Functional basis of electron transport within photosynthetic complex I

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Photosynthesis and respiration rely upon a proton gradient to produce ATP. In photosynthesis, the Respiratory Complex I homologue, Photosynthetic Complex I (PS-CI) is proposed to couple ferredoxin oxidation and plastoquinone reduction to proton pumping across thylakoid membranes. However, little is known about the PS-CI molecular mechanism and attempts to understand its function have previously been frustrated by its large size and high lability. Here, we overcome these challenges by pushing the limits in sample size and spectroscopic sensitivity, to determine arguably the most important property of any electron transport enzyme - the reduction potentials of its cofactors, in this case the iron-sulphur clusters of PS-CI (N0, N1 and N2), and unambiguously assign them to the structure using double electron-electron resonance. We have thus determined the bioenergetics of the electron transfer relay and provide insight into the mechanism of PS-CI, laying the foundations for understanding of how this important bioenergetic complex functions.

https://doi.org/10.1038/s41467-021-25527-1
The majority of life on earth is dependent on photosynthesis, which uses light energy to generate potential energy in the form of a proton gradient. The transfer of electrons is coupled to the movement of protons across a membrane by a set of exquisitely efficient molecular machines. Of these, the photosystems (PSI and PSII) and the cytochrome b_{6}f complex have been well characterised, but until recently, little information was available about an additional proton pump, photosynthetic complex I (PS-CI, previously known as NDH-1). PS-CI is a key component of cyclic electron flow (CEF) in cyanobacteria and plants. Photosynthetic organisms utilise CEF around photosystem I to increase the transmembrane proton gradient and thus ATP production, to meet the ATP:NADPH ratio required for CO_{2} fixation. In addition to its role in cyanobacteria, it has also been shown that PS-CI is important in many higher plants, including several crops. This is especially the case when ATP demands are high, such as when performing C4 type photosynthesis or sustaining growth under low light or other stresses. In this way, PS-CI is critical to yield in some crops. PS-CI was first identified as a homologue to respiratory complex I (R-CI). Recent cryo-EM structures have confirmed that PS-CI is a large multisubunit membrane protein with L-shaped architecture. It comprises 11 core subunits and seven oxygenic photosynthesis-specific subunits (OPS) which are found in both hydrophobic and hydrophilic domains. The hydrophobic arm has four Mrp (Multiple-resistance-and-pH-like Na^{+}/H^{+} antiporters which likely translocate protons) subunits in R-CI counterparts. Although the core hydrophilic subunits are very similar in structure to their R-CI counterparts, the hydrophilic domain is truncated by three subunits, including the NADH binding domain.

Electron transfer from the donor Fd to the acceptor PQ is indicated by black arrows. The putative movement of protons across (horizontal arrow) and through the membrane domain are indicated by red arrows. Electron transfer from the donor Fd to the acceptor PQ is indicated by black arrows.

**Fig. 1 Structural and functional comparison of PS-CI and R-CI.** a Structure and proposed catalysis of *T. elongatus* PS-CI (Photosynthetic complex I) (PDB: 6HUM). The 11 core subunits are coloured as indicated; the seven oxygenic photosynthesis-specific (OPS) subunits are in green; reactions are shown schematically. Electron transfer from the donor Fd to the acceptor PQ is indicated by black arrows. The putative movement of protons across (horizontal arrow) and through the membrane domain are indicated by red arrows. b Structure and proposed catalysis of *T. thermophilus* R-CI (PDB: 4HEA). The analogous subunits to PS-CI are coloured using the same key as in a; all other subunits are pale blue; reactions are shown as in a. FeS clusters in R-CI are labelled according to their EPR signals. Inset FeS clusters (Fe in red and S in yellow) of PS-CI (black, PDB: 6HUM) superimposed with structurally equivalent clusters in R-CI (Respiratory complex I) (cyan, PDB: 4HEA). Centre-to-centre PS-CI FeS cluster distances are labelled.
reduction potentials\cite{18,25}, preceded structural information by several decades, with pulse EPR later enabling a definitive assignment of the cluster properties to their spatial location in the electron transfer chain\cite{34}. Although the presence of PS-CI was discovered in 1998\cite{33,36}, there is—perhaps surprisingly—no information on the reduction potentials of the electron transfer centres. Without reduction potentials, it is difficult to even formulate a hypothesis on how this molecular machine works. The lack of this fundamental parameter for any electron transfer enzyme may be due to experimental bottlenecks, because EPR-based potentiometric titrations and detailed pulse EPR measurements typically require very large amounts of enzyme. In addition, the high magnetic anisotropy and extensive spin delocalisation of FeS clusters, whose EPR signals all overlap, make them one of the most challenging paramagnetic centres to work with.

Here, we overcome these experimental bottlenecks and not only determine the reduction potentials of the FeS clusters, but also assign their position in the electron transfer chain. We characterise the reduced FeS clusters of PS-CI from two strains of cyanobacteria using a combination of pulsed and continuous wave (CW) EPR spectroscopic methods. We determine the g values for all clusters and the reduction potentials of the two fully reducible clusters. Moreover, we provide a conclusive assignment of thermodynamic properties to structurally defined FeS counterparts, giving insight into the functional mechanism of electron transfer in this crucial enzyme, placing it into the redox map of photosynthesis, and providing an essential foundation for future work on PS-CI.

Results
Identification of three distinct reduced [4Fe-4S] clusters in PS-CI by EPR. To study the FeS clusters of PS-CI, the complex was purified from *Thermosynechococcus elongatus* using a native Histag on Ndhi\cite{14}, and from *Synechocystis* sp PCC6803 with a recombinant His-tag on Ndhi-J. The presence of the subunits was confirmed by proteomics (Supplementary Tables 1, 2). The isolated complexes were reduced using sodium dithionite and suggest the presence of three reduced FeS cluster CW EPR signals (Fig. 2a). Our recently reported high sensitivity EPR setup with a low-noise cryogenic preamplifier\cite{33} was employed to distinguish the overlapping FeS signals by performing different pulsed EPR relaxation filtering experiments\cite{38}. Relaxation filtering selectively recovers the different FeS cluster spectra based on their spin–lattice and spin–spin relaxation times (Supplementary Tables 1, 2 and Note 1). The N2 g values match very well with those for R-CI for both species of cyanobacteria\cite{24,39,40} (Supplementary Tables 3, 4). Consistent with what is observed for R-CI, this FeS signal is observed at relatively high temperatures (20 K) and long relaxation times\cite{38}. Given the structural and spectroscopic similarity between the species and R-CI N2, and in agreement with previous work on *T. elongatus* CI\cite{13}, we assign the N2 EPR signal to the [4Fe-4S] cluster closest to the quinone binding site. Notably, a FeS cluster with these g values and with reduction potential −270 ± 25 mV was previously observed in EPR spectra of chemically reduced thylakoid membranes from two species of *Nostoc*\cite{41}; however, its origin was unknown until now.

Continuous wave and pulsed EPR spectra of PS-CI in both species (Fig. 2a and Supplementary Figs. 1, 2) could be well simulated (red traces) assuming two fully reduced [4Fe-4S] clusters and a third partially reduced (approximately half of the clusters in the enzyme sample giving rise to this third set of EPR signals are reduced at this potential), which possess characteristic g values (Fig. 2a). To decrease confusion with structural or spectroscopic [4Fe-4S] nomenclature for R-CI we

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**Fig. 2** Assignment of the FeS cluster EPR signals to the structure of *T. elongatus* PS-CI using DEER spectroscopy. a Continuous-wave EPR spectra (15 K) of the FeS clusters in sodium dithionite reduced PS-CI (Photosynthetic complex I) of *Synechocystis* (blue) and *T. elongatus* (black), 2 mW, 100 kHz modulation frequency, 7 G modulation amplitude. Simulations of the total (red) and individual N2 (pink), N1 (purple) and NO (grey): *Synechocystis* g values: N2 (g\textsubscript{x,y} = 1.922, g\textsubscript{z} = 2.055), N1 (g\textsubscript{x} = 1.886, g\textsubscript{y} = 1.927, g\textsubscript{z} = 2.043), NO (g\textsubscript{x} = 1.851, g\textsubscript{y} = 1.867, g\textsubscript{z} = 2.079); *T. elongatus* g values: N2 (g\textsubscript{x,y} = 1.922, g\textsubscript{z} = 2.055), N1 (g\textsubscript{x} = 1.907, g\textsubscript{y} = 1.913, g\textsubscript{z} = 2.045), NO (g\textsubscript{x} = 1.852, g\textsubscript{y} = 1.899, g\textsubscript{z} = 2.064); See Supplementary Table 3 for full simulation parameters. b Set up of the pump pulse positions (red) and detection pulse position (black) for the corresponding DEER traces (for full experimental set up see Fig. S3) (10 K). Echo-detected field sweep of *T. elongatus* (black), the sum of simulations (red), N2 (pink), N1 (purple), NO (grey) (N2:N1:NO ratio 1.00:0.92:0.90); see Table S3 for simulation parameters. c, d *T. elongatus* orientation-selective DEER traces for the corresponding pump and probe positions (black) (10 K). The modulation depth is indicated as a scale bar. c Best-fit simulated DEER traces for model A, with the N1 cluster at 26.1 Å from N2 (red). d Best-fit simulated DEER traces for model B, with NO at 26.1 Å from N2 (blue). Schematics of the structural models are shown below. Note that the shorter distances (i.e. dipolar coupling to the middle cluster) do not contribute to the DEER traces; see Supplementary Note 2 for details on the simulation of the DEER traces and models employed. See Fig. S4 for a complete set of the DEER traces and simulations.
refer to the remaining clusters as N1 and N0 for the reduced and partially reduced clusters, respectively (Supplementary Note 1). The g values of the second fully reduced cluster N1 are similar between the two cyanobacterial species. However, in Synechocystis PS-CI N1 exhibits increased broadening, likely due to increased structural variation compared to T. elongatus\(^4^2\). N0 appears to be only partially reduced and its EPR signal is relatively broad. Although the g values of N1 and N0 are broadly consistent with those of other R-CI clusters, they do not match any one cluster well enough to assign them on the basis of homology (Supplementary Tables 3, 4). However, the PS-CI FeS cluster g values are similar between the photosynthetic species despite a wide evolutionary distance\(^4^3\); indicating that any heterogeneity between the complexes does not have a major effect on the FeS characteristics. Deconvolution of the three overlapping FeS signals in PS-CI through relaxation filtering provides unambiguous assignment of their g values.

**Assignment of the [4Fe-4S] cluster EPR signals to the structure of PS-CI using DEER.** To assign the respective PS-CI FeS cluster EPR signals to the clusters within the structure, we used double electron-electron resonance (DEER) spectroscopy (a pulsed EPR experiment that employs two microwave frequencies)\(^4^4\). The dipolar coupling between paramagnetic centres at the ‘pump’ and ‘probe’ microwave frequencies can be measured by analysing the modulation of the DEER spectra—this coupling strength is inversely proportional to the cubic distance between the centres providing structural information about the system (Supplementary Note 2)\(^4^5\). Multiple pump/probe positions that span the entire PS-CI EPR spectrum must be collected to calculate FeS cluster interaction distances (Fig. 2b), as their highly anisotropic nature and the limited bandwidth of microwave pulses results in a partial excitation of the EPR spectrum (orientation selection). The orientation-selective DEER spectra were simulated with a custom programme adapted from one previously developed for R-CI based on a local spin model\(^2^4\) (see Supplementary Note 2 and Fig. 6), taking into account the cluster positions (PDB:6HUM)\(^1^3\) and our experimentally determined g values (Fig. 2c, d). With the position of N2 fixed, there are two possible models: model A, in which N2 and N1 are 26.1 Å apart, and model B in which N2 and the partially reduced cluster N0 are 26.1 Å apart (Supplementary Fig. 4). Only model B provides a good fit at all experimental pump and probe positions, both in terms of modulation frequency and depth. This is especially apparent at field position 9, where N1 does not contribute at the detection pulse position (Fig. 2). We, therefore, assign the N0 signal to the [4Fe-4S] cluster adjacent to the Fd binding site.

**Determination of the reduction potentials of the [4Fe-4S] clusters in PS-CI.** Once the spectroscopic signatures of the clusters were assigned to structural positions, we determined the reduction potentials and therefore energetic favourability of electron transfer to and within PS-CI using small-volume potentiometric redox titrations (Fig. 3)\(^4^6\). The EPR signal intensity of each cluster at each potential was estimated based on the integration of the simulated spectra, given the FeS cluster signals overlap (Fig. 3 and Supplementary Fig. 7). The reduction potentials were estimated to be \(-220\) and \(-230\) mV ± 15 mV vs the standard hydrogen electrode (SHE) for N2 and N1, respectively, based on fitting the experimental data points to the one-electron Nernst equation. These values were consistent between cyanobacterial species. The reduction potentials are thus very similar and within experimental error not only between species but also between the clusters (Fig. 3). The clusters are therefore almost isopotential, meaning that electron transfer to N2 is as favourable as to N1.

The absence of a reduced N0 signal at \(-431\) mV using measurement parameters that maximise N0 (Supplementary Fig. 8) indicates that the reduction potential must be below \(-550\) mV vs SHE. Such a low reduction potential of N0 will result in N2 and N1 being preferentially reduced, preventing the backflow of electrons to form dangerous oxygen radicals in the cytosol. This is particularly important given the unknown contribution of PS-CI to free radical production, a process that initiates multiple defence and developmental signalling cascades in photosynthetic organisms\(^4^7,4^8\). R-CI is a notorious generator of the superoxide radical\(^4^9,5^0\), and it has recently been shown that blocking reverse electron transfer from the quinone site to the terminal flavin moiety prevents ROS production, protecting against cardiac ischaemia-reperfusion injury\(^5^1\). PS-CI lacks this flavin cofactor, but the terminal FeS cluster is so solvent-exposed that reverse electron transfer could also result in considerable free radical production\(^1^3,1^4,2^9\).

**Fig. 3 The reduction potentials of PS-CI N2 and N1.** Small-volume potentiometric titrations of PS-CI (Photosynthetic complex I) from a Synechocystis and b T. elongatus. The fraction of oxidised cluster (N2 or N1) was determined from the integration of the simulated continuous wave EPR spectra (insets, simulations in red) normalised against fully reduced N2 or N1. Data points were fitted with the one-electron Nernst equation using the indicated midpoint potentials (\(E_m\)). The g -2 signal likely originates from the redox mediators required to perform the titration.
Discussion
We were intrigued to find two clusters with equal and relatively positive $E_{m}$ adjacent to each other as electrostatic repulsion would suggest this to be energetically unfavourable. Moreover, our results are contrary to what is observed in R-CI, where with the exception of the $E. coli$ enzyme (and potentially Thermotoga thermophilus where the N2 signal is not observed) cluster N2 has the most positive reduction potential of the EPR-visible Fe-S clusters, with the adjacent cluster remaining oxidised upon NADH reduction. Hence, the two adjacent iso-potential clusters in PS-CI challenges the mechanistic principle of alternating high- and low-potential clusters in electron transfer relays. In PS-CI the redox potentials are very similar between the two species indicating this property is conserved. The iso-potential nature of the clusters suggests that reduction of cluster N2 is unlikely to be involved in the reaction that couples electron transfer and proton translocation, in line with what is known about R-CI. On the other hand, and contrary to R-CI, N2 and N1 in PS-CI may both be electron sinks, thereby facilitating the likely rate-limiting two-electron PQ reduction required for activity, from the one-electron donor Fd.

In the cell, the very negative reduction potential of N0 would limit reduction of PS-CI until the Fd pool is in a highly reduced state (at least 100 times more abundant than PS-CI based on copy per cell estimates), with the close proximity of Fd to N0 providing an electron tunnelling pathway from Fd to N1/N2. It is possible that the potential is altered by the binding of loosely associated subunits (such as NDH-V) as is observed in R-CI, or the substrate itself, as seen in photosystem I. A purified plasmid was used for the transformation of wild type Synechocystis sp. PCC6803, followed by selection on agar-BG11 supplemented with kanamycin at a concentration of 50 µg/ml at 30 °C under continuous illumination of 50 µm2 s-1. Large scale growth (70 L of culture) and purification were performed as described. Cells were resuspended in 20 mM Li2SO4, 20 mM Na2HPO4, 5 mM KCl, 0.5 mM MgCl2, 10 mM CaCl2, 25% glycerol (hereafter BB) with a final concentration of 1% (w/v) n-Dodecyl β-D-maltoside. The suspension was centrifuged at 50,000 x g for 30 min at 4 °C. The supernatant was passed down a 25 mL Ni-NTA column equilibrated in BB (+10 mM imidazole, 0.03% DDM). PS-CI was eluted in BB supplemented with 200 mM imidazole and desalted by PD-10 column prior to EPR sample preparation. PS-CI was purified from Thermosynechococcus elongatus as previously described using a native His-tag and Ni-NTA resin.

Methods
Purification of photosynthetic complex I. PS-CI was purified from Synechocystis sp. pcc 6803 by introducing a His-tag to the Ndh-J subunit. A synthetic DNA sequence corresponding to a fragment of the Synechocystis sp. PCC6803 genome stretching 400 b.p. both upstream and downstream of the Ndh-J gene was synthesised (GENEWIZ, Leipzig, Germany) (Supplementary Fig. 9). The additional sequence was incorporated, encoding a 6X His-tag in the frame at the C-terminal end of the gene for the Ndh-J protein, connected to the protein by a Factor Xa site. This was followed by both rrev T1 terminator and 1778E terminator sequence. The sequence was inserted into pEX-K4, and a fragment containing the KanR gene for kanamycin resistance sub-cloned onto the Stul and SacI restriction cloning sites downstream, but anticoding, to the Ndh-J gene. All enzymes for molecular biology were from New England Biolabs (Ipswich, MA, USA). A purified plasmid was used for the transformation of wild type Synechocystis sp. PCC6803, followed by selection on agar-BG11 supplemented with kanamycin at a concentration of 50 µg/ml at 30 °C under continuous illumination of 50 µm2 s-1. Large scale growth (70 L of culture) and purification were performed as described. Cells were resuspended in 20 mM Li2SO4, 20 mM Na2HPO4, 5 mM KCl, 0.5 mM MgCl2, 10 mM CaCl2, 25% glycerol (hereafter BB) with a final concentration of 1% (w/v) n-Dodecyl β-D-maltoside. The suspension was centrifuged at 50,000 x g for 30 min at 4 °C. The supernatant was passed down a 25 mL Ni-NTA column equilibrated in BB (+10 mM imidazole, 0.03% DDM). PS-CI was eluted in BB supplemented with 200 mM imidazole and desalted by PD-10 column prior to EPR sample preparation. PS-CI was purified from Thermosynechococcus elongatus as previously described using a native His-tag and Ni-NTA resin.

Liquid chromatography-mass spectrometry. In-gel digestion was carried out using sequencing-grade trypsin (Promega) according to standard protocols, without reduction and carboxymethylation of cysteines. Chromatographic separation of peptides was performed using an Ultimate 3000 RSLC Nano system (Dionex, part of Thermo Fisher Scientific). The sample (3 µL) was loaded on a trapping column (C18 PepMap 100, 300 µm x 5 mm, 5 µm particle size, 100 Å pore size; Thermo Scientific) and desalted for 5 min using 2.5% acetonitrile/0.05% tri-fluoroacetic acid in ultrapure water at a flow rate of 10 µL/min. Then the trap column was switched in line with the separation column (Acclaim PepMap100 C18, 75 µm x 50 cm, 2 µm particle size, 100 Å pore size, Thermo Scientific). The mobile phase for peptide elution consisted of 0.1% (v/v) formic acid in ultrapure water (A) and 80% acetonitrile/0.1% (v/v) formic acid in ultrapure water (B). Peptides were eluted at a flow rate of 300 nL/min with the following gradient profile: 2.5% B over 5 min, 2.5–45% B over 40 min, 45–99% B over 5 min and 99% B over 20 min. Afterwards the column was re-equilibrated with 2.5% B for 45 min. The LC system was coupled via a nanospray source to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). MS full scans (m/z 300–1600) were acquired in positive ion mode by FT-MS in the Orbitrap at a resolution of 70,000 (FWHM) with internal lock mass calibration on m/z 445.12003. The 12 most intense ions were fragmented with 27% normalised collision energy at a resolution of 17,500. Maximum injection time of 50 ms. Automatic gain control (AGC) was enabled with target values of 3 × 106 and 5 × 104 for MS full scans and MS/MS, respectively. One microscan was acquired per MS/MS spectrum and the maximum fill time was 55 ms. Dynamic exclusion was enabled with an exclusion duration of 30 s and an exclusion mass width of ± 5 ppm. Ions with unassigned charge states, singly charged ions as well as ions with charge state >6 were rejected. For peptide and protein identification spectra files (RAW-files) were searched using MaxQuant (version 1.6.14.0) with default parameters for non-

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CW EPR spectroscopy. EPR measurements were performed using an X-band Bruker Elexys E580 Spectrometer (Bruker BioSpinGmbH, Germany) equipped with a closed-cycle cryostat (Cryogenic Ltd, UK) using Xper software. All T. elongatus and Synechocystis samples were measured in an X-band split-ring resonator (ER 4118X-MS2). The field was calibrated using a DPPH standard (Bruker). The Synechocystis fully reduced sample was measured using an ER 4118XM5D5 resonator. Baseline spectra from samples containing only buffer or an electrochemical glass cell equipped with a 4 cM water bath. Once equilibrated under nitrogen while stirring, 30 µM of the redox mediators methylene blue, indigotrisulfonate, indigodisulfonate, anthraquinone-2-sulfonate, benzyl viologen and methyl viologen (Sigma Aldrich) were added. The potential was measured using an Ag/AgCl mini-reference electrode (DRI-REF-2, World Precision Instruments) and a platinum working electrode (Scientific Glassblowing Service, University of Southampton; Pt from Goodfellow) and connected to an EmSTAT3 + potentiostat (PalmSens). Samples (≤10 µL) were transferred to a 1.6 mm OD Suprasil quartz EPR tubes (Goss Scientific) at the indicated potentials and flash-frozen in ethanol cooled from outside the glovebox by a dry ice aceton bath, before being transferred to liquid nitrogen. All reduction potentials were given relative to the potential of the SHE. The reference electrode potential was determined to be +201 mV vs. SHE using quinhydrone (Sigma Aldrich) as an external standard.

Simulation of EPR spectra and analysis. CW and EDFS data were analysed and simulated with EasySpin esitt using Monte Carlo simulation in Matlab. All spectra, which were consistent amongst different protein batches, were simulated using the parameters reported in Supplementary Table 3. ‘Nernst plots’ (Fig. 3) were fitted using the integrated area of the simulated signals (due to EPR signal overlap), plotted against the reduction potential of the samples, normalized to the maximum intensity signal resulting from the fully reduced sample. The one-electron Nernst equation was fitted to the experimental data points using the Matlab curve-fitting toolbox. The mid-point reduction potential error was calculated based on the 95% confidence intervals for the regression over the linear section of the Nernst curve. For details of DEER, trace simulations see Supplementary Note 2 and the associated supplementary figures and tables.

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Acknowledgements
K.H.R. thanks the London Interdisciplinary Doctoral Programme for a studentship. The EPSRC (EP/P510841/1) seed funding to M.M.R and G.T.H as part of global challenges award. EP/J031425/1 to the Centre for Pulse EPR at Imperial College, EP/L011972/1 to the Centre for Advanced ESR at the University of Oxford and EP/P510270/1 to M.S., BBRSC (BB/R004838/1) to G.T.H), John Fell Fund (0007019 to W.K.M.), DFG priority programme 2002 (NO 836/4-1 to M.M.N.) and Leverhulme Trust (RPG-2018-183 to M.M.R.) are gratefully acknowledged for funding.

Author contributions
K.H.R. performed all data analysis and research except those specified below. G.T.H., M.M.R. and K.H.R. designed the research with assistance from J.J.W. and G.M. G.T.H. and M.M.R. directed the research. K.H.R. and J.T. purified PS-CI from T. elongatus cells grown by J.T. and M.M.N. A.M.E. cloned the NdhJ-His Synechocystis sp pcc 6803 strain. W.K.M. performed DEER experiments 1–5. M.H. performed the mass-spectrometry on PS-CI purified from Synechocystis sp pcc 6803. K.H.R. and M.S. measured DEER traces 6–9 and the relaxation filtered EPR using the HEMT probe designed by M.S. and J.J.L.M. K.H.R. M.M.R. and G.T.H. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-25527-1.

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Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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