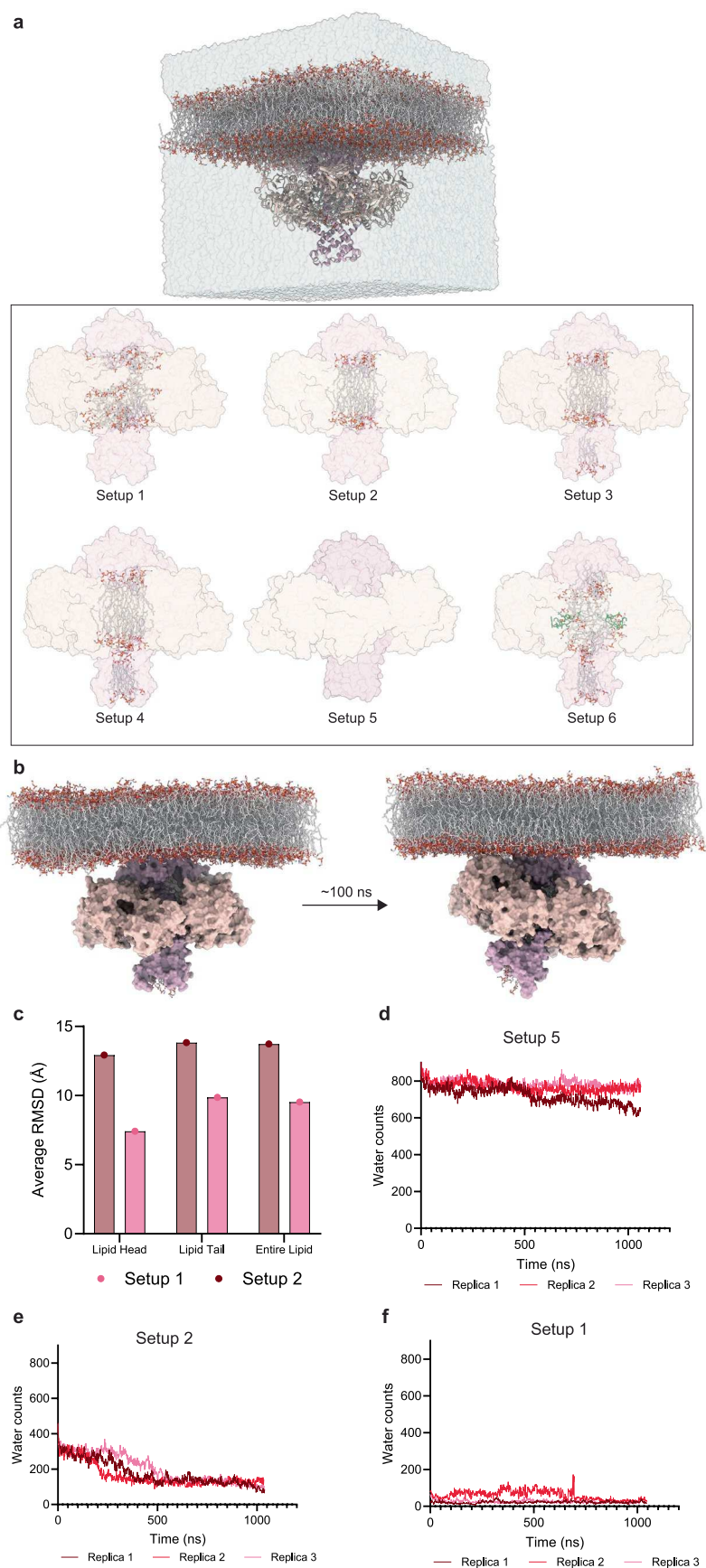
fractionations shown in panels b and c ($n = 1$). **e**, NADH oxidase activity staining of a duplicate of the gel from panel d ($n = 1$). The fraction containing activity at a size attributable to Ndh-Ncp, which was used for negative-stain EM is indicated with a star. **f**, Regions of Negative stain EM images fractionated *B. subtilis* lysates with NADH oxidase activity, showing particles of a size and shape consistent with Ndh-Ncp filaments (left panels), compared to recombinantly produced Ndh-Ncp filaments (right panels) (representative particles from $n = 50$ micrographs). **g**, A cartoon representation of the Ndh-Ncp filament, showing its dimensions correspond to the particles in panel f.



Extended Data Fig. 4 | See next page for caption.

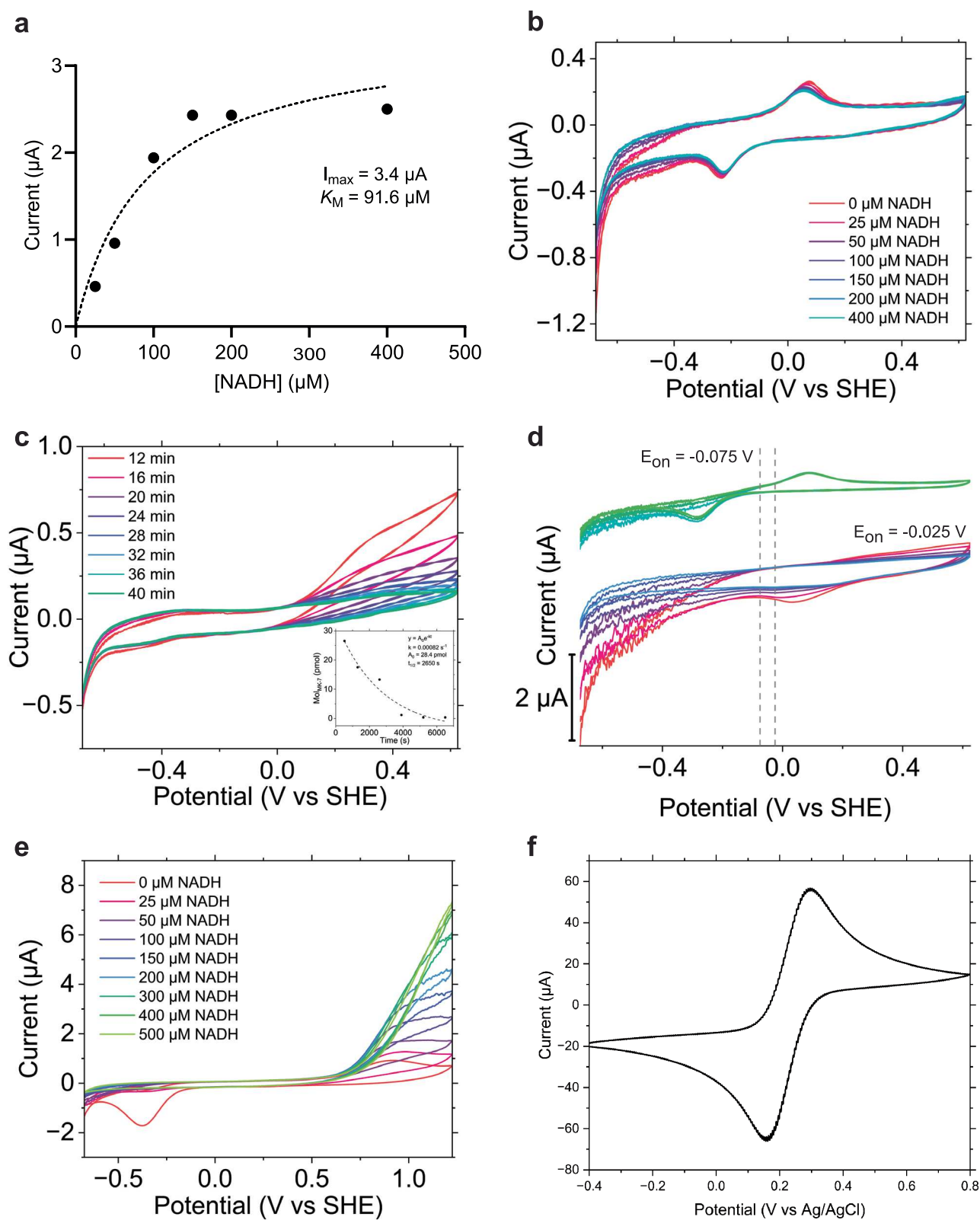
Extended Data Fig. 4 | Analysis of recombinant Ndh-Ncp incubated with *B. subtilis* membranes. **a**, A scheme of the experimental workflow for the incubation and re-isolation of recombinantly produced Ndh-Ncp with *B. subtilis* membranes (one representative micrograph is shown from $n = 10$ (top left) or $n = 20$ (bottom left) micrographs). Aspects of this figure were generated with BioRender. **b**, Negative stain EM and SDS-PAGE of the reisolated Ndh-Ncp complex utilised for lipidomic analysis. Data from one representative experiment is shown, with three independent experiments conducted to provide material for analysis (one representative micrograph shown from $n = 10$ micrographs). **c**, The relative mass spec peak intensity ratio of MK-7 to a putatively identified DGP-14-18:1 species in Ndh-Ncp complex reisolated after incubation with *B. subtilis* membranes compared to the membranes used for incubation. **d**, The relative mass spec peak intensity ratio of MK-7 to putatively identified dihexosyldiacylglycerol (Hex₂DG) species in Ndh-Ncp complex reisolated

after incubation with *B. subtilis* membranes compared to the membranes used for incubation. **e**, The relative mass spec peak intensity ratio of all UQ and MK assigned species to DGP-14-18:1 in Ndh-Ncp complex reisolated after incubation with *B. subtilis* membranes compared to the membranes used for the incubation. **f**, The relative mass spec peak intensity ratio of all UQ and MK assigned species to Hex₂DG species in Ndh-Ncp complex reisolated after incubation with *B. subtilis* membranes compared to the membranes used for the incubation. **g**, Additional negative-stain EM images of Streptactin isolated Ndh-Ncp after incubation with *B. subtilis* membranes, showing attachment to membrane vesicles (one representative micrograph shown from $n = 20$ micrographs). Lipidomics data in panels **c**, **d**, **e** and **f**, are derived from three independent experiments ($n = 3$) for Ndh-Ncp complexes and two independent experiments ($n = 2$) for *B. subtilis* membrane, with all samples analysed concurrently. Error bars represent $\pm$ SD of the mean.



Extended Data Fig. 5 | Molecular dynamic simulation setups. a, Model systems constructed in this work. Bottom inset shows the initial lipid arrangement inside the Ncp tube for every simulation setup (Table S3), showing the lipids (white) and MK-7 (green). **b**, Tilting of the Ndh-Ncp complex over 100 ns of the simulation

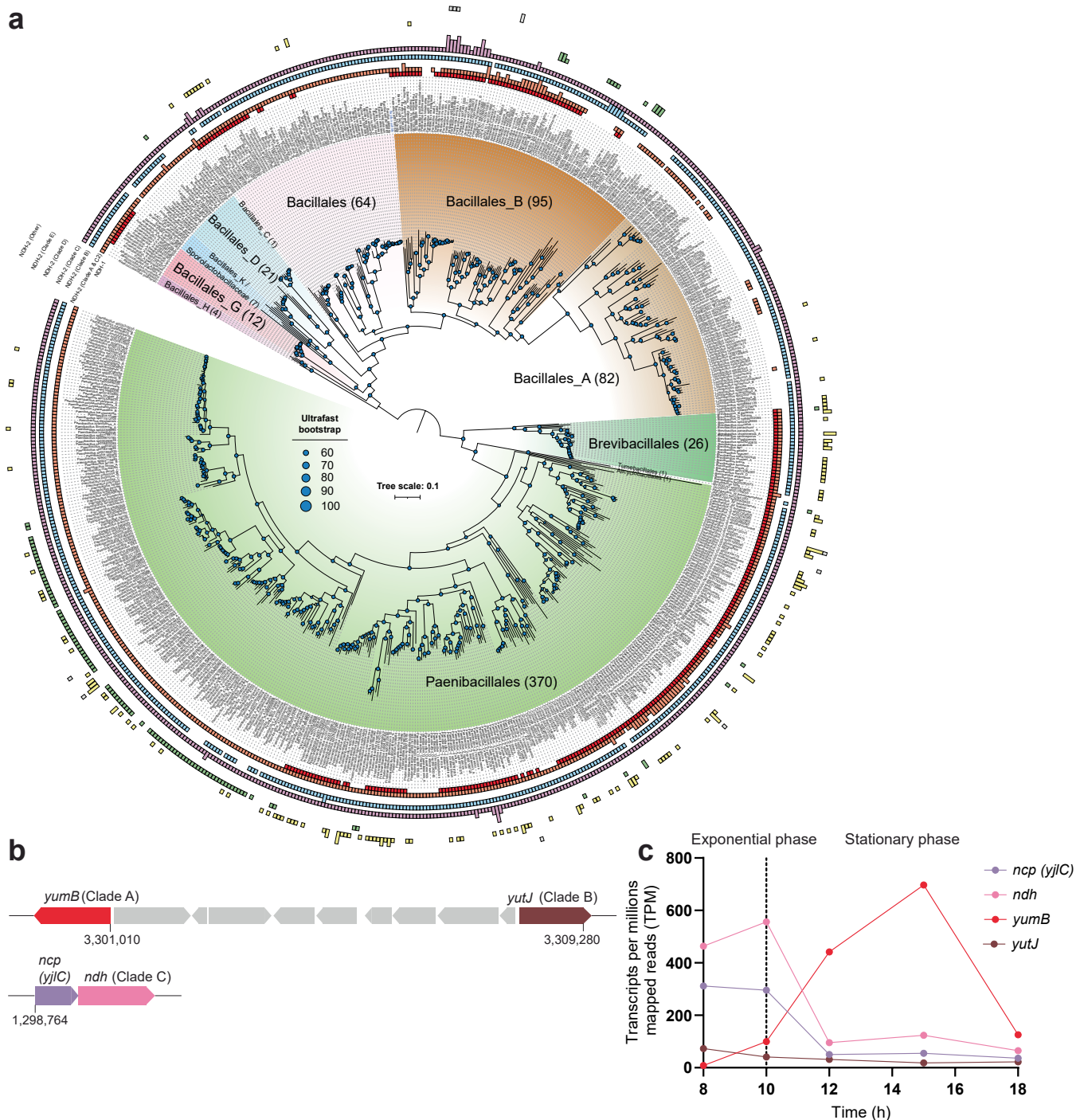
trajectory. **c**, The average RMSD (Å) of lipid movement across the simulations in setups 1 and 2, showing lipids are more mobile in setup 2. **d-f**, The number of water molecules within the Ncp chamber over the course of the simulations for setup 5 (no lipids) and setups 1 and 2.



Extended Data Fig. 6 | See next page for caption.

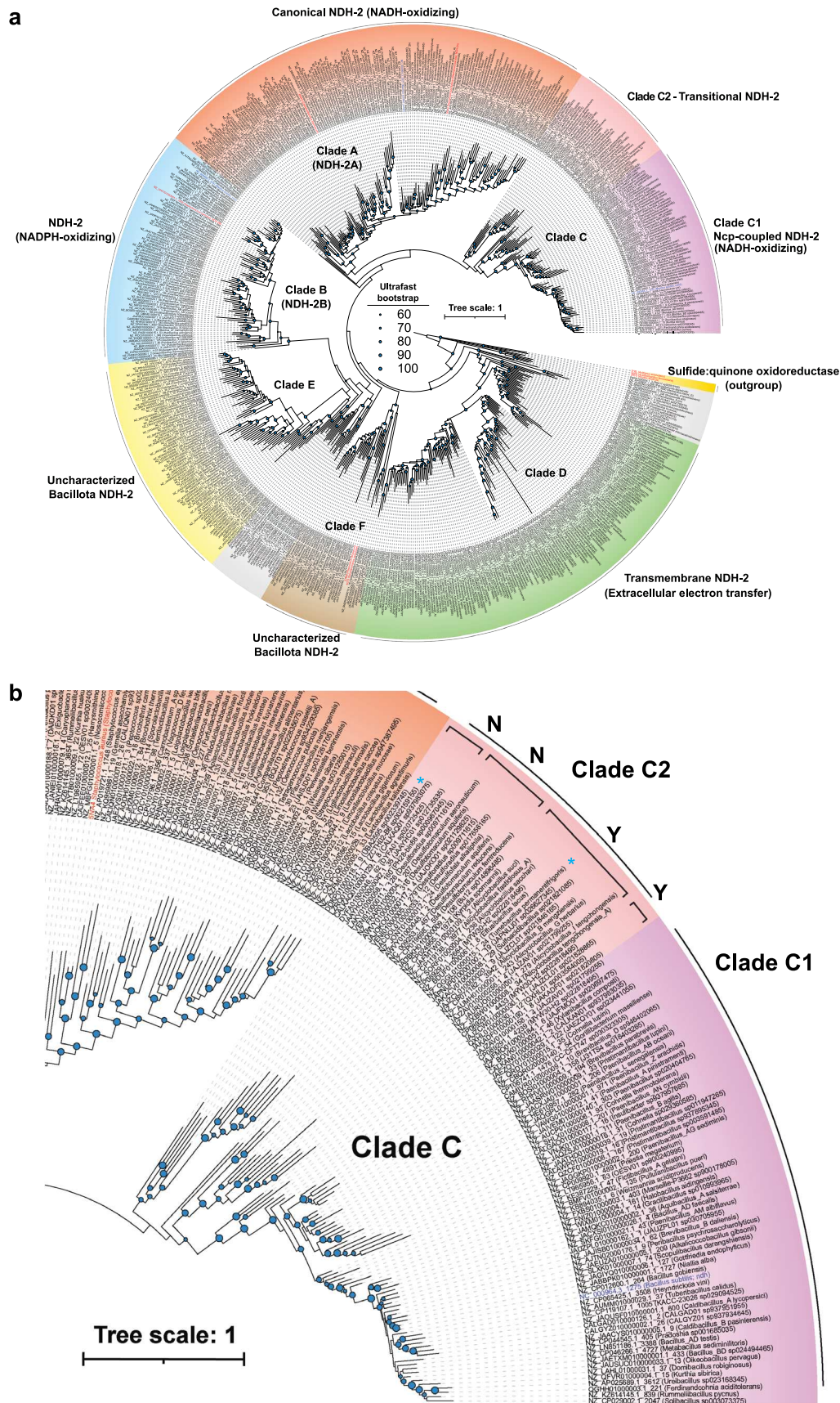
Extended Data Fig. 6 | Cyclic voltammetry of Ndh-Ncp. **a**, A plot of the current values at +0.3 V vs SHE were extracted from the reductive sweeps (from Fig. 4i) and plotted against NADH concentration (solid circles), then fit to the Michaelis-Menten equation (dashed line). **b**, Cyclic voltammograms of NADH titration in the absence of Ndh-Ncp, with MK-7 present **c**, Cyclic voltammograms of Ndh-Ncp associated with the bilayer-coated electrode, recorded at 400 μ M NADH from -0.675 to +0.625 V vs SHE at 10 mVs⁻¹ in the absence of MK-7 at 4-minute intervals, inset of the quinone decay plot calculated from the reductive peak of Extended Data Fig. 6d (bottom). **d**, Cyclic voltammograms of Ndh-Ncp associated with

the bilayer-coated electrode in the absence of NADH, in the presence (top) and absence (bottom) of exogenous MK-7. **e**, Cyclic voltammograms recorded at varying NADH concentrations from -0.675 to +1.225 V vs SHE at 10 mVs⁻¹ in the presence of ubiquinone-8. **f**, Cyclic voltammogram with a clean, blank electrode recorded in 0.2 M Tris buffer, pH 7.00 in the presence of 10 mM potassium ferricyanide to calibrate the Ag/AgCl reference electrode against the known midpoint potential of potassium ferricyanide. The scan was recorded from -0.4 to +0.8 V vs Ag/AgCl at 100 mVs⁻¹. All data are derived from a single representative experimental, performed after multiple rounds of optimisation.



Extended Data Fig. 7 | Ndh-Ncp encoding Bacillota are taxonomically widespread and co-encode multiple NDH-2 enzymes with distinct physiological functions. a, Mid-point rooted maximum-likelihood phylogenomic tree of 684 Ndh-Ncp encoding Bacillota species genomes from GTDB R09-RS220 using concatenated 120 GTDB bacterial marker genes and WAG + G20 model. Outer-rings show the copy numbers of Complex I (NDH-1) and various NDH-2 clades encoded by each species. Branch circles show ultrafast bootstrap support values with 1000 replicates. The scale bar corresponds to the

expected number of substitutions per site. **b**, Canonical genetic arrangement of the NDH-2 from clade A (*yumB*) and NDH-2 from clade B (*yutJ*), compared to NDH-2 from clade C (*ndh*) and Ncp, using *B. subtilis* as an example. Canonical NADH-oxidizing NDH-2 (Clade A) and NADPH-oxidizing NDH-2 (Clade B) are commonly co-encoded in close genetic proximity (typically 4–20 genes apart) in Bacillota genomes. Clade C1 NDH-2 is always co-encoded with Ncp in the immediate genetic proximity **c**, Time-course transcriptomic profiling of NDH-2 and Ncp expression in *B. subtilis* grown in lysogeny broth, from Jun et al.⁵².

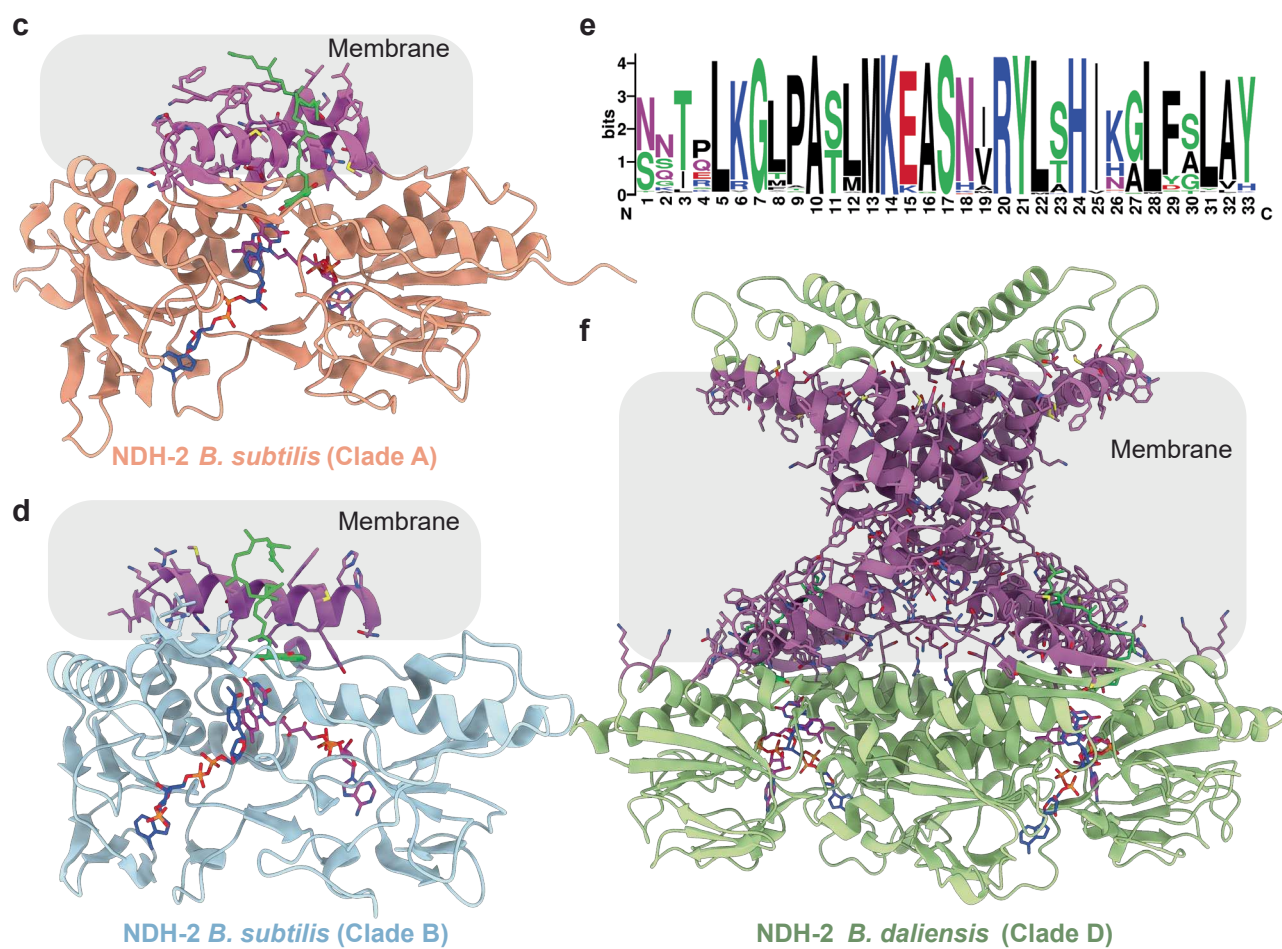
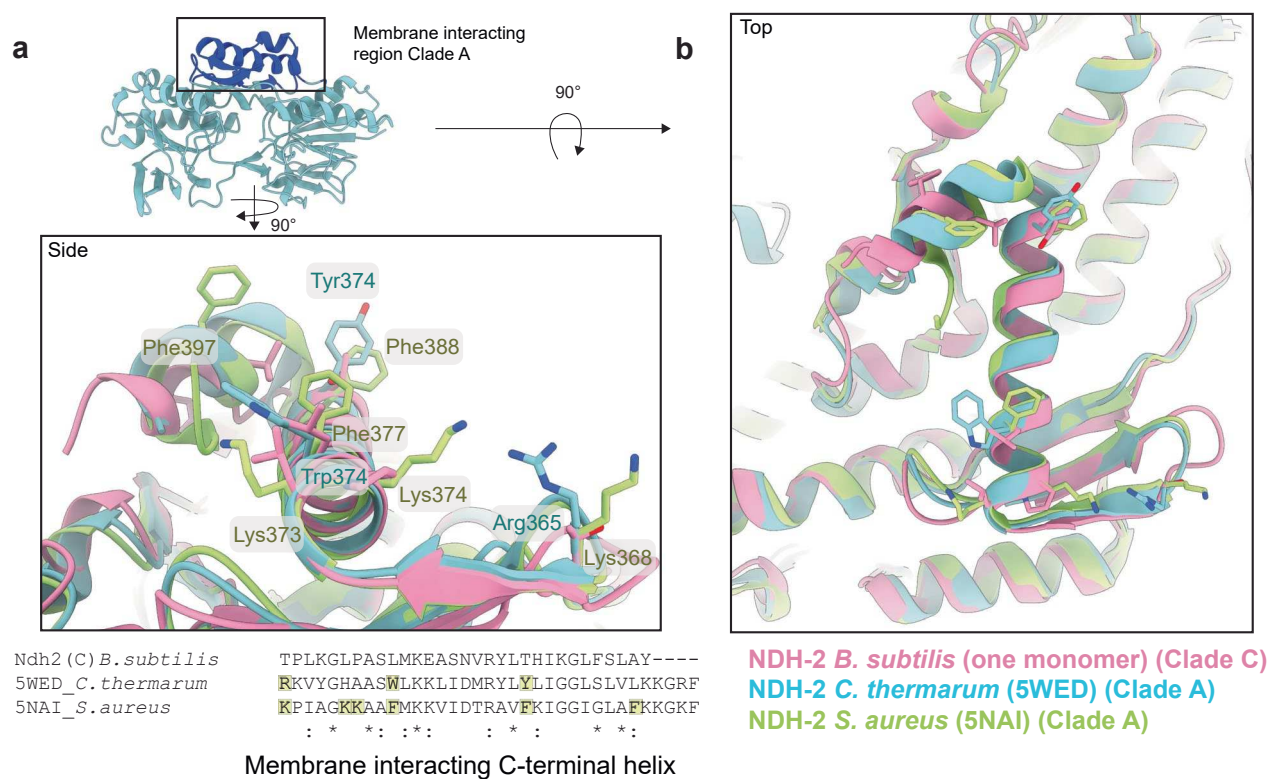


Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | Expanded phylogenetic tree of NDH-2 across Bacillota.

a, Maximum-likelihood tree constructed based on 683 cluster-dereplicated NDH-2 amino acid sequences from Bacillota (Ncp-coupled Ndh: 80% identity; Other NDH-2: 60% identity) using the LG + I + R10 model and rooted using sulfide:quinone oxidoreductases (Sqr) as an outgroup. Taxonomy of the genomes follows GTDB R09-RS220. Coloured tip labels show reference

NDH-2/Sqr sequences (red) and the three *Bacillus subtilis* NDH-2 (blue). Branch circles show ultrafast bootstrap support values with 1000 replicates. The scale bar corresponds to the expected number of substitutions per site. **b**, Zoom of clade C NDH-2 with annotations on clade C2 showing Ndh with a DUF1641-domain protein present elsewhere in the genome (Y) or absent (N). Those annotated with a blue asterisk are outliers.



Extended Data Fig. 9 | See next page for caption.

Extended Data Fig. 9 | Membrane interaction modes of NDH-2 from Bacillota.

a, Overlay of NDH-2 from *B. subtilis* (Clade C1 Ndh, this study), *C. thermarum* (Clade A, PDB: [5WED](#)) and *S. aureus* (Clade A, PDB: [5NAI](#)). The side view of the key residues for membrane interaction from these helices are shown. *S. aureus* (green) and *C. thermarum* (blue) have key aromatic residues that are absent in *B. subtilis* Ndh (pink). **b**, The top view of the structures shows the key residues, with the sequence alignment below demonstrating the conservation of these

residues in the clade A NDH-2. **c**, Model of clade A NDH-2 (*B. subtilis*) with membrane-interacting region shown in purple **d**, Model of clade B NDH-2 (*B. subtilis*) **e**, WebLogo of the C-terminus of the Ndh from clade C1 that couple to Ncp from Bacillota **f**, Model showing the membrane-interacting region of clade D NDH-2 (*B. dاليensis*). Notably, clade D NDH-2 has transmembrane helices to completely integrate into the membrane.

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	EPU v3.6 (Thermo Fisher Scientific) for single-particle cryo-EM data acquisition, Refeyn DiscoverMP for mass photometry, GROMACS 2022.3/4 and NAMD 2.14 for molecular dynamics simulations, Prodigal v2.6.3, DRAM v1.2.4, BLAST+ v2.16.0, DIAMOND v2.1.10 were used for genome surveys, Thermo Scientific SII for XCalibur v1.8.0.530 and Tune Application v5.1.761.17 for lipidomics, Iviumsoft 4.1148 for electrochemistry
Data analysis	Microsoft Excel 2016, GraphPad Prism 10.6, UCSF MotionCor3, crYOLO (1.7.6), CTFFIND-4.1, RELION 5.0, TOPAZ, AFIS.py, cryoSPARC 4.6.0, UCSF ChimeraX 1.8, Phenix 1.2, Pymol 3.1.8, WinCoot 0.9.8.1, AlphaFold3 (AlphaFold Server), Chai1 (online server) VMD v1.9.4, Openstructure software package, mzMine 4.3, TraceFinder 5.2 SP3, Muscle v5.3, IQ-TREE v2.4.0, iTOL v7, MestReNova 15.0.1-36756 for NMR analysis

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The cryo-EM structures are available in the PDB and EMDDB under the codes PDB: 9PXK, EMD-71972 (Ndh-Ncp with MK-8/NADH), PDB: 9PXL, EMD-71973 (Ndh-Ncp) and PDB: 9PXM, EMD-71974 (Ndh-Ncp filament). All phylogenetics sequences and accession numbers are found in Supplementary data file S4. Raw and processed lipidomics data have been deposited here: <https://doi.org/10.5281/zenodo.21271664> Molecular dynamics simulation data have been deposited here: <https://doi.org/10.5281/zenodo.21226551>. Source data containing raw and uncropped gels and numerical values for all graphs are published in the Source Data file associated with the manuscript. All other data are provided with the manuscript.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Reporting on race, ethnicity, or other socially relevant groupings

Population characteristics

Recruitment

Ethics oversight

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to predetermine sample size. Sample sizes follow established conventions for each experiment type and were chosen to ensure reproducibility while reflecting the large effect sizes observed. For growth assays, lipidomics and activity assays (kinetics, staining and UV- Vis), experiments were conducted in n = 3 independent replicates, with the exception of B. subtilis and E. coli membrane lipidomics that were n = 2 independent replicates. For negative stain TEM micrographs, data were collected with a minimum of 10 independent micrographs, dependent on number of particles within the micrograph (more micrographs were collected for sparsely populated gridholes). For single-particle cryo-EM, no target particle number was predetermined; data were collected to the extent needed to reach high resolution, and final reconstructions used [356,549 / 389,955 / 341,297] particles for the Ndh-Ncp, Ndh-Ncp filament, and Ndh-Ncp NADH/ MK-8 structures respectively, yielding gold-standard resolutions of 2.11–3.09 Å sufficient for de novo model building. Molecular dynamics used three independent ~1 µs replicas, sufficient to assess the movement and association of lipids to the complex.
Data exclusions	Optimisation experiments were conducted for activity assays (including electrochemistry, kinetics, UV-Vis spectra and native PAGE activity stain) and these were not included in the final manuscript but are consistent with the data presented.
Replication	Independent biological replicates were used to verify findings, and in some instances orthogonal experiments were used. For cryo-EM structures, substrates were confirmed to bind Ndh-Ncp through UV-Vis, kinetics and electrochemistry experiments. Experiments were conducted with independent biological replicates of n = 3 or n = 2. Two cryo-EM data collections were carried out on independent samples and three structures were refined from independent half-maps and validated by gold-standard FSC (0.143). The Ndh-Ncp multimer and filament formation was supported by analytical size-exclusion, native PAGE and native PAGE activity assays, mutagenesis, mass photometry and native B. subtilis purification and recombinant E. coli purification. Molecular dynamics simulations were carried out n = 3 times as independent simulations. All replication attempts gave consistent results, and no findings failed to reproduce.
Randomization	Randomisation is not relevant to this study. Microbial strains are derived from single clonal cultures and then grown from this culture into independent replicates, so there are no pre-existing differences between samples to require randomisation.

Blinding

Blinding was not relevant to this study. The experiments are mechanistic and biochemical rather than subjective-outcome assessments, and the measurements are quantitative readouts collected by automated instruments (optical density for growth, absorption for kinetics/UV-Vis, mass spectrometry for lipidomics, detector counts for cryo-EM, detector readouts for electrochemistry and mass photometry, that are not susceptible to observer expectation. Computational pipelines for cryo-EM reconstruction and molecular dynamics applied identical, predefined parameters to all conditions, removing scope for selective analysis.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Plants

Seed stocks

N/A

Novel plant genotypes

N/A

Authentication

N/A