Purified F-ATP synthase forms a Ca\(^{2+}\)-dependent high-conductance channel matching the mitochondrial permeability transition pore

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The molecular identity of the mitochondrial megachannel (MMC)/permeability transition pore (PTP), a key effector of cell death, remains controversial. By combining highly purified, fully active bovine F-ATP synthase with preformed liposomes we show that Ca\(^{2+}\) dissipates the H\(^+\) gradient generated by ATP hydrolysis. After incorporation of the same preparation into planar lipid bilayers Ca\(^{2+}\) elicits currents matching those of the MMC/PTP. Currents were fully reversible, were stabilized by benzodiazepine 423, a ligand of the OSCP subunit of F-ATP synthase that activates the MMC/PTP, and were inhibited by Mg\(^{2+}\) and adenine nucleotides, which also inhibit the PTP. Channel activity was insensitive to inhibitors of the adenine nucleotide translocase (ANT) and of the voltage-dependent anion channel (VDAC). Native gel-purified oligomers and dimers, but not monomers, gave rise to channel activity. These findings resolve the long-standing mystery of the MMC/PTP and demonstrate that Ca\(^{2+}\) can transform the energy-conserving F-ATP synthase into an energy-dissipating device.
The permeability transition (PT) is a Ca\(^{2+}\)-dependent permeability increase of the mitochondrial inner membrane leading to depolarization and cessation of ATP synthesis. The cutoff of about 1500 Da allows inner membrane permeabilization to ions and solutes with matrix swelling. Known since the early days of research on isolated organelles, the molecular basis of the PT has remained a mystery for >60 years\(^1\). The PT is mediated by opening of a channel, the permeability transition pore (PTP), also called mitochondrial megachannel\(^2\) (MMC) or multiconductance channel\(^3\) because of its electrophysiological properties\(^4\). Following the observation that the PTP activator cyclophilin (CyP) D interacts with the F-ATP synthase in a cyclosporin (Cs)A-sensitive manner\(^5\) (which matches MMC/PTP activation by CyP/D and desensitization by CsA), the possibility that F-ATP synthase generates channels has been investigated with encouraging results\(^6\)-\(^12\). Given that no obvious structural features would predict that the F-ATP synthase can form channels\(^13\)-\(^28\) and that a tightly coupled F-ATP synthase is essential to power ATP generation\(^2\), this hypothesis may appear unlikely. However, recent cryo-EM studies have revealed a far more complex structural organization than anticipated both for the yeast dimeric Fo domain\(^18\) and for the porcine F-ATP synthase tetramer\(^20\). Furthermore, F-ATP synthases from a variety of sources catalyze hydrolysis of Ca\(^{2+}\)-ATP\(^{22,25}\); yet no proton gradient forms during ATP hydrolysis\(^22,24,25\), a finding that is compatible with Ca\(^{2+}\)-dependent opening of a dissipative pathway. In previous studies a solid link was established between F-ATP synthase and formation of the MMC/PTP through detection of currents upon reconstitution into planar bilayers of F-ATP synthase extracted from mitochondria or from native gels\(^7\)-\(^10\). In spite of extensive pharmacological evidence pointing at the F-ATP synthase as being responsible for current formation, the conclusions of these studies are limited by the presence of additional proteins. To overcome these limitations we have used two complementary strategies. In the first, we have produced selective mutants of F-ATP synthase and assessed the consequences of the mutations on Ca\(^{2+}\)-sensitivity, inhibition by H\(^{+}\) and modulation by specific reagents of the MMC/PTP\(^11,12,26-28\). In the second strategy, the results of which are described in the present manuscript, we have employed highly pure and stable F-ATP synthase from large-scale preparations from bovine hearts (Supplementary Fig. 1). These preparations, which displayed oligomycin-sensitive ATPase activity and catalyzed H\(^{+}\) translocation after incorporation into preformed liposomes, were used to address the question of whether F-ATP synthase can give rise to bona fide channels after incorporation into planar lipid bilayers, and whether these channels match the known features of the MMC/PTP.

**Results**

**Characterization of bovine F-ATP synthase.** F-ATP synthase was purified from bovine heart mitochondria by a combination of sucrose density gradient centrifugation and ion-exchange chromatography employing the mild, high-affinity detergent laurylmaltose-neopentyl glycol (LMNG)\(^15,29,30\). Clear-native gel electrophoresis documented the presence of monomeric, dimeric and oligomeric forms of the enzyme complex (Fig. 1a), which were also visualized by negative stain EM (Fig. 1b). Denaturing sodium dodecyl sulfate (SDS) gel electrophoresis (Fig. 1c) and mass spectrometry (Supplementary Tables 1 and 2) allowed detection of all the expected subunits, including the labile 6:8PL and DAPIT. The preparation contained very low amounts of contaminating proteins (Supplementary Table 2; see Data Availability for access to mass spectrometry proteomics data) and catalyzed oligomycin-sensitive ATP hydrolysis (Fig. 1d, e) with the typical concentration-dependence on ATP and Mg\(^{2+}\) (Supplementary Fig. 3a, b). In these measurements we used the standard assay based on coupled enzymatic reactions, which cannot be utilized to study Ca\(^{2+}\)-ATPase activity because of the Mg\(^{2+}\) requirement of pyruvate kinase (Supplementary Fig. 3c, d). With a method based on direct detection of Pi release it became clear that the bovine F-ATP synthase was also capable to hydrolyze Ca\(^{2+}\)-ATP (Supplementary Fig. 4). The total amount of phospholipids per F-ATP synthase monomer in the Foros column fractions relevant to this study were 150 ± 54 (n = 8). Of these, 57 ± 5% was phosphatidylethanolamine, 16 ± 2% was phosphatidylcholine and 21 ± 6% was cardiolipin. The latter value compares quite well with the in situ cardiolipin content of the inner mitochondrial membrane of about 18%\(^31\).

This preparation was then incubated with preformed liposomes, a procedure that yielded high density of incorporation, as detected by both cryo-EM (Fig. 1f) and negative stain EM (Fig. 1, compare g and h). Morphometric analysis of (proteo)liposomes revealed a clear preference for incorporation into smaller liposomes, with a distribution centered at a diameter of about 40 nm (Fig. 1i, j). Re-solubilization of the F-ATP synthase proteoliposomes with digitonin and subsequent analysis by BN-PAGE indicates the presence of monomeric, dimeric and oligomeric F-ATP synthase (Supplementary Fig. 5). After reconstitution into proteoliposomes F-ATP synthase was catalytically active and sensitive to oligomycin (Fig. 2a); and upon addition of 2.5 mM ATP it catalyzed H\(^{+}\) translocation with the establishment of a H\(^{+}\) gradient, as assessed by 9-amino-6-chloro-2-methoxyacridine (ACMA) quenching (Fig. 2b, c). The H\(^{+}\) gradient was stable and could be rapidly dissipated by the addition of nigericin (Fig. 2c). Given that Mg\(^{2+}\) and adenine nucleotides are effective inhibitors of the PTP, we also performed these experiments with 0.125 mM Mg\(^{2+}\)-ATP for testing the effect of ATP inducers after proton pumping. Addition of Ca\(^{2+}\)-alone slightly perturbed the H\(^{+}\) gradient (Fig. 2d), while the PTP agonists benzodiazepine (Bz)-423 and phenylarsine oxide (PhAsO) caused a slow, partial release of the H\(^{+}\) gradient (Fig. 2e), which is probably due to inhibition of F-ATP synthase by Bz-423\(^32\). Bz-423 is a small organic compound (MW 441 Da) that was discovered in a screening of molecules able to selectively kill autoreactive B lymphocytes\(^33\) and later shown to selectively bind F-ATP synthase in a phage display assay\(^32\). Since Bz-423 binds F-ATP synthase subunit OSCP\(^34\) at the same site as CyP/D, resulting in MMC/PTP activation\(^7\), in this study we have used Bz-423 rather than CyP/D as the standard agonist because of its availability, stability and lack of interference with the experimental set-up. PhAsO is a dithiol cross-linker that mimicks the effect of oxidants\(^35\) and has been extensively characterized in studies of the PT in mitochondria\(^1\). When 2 mM Ca\(^{2+}\)-was also added to the proteoliposomes, together with Bz-423 and PhAsO, immediate and complete collapse of the H\(^{+}\) gradient was observed (Fig. 2f). Thus, when treated with well-characterized MMC/PTP inducers in the presence of Ca\(^{2+}\)-, F-ATP synthase proteoliposomes energized via ATP hydrolysis-powered acidification undergo full permeabilization, suggestive of MMC/PTP opening.

**Channel formation by F-ATP synthase.** Next, we tested whether F-ATP synthase forms channels after fusion of the proteoliposomes into planar lipid bilayer membranes. Since Ca\(^{2+}\)- alone is sufficient to elicit MMC activity in patch clamp experiments in mitoplasts\(^4\), we firstly addressed the key question of whether Ca\(^{2+}\)- is necessary and sufficient to trigger channel activity by F-ATP synthase. Proteoliposomes were added to the cis chamber, followed by a period of up to 5 min of recording, during which no
currents were seen. In the experiment reported here, 0.1 mM Ca\(^{2+}\) was then added to the cis side, and a small-conductance channel activity was detected, the amplitude of which increased as the Ca\(^{2+}\) concentration was raised to 1 mM (Fig. 3a). Addition of 1 mM EGTA abolished channel openings, which were restored by the subsequent addition of Ca\(^{2+}\) in excess of EGTA, similarly to MMC measured in the native IMM\(^{36}\). It should be noted that we did not apply an osmotic gradient, which promotes fusion of proteoliposomes to the planar lipid bilayer\(^{37}\); and that liposomes with diameter lower than 100 nm have a low probability of fusion\(^{38}\). Thus, in spite of the large amount of F-ATP synthase incorporated into the liposomes, in the electrophysiological

Fig. 1 Characterization of bovine F-ATP synthase. a Clear-native gel electrophoresis indicates the presence of monomeric, dimeric, and oligomeric bovine F-ATP synthase, and the absence of subcomplexes or other smaller protein complexes. Lane M: molecular weight markers (kDa); Lane 1: F-ATP synthase (30 \(\mu\)g). b Negative stain EM documents the presence of bovine F-ATP synthase monomers (blue rectangle), dimers (red circle), and tetramers (yellow rectangle). Scale bar 50 nm. c Subunit composition was determined by denaturing SDS gel electrophoresis. All expected subunits were detected, including the very weakly associated 6.8PL and DAPIT, see also data from mass spectrometry (Supplementary Table 1). Lane M: molecular weight markers (kDa); Lane 1: F-ATP synthase (30 \(\mu\)g). d, e NADH oxidation-coupled enzymatic assay showing that soluble F-ATP synthase (bFOF1 Sol, 10 \(\mu\)g added where indicated) has ATPase activity (addition of ATP 2.5 mM where indicated) that can be fully inhibited by oligomycin A (2 \(\mu\)M where indicated). Traces are representative of three independent experiments. f Cryo-EM image of a bovine F-ATP synthase proteoliposome. The clearly visible tram-track features (red double arrow) confirm the formation of lipid bilayers, and F\(_1\) domains protruding out of the membrane are easy to identify at the edge of the proteoliposome (white arrowheads). Scale bar 50 nm. g Negative stain EM of liposomes before and h after reconstitution of bovine F-ATP synthase. After reconstitution, F\(_1\) domains (open triangles) can be clearly distinguished from naked liposomes (closed triangles). Scale bar, 50 nm. i-j Nine-hundred and ninety-nine liposomes from 19 electron microscopy images taken after incorporation of F-ATP synthase were scored for the presence of F\(_1\) and their size measured with the aid of ImageJ software. Source data for panels d, e, i, j are provided as a Source Data file.
recordings we observed few events, which is a desirable feature if single-channel activity is to be assessed. To further explore the frequency of fusion events, we also used proteoliposomes of the same size, composition, and lipid-to-protein ratio where gramicidin D (which forms channels with high efficiency) had been incorporated with the same protocol used for the F-ATP synthase. Although the two proteins obviously differ substantially, also in this case activity of well-resolved single channels could be detected (Supplementary Fig. 6). Overall, with F-ATP synthase proteoliposomes we observed channel activity in 13 out of 16 experiments (81%) and with gramicidin D in 6 out of 8 (75%).

Given that in these protocols the majority of F-ATP synthase molecules appears to remain in proteoliposomes not fused to the planar membrane, in subsequent electrophysiological experiments we have mostly added the purified, LMNG-stabilized preparation directly to the cis chamber, a procedure that yielded channel activity in 103 out of 131 experiments (79%).

In addition to Ca\(^{2+}\), a variety of additional inducers, such as Bz-423 and PhAsO are required for PTP opening and for detection of high-conductance channels in gel-purified preparations\(^7,9,10\). After confirming ohmic resistance of the lipid bilayer, we added 3 mM Ca\(^{2+}\), 0.1 mM PhAsO, and 0.2 mM Bz-423, conditions that elicited complex channel activity with a maximum conductance of about 1.3 nS and multiple conductance substates (Fig. 3b and Supplementary Fig. 7). As already mentioned, these substates are typical of the channel in the native membrane\(^2,3\), and are consistent with the alternative denomination of the MMC as mitochondrial multiconductance channel\(^3\). Activity could also be
elicited within about 3 min of the addition of Ca\(^{2+}\) in the presence of Bz-423 only (Supplementary Fig. 8). Channel activity was inhibited by the well-known MMC/PTP inhibitors Ba\(^{2+}\) (Fig. 3c and Supplementary Fig. 9a) and Mg\(^{2+}\)-ADP (Fig. 3d and Supplementary Fig. 9b) as well as by GdCl\(_3\) (Fig. 3e and Supplementary Fig. 9c), a general cation channel blocker able to inhibit MMC/PTP in mitoplasts\(^ {12}\).

We next analyzed the current–voltage relationship of the reconstituted F-ATP synthase channels. Bz-423 and Ca\(^{2+}\) were added; after a stable activity had been reached, currents were recorded at \(V_{\text{cis}}\) ranging from \(-80\) to \(+20\) mV. An essentially linear current–voltage relationship was observed (Fig. 4a) with an increased propensity of the channels for open states at more depolarized voltages (Supplementary Fig. 10). To test whether F-ATP synthase insertion in the planar membrane occurred with a preferential orientation, we added it to the cis side while Ca\(^{2+}\) (up to 3 mM) and 0.15 mM Bz-423 were added to the trans side. No channel activity was detected, while the subsequent addition of the same agonists to the cis side readily elicited channel activity (Fig. 4b). This set of findings indicates that the F-ATP synthase inserts into the membrane with its hydrophobic F\(_0\) domain, leaving the large hydrophilic F\(_1\)-sector accessible from the cis side as observed in the proteoliposomes (Fig. 1f, h). We also examined the electrophysiological behavior of samples cut out from BN-PAGE. As was the case for proteins eluted from gels after separation of total mitochondrial extracts\(^ {7}\), currents were detectable after insertion of proteins extracted from dimer and oligomer, but not monomer bands (Fig. 4c). Maximal conductance of channels formed by dimers and oligomers was not significantly different (Supplementary Fig. 11). Channel activity was observed in 9 out of 10 experiments for oligomers (90%), 8 out of 9 for dimers (89%), and 0 out of 7 (0%) for monomers.

The ANT and VDAC have long been suspected to participate in MMC/PTP formation, also based on their channel-forming activity assessed by electrophysiology\(^ {39-41}\). Given that our preparation contains low amounts of both proteins (Supplementary Table 2) we tested the effect of bongkrekate (BKA) (Supplementary Fig. 12a) and of König’s polyanion (Supplementary Fig. 12b), the selective inhibitors of ANT\(^ {42}\) and of VDAC\(^ {40}\), respectively. No inhibition was observed in either case, demonstrating that neither ANT nor VDAC contributes to MMC/PTP-like activity of membrane-reconstituted F-ATP synthase.

We also tested if Bz-423 is capable of stabilizing the Ca\(^{2+}\)-activated channel in its full-conductance state. Reliable single-channel current analysis is negatively affected by flickering and by the presence of multiple current levels, typical MMC behaviors...
that are both observed in our recordings. To overcome this potential problem, we measured the power spectrum area (PSA), which provides a generally applicable estimate of channel activity based on the integral of the power spectrum over the entire frequency range of the acquired current signal. We developed an algorithm (Supplementary Tables 3 and 4) that allows to calculate the corresponding PSA, open probability (Po), and conductance. These experiments revealed that Bz-423 was not able to trigger channel opening per se, yet it allowed channels to reach a conductance matching that of PTP/MMC when Ca²⁺ added to the cis side of the planar lipid bilayers. Channel activity was assessed in the presence of Ca²⁺ up to 3 mM and 0.15 mM Bz-423 added to the cis side. Both dimers (n = 8) and tetramers (n = 9) elicited channel activity while monomers elicited no activity (n = 7). Representative traces are shown at V_cis = −60 mV. The closed state is denoted with C, the most frequent subconductance state with an asterisk, and the maximal open state with O. The source data underlying panel a are provided as a Source Data file.

**Discussion**

In this manuscript, we have provided solid evidence that, after treatment with Ca²⁺, highly purified bovine F-ATP synthase can give rise to high-conductance channels. Channel openings are stabilized by MMC/PTP agonists and inhibited by MMC/PTP inhibitors. These results are consistent with previous electrophysiological work⁷⁻¹⁰, but represent a key step forward because of the purity, completeness and intactness of the bovine F-ATP synthase complexes. Of specific importance is the demonstration that the preparation displays oligomycin-sensitive catalytic activity and H⁺ pumping activity after reconstitution into liposomes, and that the bulk proton gradient is fully dissipated in all proteoliposomes by the addition of Ca²⁺ and PTP agonists, which rules out a minor contaminating protein as the agent of dissipation. The electrophysiological experiments indicate that permeabilization is due to opening of high-conductance channels that possess all the properties of the MMC/PTP, including (i) full reversibility of Ca²⁺-dependent channel openings, (ii) sensitivity to well-characterized MMC/PTP activators and inhibitors in lipid bilayers, and (iii) conductance values and presence of substates of the measured channels.¹⁴ Unique findings of the present work are the characterization of the single-channel activity elicited by Ca²⁺ alone and the definition of the minimum Ca²⁺ concentration required to trigger channel activity in vitro. Of note, no effects were elicited when the protein was added to the cis side and agonists to the trans side. Thus, the F-ATP synthase inserted in the hydrophobic F₉ sector facing the cis compartment; and the effect of agonists cannot be ascribed to unspecific effects on the lipid bilayer. Based on these results we see very little room for doubt that the permeabilization process in the liposomes and the currents measured in our experimental set-up are elicited by the bovine F-ATP synthase. We therefore conclude that F-ATP synthase is responsible for the formation of the MMC/PTP, as also strongly supported by our recent site-specific mutagenesis studies.¹¹⁻¹³,¹⁶⁻²⁸

Native gel-eluted dimers and tetramers, but not monomers gave rise to currents after direct addition of the eluate to the cis side of the planar bilayer. The importance of this experiment lies in the fact that it further rules out the possibility that an unknown and difficult-to-detect contaminating protein may be...
responsible for the recorded channel activity. Indeed, a putative contaminating channel of a size similar to F-ATP synthase might have co-migrated with the dimer or with the oligomer, but not with both; and a contaminating channel with high affinity for F-ATP synthase should have co-migrated with the monomer as well. Hence, the possibility that a protein different from F-ATP synthase is the underlying cause of the recorded currents becomes negligible. The absence of channel activity for the gel-eluted monomer and the equivalence of channel activity for the gel-eluted dimer and oligomer might suggest that the minimal channel-forming unit is the F-ATP synthase dimer. There are, however, several caveats to this interpretation. For example, the gel elution process might have stripped off factors essential for channel formation (such as structural lipids) from the monomers but not from the dimers and oligomers; or dimers reconstituted into the bilayer might have self-assembled into oligomers before the actual channel formation. Indeed, self-oligomerization of purified F-ATP synthase after lipid bilayer reconstitution has already been demonstrated for the yeast dimer by combining the mild GREcon membrane reconstitution approach with cryo-electron tomography of the resulting proteoliposomes. Finally, channel formation itself might change the oligomeric state via monomerization. Thus, since we did not detect the oligomeric state and electric currents in the same experiment, the conformation of F-ATP synthase during PTP formation remains an open question.

Oligomycin sensitivity and acidification of the internal volume indicate that close to all F-ATP synthase complexes incorporated in the liposomes exhibit their core function, i.e., coupled ATP hydrolysis and H⁺ pumping; and characterization of the proteoliposomes clearly shows insertion of many F-ATP synthase complexes per liposome. Therefore, the detection of single-channel activity is surprising. A possible explanation is that only very few of the liposome-reconstituted F-ATP synthase complexes are MMC/PTP-competent, e.g., as a consequence of conformational heterogeneity. Alternatively, several complexes might participate in the measured currents with each single complex having only a low open probability, thus contributing to a few or even a single event during the total recording time. Irrespective of the underlying mechanism, however, it should be noted that the single-channel recordings reported here match the observation of MMC activity of patched mitoplasts, which should contain a high density of F-ATP synthase oligomers. Yet, single-channel activity can be observed also in the native membrane.

PTP formation has never been reported in prokaryotes. Mammalian oligomeric F-ATP synthase possesses several transmembrane subunits, which do not have homologs in the strictly monomeric chloroplast or bacterial F-ATP synthases, namely e, f, g, DAPIT, 6.8PL, and the newly found k subunit. Therefore,
these are good candidates for playing a role in MMC/PTP formation. The recently published single particle cryo-EM structure of the porcine F$_1$F$_0$-binder tetramer is the first structure of a mammalian F-ATP synthase where it is safe to assume that no subunits have been lost during the process of structure determination. The structure revealed an intricate system of interactions between the monomers in the membrane-spanning F$_0$ domains, where the g-g and e-e interactions stand out as central. An unexpected finding of the structure is that the lumen of the c-ring seems to be filled by protein, putatively assigned to 6.8PL. This subunit makes a clear contact with the c-terminus of the ring seems to be questioned in recent studies of cells where the genes encoding for subunits c, b, and OSCP are deleted. The structure revealed an intricate system of interactions between the monomers in the membrane-spanning F$_0$ domains, and in our reading the most recent study supports the conclusion that the PTP does originate and is of limited extent, consistent with the lack of a bona fide interaction with other proteins.

Formation of the MMC/PTP from F-ATP synthase has been questioned in recent studies of cells where the genes encoding for subunits c, b, and OSCP, and f, e, DAPT, and 6.8PL have been deleted. The key argument is that the PT persists in the absence of F-ATP synthase, given that deletion of any of these subunits prevents assembly of functional F-ATP synthase and of the respiratory chain. We have addressed in detail the reasons why we think that the experiments of the first two publications are not conclusive, and in reading the most recent study, supports the conclusion that the PTP does originate from F-ATP synthase. Indeed, in wild-type cells Ca$^2^+$-induced swelling in sucrose is immediate and complete in about 30 s, while mitochondrial swelling in cells lacking F-ATP synthase starts after a lag of about 30 s, proceeds at a very slow rate and is of limited extent, consistent with the lack of a bona fide PTP. We suspect that residual swelling activity and occurrence of Ca$^2^+$-dependent Ca$^2^+$ release in these cells is due to opening of the ANT channel, as suggested by its sensitivity to the selective ANT inhibitor BKA, which does not directly inhibit the MMC/PTP nor the activity of the reconstituted F-ATP synthase channel (this manuscript).

While the main characteristics of the channel activity described here and those of MMC are in good agreement, differences also exist like the minimum Ca$^2^+$ concentration needed to activate the MMC/PTP to full conductance, which is lower in mitoplasts from rat liver mitochondria. This in turn suggests that additional factors—such as membrane curvature, lipid composition and interaction with other proteins—may contribute to regulation of the pore in intact, native membranes. Understanding the actual mechanism of pore formation will have to await experiments that are able to simultaneously detect the oligomeric state and channel opening, and the structural description of the open and closed channel states, and at the atomic level.

**Methods**

**Purification of F-ATP synthase from bovine heart.** Purification of the laurylmaltose-neopentyl glycol (LMNG) stabilized F-ATP synthase complex was conducted as previously described. Briefly, fresh bovine hearts were obtained by hydrating the lipid monolayer with 1 ml of buffered solution (150 mM KCl, 10 mM Hepes, pH 7.0, containing 660 mM sucrose, adjusting the protein concentration to ~23 mg/ml. The suspension was kept in 40 ml HEPES pH 7.8, 2 mM MgCl$_2$, 0.1 mM EDTA, and 0.1 mM DTT and solubilized on ice via addition of deoxycholate and decylmaltoside to final concentrations of 0.3% (wt/vol) and 0.4% (wt/vol), respectively. Subsequently, the suspension was centrifuged at 176,000 × g for 50 min. The supernatant applied to the superstep step gradient of 0.1 M HEPES pH 7.8, 0.1 mM EDTA, 0.2% wt/vol decylmaltoside and 2.0 M, 1.1 M, 0.9 M sucrose) and centrifuged at 176,000 × g for 15.5 h. Fractions exhibiting ATPase activity determined by an ATP-regenerating enzyme-coupled assay were then loaded onto a Poros 20QH ion-exchange column. The detector was exchanged to LMNG using a double gradient from 0.2% to 0% decylmaltoside and 0%–0.05% LMNG for 80 min at 1 ml/min. Complexes were eluted by a linear concentration gradient of 0–240 mM KCl in 40 mM HEPES pH 7.8, 150 mM sucrose, 2 mM MgCl$_2$, 0.1 mM EDTA, 0.1 mM DTT, and 0.05% (wt/vol) LMNG. Shortly after elution, F-ATP synthase fractions containing high amounts of native phospholipids were determined by anion exchange chromatography were flash-frozen in aliquots of about 500 µl for later use.

**Lipid analysis of the purified F-ATP synthase preparation.** Conditions for the extraction of bovine F-ATP synthase were optimized to retain native phospholipids including cardiolipin. To this end, the total amount of phospholipids and relative amount of lipid species were monitored during purification as previously described. The total amount of phospholipid content in the preparation was determined by absorbance spectrometry (V–630 BIO spectrometer) at 600 nm using the phospholipid C–TestWako kit from Wako Fine Chemicals. The relative amount of native cardiolipin (CL), phosphoethanolamine (PE), and phosphocholine (PC) was analyzed by thin-layer chromatography. To extract the phospholipids, chloroform, methanol, and water were used at 6:3:2.5:0.2 and then at 75:22:3:0.1 ratios. The developed plate was completely dried and stained with 0.005% Primuline in 80% acetone. After air-drying, spots were scratched out and heated with perchloric acid at 155 °C for 3 days to digest the extracted lipids completely. A 0.22 ammnnion molybdate solution was added as Fiske-Subbarow reagent and mixed with digested lipids, and the mixture was kept at 100 °C without color change. Phosphorus content was determined by absorbance spectrometry.

**Gel electrophoresis and protein elution from gels.** To confirm the subunit composition and intactness of the bovine F-ATP synthase after freezing and thawing for transport or for preparation of proteoliposomes, aliquots were subjected to denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing clear-native PAGE. For the experiments of Fig. 4c, 0.4 mg of purified F-ATP synthase were separated by BN-PAGE; monomers, dimers, and oligomers were identified, cut and eluted as described. Briefly, bands were excised, digested with 25 µM tricine, 7.5 mM Bis-Tris, 1% (w/v) n-heptyl β-D-thioglucopyranoside, pH 7.0, and supplemented with 8 mM ATP-Tris and NADH. After overnight incubation at 4 °C, the precipitate was centrifuged at 20,000 × g for 10 min at 4 °C and supernatants were used directly for reconstitution in electrophysiologial studies.

**Negative staining electron microscopy.** To remove excess free LMNG an aliquot of 100 µl of purified bovine F-ATP synthase was subjected to a GraDeR run as described. Subsequently a 2.5 µl aliquot was applied onto freshly glow-discharged, carbon-coated 400 mesh copper grids (Veco). After blotting (Whatman #1), the samples were stained by using a 2% uranyl acetate solution and air-dried. Images were taken with a JEM1010 transmission electron microscope (JEOL) equipped with a 4 × 4K Tietz CMOS TemCamF416 (TVIPS, Gauting, Germany) at 100 kV and 20 pa/cm$^2$, an exposure time of 2 s and a magnification of 40,000 ×, corresponding to a pixel size of 3.83 Å.

**Protein**liposome preparation. Reconstitution was performed according to a published protocol. Liposomes were prepared from purified soybean asolectin (L-α-phosphatidylycholine, Sigma). Lipids were dissolved in chloroform (5 mg/ml) until a homogeneous mixture was obtained, the solvent was then evaporated under a dry nitrogen stream to yield a thin layer of lipids at the bottom of a glass tube. The lipid film was thoroughly dried by placing the tube in a vacuum pump overnight to remove residual chloroform. Large multilamellar vesicles (LMV) were obtained by hydrating the lipid film with 1 ml of buffered solution (150 mM KCl, 10 mM Hepes, pH 7.4) and gently agitating at room temperature. Large unilamellar vesicles (LUV) were then prepared from LMV by extrusion through a polycarbonate filter with a pore size of 100 nm (extruder and membrane by Avanti Polar Lipids). F-ATP synthase or gramicidin was inserted into freshly made liposomes by direct incubation of the liposome solution for 30 min at 4 °C at a protein:asoxeclon ratio of 1:10 (w/v). After incubation, the solution was centrifuged.
at 30,000 x g for 5 min, the supernatant removed, and fresh buffer added to resuspend the proteoliposomes. For biochemical assays, bovine heart lipids containing 4% brain LDAO and 0.1% 1,2-dimyristoyl-sn-glycero-3-phospho-(O-β-D-glucohexopyranosyl)-ethanol (Avanti Polar Lipids) in reconstitution buffer (50 mM MOPS pH 7.4, 30 mM NaCl, 100 mM KCl, 1.5 mM MgCl$_2$) were extruded 13 times through a 400 μm polycarbonate membrane at 38 °C. Liposomes were then incubated with the purified protein for 30 min at 25 °C with continuous mixing by inversion before use.

**Cryo-EM of liposome-reconstituted F-ATP synthase.** Pre-irradiated 200 mesh Quantifoil R2/2 molybdenum grids were glow-discharged and 3-μl droplets of proteoliposome solution added to the grid. Excess solution was blotted with a GP Leica (Leica) followed by plunge freezing into liquid ethane after blotting for 10 s at 80% humidity and 20 °C. Data collection was performed on a JEM-3000SFF (JEOL) electron microscope at 300 kV with a field emission gun and a magnification of ×450.5. The specimen stage temperature was maintained at ~80–100 K. The images were recorded on a K2 summit direct electron detector camera (Gatan) operated in a counting mode with a pixel size of 1.23 Å at the specimen level. Each image included 24 fractionated frames and image stacks were binned 2 x 2 by Fourier cropping, resulting in a pixel size of 2.47 Å. The stacked frames were subjected to motion correction with MotionCor2$^{61}$.

**Negative stain EM of liposome-reconstituted F-ATP synthase.** One droplet of solution (about 25 μl) containing freshly prepared proteoliposomes was placed on 400 mesh holey film grid, stained with 1% uranyl acetate and observed with a Tecnai G2 (FEI) transmission electron microscope operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System) digital camera.

**ATPase activity in solution and after reconstitution.** ATP hydrolysis activity was measured at 38 °C with stirring at 1000 rpm using an ATP-regenerating assay$^{62}$. Unless otherwise specified, the assay mixture contained 50 mM MOPS (pH 7.4), 30 mM NaCl, 100 mM KCl, 3 mM phosphoenolpyruvate, 1.5 mM MgCl$_2$, 0.25 mM NADH, 0.57 μM pyruvate kinase, 3.2 U/ml lactate dehydrogenase, and 2 mM ATP. MgCl$_2$, CaCl$_2$, and ATP concentrations were varied as indicated in the figure legend. The reaction was initiated by the addition of 10 μl of F-ATP synthase either in solution or after reconstitution into bovine heart lipids, into 1 ml of assay mixture. Where reconstituted enzyme was used, 1 μl valinomycin was included in the assay. The rate of NADH oxidation was monitored continuously at 340 nm using a modified Cary 60 spectrophotometer (Agilent). Where indicated, 2 μM oligomycin was added. The activity that hydrolyzed 1 μmol of ATP per min is defined as 1 unit.

**Determination of ATPase activity using Pi release.** ATP hydrolysis activity was measured in an assay containing 10 μg bovine F-ATP synthase, 50 mM MOPS (pH 7.4), 30 mM NaCl, and 100 mM KCl. When used, MgCl$_2$ and CaCl$_2$ concentrations were 2 mM, respectively. The reaction was initiated by the addition of sodium-ATP to a final concentration of 2 mM in a 1 ml assay mixture. ATPase activity was measured at 38 °C with stirring at 1000 rpm using a colorimetric assay that detects inorganic phosphate liberated from ATP$^{63}$. Nonenzymatic degradation of ATP was measured at 38 °C with stirring at 1000 rpm using a colorimetric assay that detects an increase in the 5A chromophore at 340 nm using a modified Cary 60 spectrophotometer (Agilent). Where indicated, 2 μM oligomycin was added. The activity that hydrolyzed 1 μmol of ATP per min is defined as 1 unit.

**ATP-dependent proton translocation.** ATP-dependent proton translocation was determined at 38 °C based on the quenching of ACMA. The 1:5 ml reaction mixture contained 50 mM MOPS (pH 7.4), 30 mM NaCl, 100 mM KCl, 3 mM phosphoenolpyruvate, 1.5 mM MgCl$_2$, 0.25 mM NADH, 0.57 U/ml pyruvate kinase, 3.2 U/ml lactate dehydrogenase, 1 μM ACMA, 1 μM valinomycin, and 10 μg F-ATP synthase complexes reconstituted in bovine heart lipids. Where indicated, 2.5 mM ATP was added. In some experiments, 0.125 mM Mg$_2$ATP was used in a reaction mixture containing no MgCl$_2$, together with 30 μg F-ATP synthase complexes reconstituted in bovine heart lipids. After the fluorescence signal had stabilized, the reaction was initiated by the addition of the concentrated neutralized ATP indicated in the figure legends. Fluorescence was measured with an excitation wavelength of 410 nm and an emission wavelength of 480 nm (slit width, 10 nm) in a modified Cary Eclipse photospctrophotometer (Agilent).

**Protein digestion and liquid chromatography-mass spectrometry (LC-MS/MS) analysis.** The preparation of F-ATP synthase complex obtained as above was loaded into a NanoEase trap column (Supelco) and eluted with 2 mM ATP. MgCl$_2$, CaCl$_2$, and ATP concentrations were varied as indicated in the figure legend. The reaction was initiated by the addition of 10 μl of F-ATP synthase either in solution or after reconstitution into bovine heart lipids, into 1 ml of assay mixture. Where reconstituted enzyme was used, 1 μl valinomycin was included in the assay. The rate of NADH oxidation was monitored continuously at 340 nm using a modified Cary 60 spectrophotometer (Agilent). Where indicated, 2 μM oligomycin was added. The activity that hydrolyzed 1 μmol of ATP per min is defined as 1 unit.

**LC-MS/MS and data analysis.** LC-MS/MS analysis was carried out using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled online to a nano-HPLC Ultimate 3000 (Dionex-Thermo Fisher Scientific). Samples were loaded into a NanoEase trap column (Supelco) and eluted with 2 mM ATP. MgCl$_2$, CaCl$_2$, and ATP concentrations were varied as indicated in the figure legend. The reaction was initiated by the addition of the concentrated neutralized ATP indicated in the figure legends. Fluorescence was measured with an excitation wavelength of 410 nm and an emission wavelength of 480 nm (slit width, 10 nm) in a modified Cary Eclipse photospctrophotometer (Agilent).

**Electrophysiology.** Electrophysiological properties of F-ATP synthase were assessed by means of single-channel recording following protein insertion into artificial planar lipid bilayers either from proteoliposomes (4 μg of protein), by direct addition of the purified protein (4 μg of protein) or from gel-purified proteins (1–2 μg of protein) as specified in text and legends. Proteoliposomes prepared as described or purified protein were added at a final concentration of 2.22 mM F-ATP synthase (based on the molecular mass of the monomer). For gel elution, 16 μg of purified protein were loaded in each lane followed by separation in BN-PAGE. Membranes were prepared by painting a solution of soybean aselichen (10 mg/ml in decane, Sigma) across a 250-μm-diameter hole on a teflon partition separating two compartments filled with a recording solution (150 mM KCl, 10 mM HEPES, pH 7.4) before membrane painting. The two compartments are identified as cis and trans, and all voltages refer to the cis side, zero being the trans...
Mean conductance ($g_{mean}$) was calculated from the average of the whole current signal in the open state on the basis of Ohm's law. Maximal conductance ($G_{max}$) was calculated on the basis of the maximal stable current level (i.e., events lasting at least 10 ms) in the recording interval. $G_{max}$ and $g_{mean}$ were calculated for each experimental condition indicated in the text. In the voltage-dependence experiments data were corrected for the shift of about 13 pA caused by the imposed Ca$^{2+}$ gradient. Data are represented as mean ± SEM, and fits were calculated with Origin (OriginLab). PSA and Po distributions as a function of Ca$^{2+}$ concentration were fitted using the Hill equation68. Statistical comparison of data was assessed with the Mann–Whitney analysis.

Protein assay. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Sigma) with bovine serum albumin as the standard. These studies have complied with all relevant ethical regulations for animal testing and research.

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

Data availability

Data supporting the findings of this manuscript are available from the corresponding authors upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE67 partner repository with the dataset identifier PXD015108. The source data underlying Fig. 1d, e, i, j, 2a, c, d, 3a, 4a, 5a, b, e, f and Supplementary Figs. 3a-d, 4, 8, and 11 are provided as a Source Data file.

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References

1. Bernardi, P., Rasola, A., Forte, M. & Lippe, G. The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology. Physiol. Rev. 95, 1111–1155 (2015).

2. Petronilli, V., Szabó, I. & Zoratti, M. The inner mitochondrial membrane contains ion-conducting channels similar to those found in bacteria. FEBS Lett. 259, 137–143 (1989).

3. Kinnally, K. W., Campo, M. L. & Tedeschi, H. Mitochondrial channel activity studied by patch-clamping mitoplasts. Biochim. Biophys. Acta 209, 1148–1159 (1979).

4. Szabó, I. & Zoratti, M. Mitochondrial F$_{1}$F$_{0}$-ATP synthase as the molecular target of the immunomodulatory benzodiazepine Bz-21. Structure and conformational states of the yeast ATP synthase by cryo-EM. eLife Sci. 4, e01180 (2015).

5. Giorgio, V. et al. Cyclophilin D modulates mitochondrial F$_{0}$F$_{1}$-ATP synthase. Nature 521, 237–240 (2015).

6. Bonora, M. et al. The unique histidine in OSCP subunit of F$_{0}$F$_{1}$-ATP synthase triggers the permeability transition pore, a voltage-dependent mitochondrial megachannel. J. Biol. Chem. 294, 10987–10997 (2019).

7. Giorgio, V. et al. Argument 107 of yeast ATP synthase subunit g mediates sensitivity of the mitochondrial permeability transition to phenylglyoxal. J. Biol. Chem. 293, 14632–14645 (2018).

8. Guo, L. et al. Mitochondrial megachannel of pea stem mitochondria. Front. Physiol. 9, 1626 (2018).

9. Carraro, M. et al. Channel formation by yeast F-ATP synthase and the role of dimerization in the mitochondrial permeability transition. J. Biol. Chem. 289, 15980–15985 (2014).

10. von Stockum, S. et al. F-ATPase of D. melanogaster forms 53 picoisomet (53-pS) channels responsible for mitochondrial Ca$^{2+}$-induced Ca$^{2+}$ release. J. Biol. Chem. 290, 4537–4544 (2015).

11. Giorgio, V. et al. Ca$^{2+}$ binding to F-ATP synthase β subunit triggers the mitochondrial permeability transition. EMBO Rep. 18, 1065–1076 (2017).

12. Carraro, M. et al. High-conductance channel formation in yeast mitochondria is mediated by F-ATP synthase e and g subunits. Cell. Physiol. Biochem. 50, 1840–1855 (2018).

13. Rubinstein, J. L., Walker, J. E. & Henderson, R. Structure of the mitochondrial ATP synthase by electron cryomicroscopy. EMBO J. 22, 6182–6192 (2003).

14. Allegrisi, M. et al. Horizontal membrane-intrinsic α-helices in the stator subunit of an F$_{0}$-ATP synthase. Nature 521, 237–240 (2015).

15. Zhou, A. et al. Structure and conformational states of the bovine mitochondrial ATP synthase by cryo-EM. eLife Sci. 4, e01180 (2015).

16. Hahn, A. et al. Structure of a complete ATP synthase dimer reveals the molecular basis of inner mitochondrial membrane morphology. Mol. Cell 63, 445–456 (2016).

17. Mitchell, P. Keilin’s respiratory chain concept and its chemiosmotic consequences. Science 200, 1148–1159 (1979).

18. Papageorgiou, S., Melandri, A. B. & Solaini, G. Relevance of divalent cations to ATP-driven proton pumping in beef heart mitochondrial F$_{1}$F$_{0}$-ATPase. J. Bioenerg. Biomembr. 30, 533–541 (1998).

19. Nathanson, L. & Gromet-Elhanan, Z. Mutations in the β-subunit Thr$^{559}$ and Glu$^{284}$ of the Rhodopseudomonas rubrum F$_{1}$F$_{0}$-ATP synthase reveal differences in ligands for the coupled Mg$^{2+}$- and decoupled Ca$^{2+}$-dependent F$_{0}$-ATPase activities. J. Biol. Chem. 275, 901–905 (2000).

20. Tesk, N. & Zoratti, M. The mitochondrial ATP synthase by cryo-EM. J. Biol. Chem. 293, 14632–14645 (2018).

21. Antonelli, M. et al. The unique histidine in OSCP subunit of F$_{0}$F$_{1}$-ATP synthase mediates inhibition of the permeability transition pore by acidic pH. EMBO Rep. 19, 257–268 (2018).

22. Guo, L. et al. Mitochondrial megachannel of pea stem mitochondria. Front. Physiol. 9, 1626 (2018).

23. Giorgio, V. et al. Channel formation by yeast F-ATP synthase and the role of dimerization in the mitochondrial permeability transition. J. Biol. Chem. 289, 15980–15985 (2014).

24. Giorgio, V. et al. Cyclophilin D modulates mitochondrial F$_{0}$F$_{1}$-ATP synthase by interacting with the lateral stalk of the complex. J. Biol. Chem. 284, 33982–33988 (2009).

25. De Col, V. et al. The unique histidine in OSCP subunit of F$_{0}$F$_{1}$-ATP synthase triggers the permeability transition pore. Proc. Natl Acad. Sci. USA 110, 5887–5892 (2013).

26. Alavian, K. N. et al. An uncoupling channel within the c-subunit ring of the F$_{0}$F$_{1}$ ATP synthase is the mitochondrial permeability transition pore. Proc. Natl Acad. Sci. USA 111, 10580–10585 (2014).
38. Woodbury, D. J. & Miller, C. Nystatin-induced liposome fusion. A versatile approach to ion channel reconstitution into planar bilayers. *Biophys. J.* 58, 833–839 (1990).

39. Brustovetsky, N., Klingenber, M. Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca^{2+}. *Biochemistry* 35, 8483–8488 (1996).

40. Colombini, M., Yeung, C. L., Tung, I. & Konig, T. The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion. *Biochem. Biophys. Acta* 905, 279–286 (1987).

41. Brustovetsky, N., Tropschug, M., Heimpel, S., Heidkamper, D. & Klingenber, M. A large Ca^{2+}-dependent channel formed by reconstituted ADP/ATP carrier from *Neurospora crassa* resembles the mitochondrial permeability transition pore. *Biochemistry* 41, 11804–11811 (2002).

42. Hagen, P. J. & Zaug, H. A. Bongkrekic acid. An inhibitor of the adenine nucleotide translocase of mitochondria. *J. Biol. Chem.* 245, 1319–1326 (1970).

43. Althoff, T., Davies, K. M., Schulze, S., Joos, F. & Kühnlbrandt, W. GLecon: a method for the lipid reconstitution of membrane proteins. *Angew. Chem. Int. Ed. Engl.* 51, 8343–8347 (2012).

44. Blum, T. B., Hahn, A., Meier, T., Davies, K. M. & Kühnlbrandt, W. Dimers of mitochondrial ATP synthase induce membrane curvature and self-assemble into rows. *Proc. Natl Acad. Sci. USA* 116, 4250–4255 (2019).

45. Hahn, A., Vonck, J., Mills, D. J., Meier, T. & Kühnlbrandt, W. Structure, mechanism, and regulation of the chloroplast ATP synthase. *Science* 360, pii: eaat4318 (2018).

46. Sobti, M. et al. Cryo-EM reveals distinct conformations of E. coli ATP synthase on exposure to ATP. *eLife* 8, e43864 (2019).

47. Gerle, C. On the structural possibility of pore-forming mitochondrial F1, ATP synthase. *Biochim. Biophys. Acta* 1857, 1191–1196 (2016).

48. He, J. et al. Persistence of the mitochondrial permeability transition in the absence of subunit c of human ATP synthase. *Proc. Natl Acad. Sci. USA* 114, 3409–3414 (2017).

49. He, J., Carroll, J., Ding, S., Fearnley, I. M. & Walker, J. E. Permeability transition in human mitochondria persists in the absence of peripheral stalk subunits of ATP synthase. *Proc. Natl Acad. Sci. USA* 114, 9086–9091 (2017).

50. Carroll, J., He, J., Ding, S., Fearnley, I. M. & Walker, J. E. Persistence of the permeability transition pore in human mitochondria devoid of an assembled ATP synthase. *Proc. Natl Acad. Sci. USA* 116, 12816–12821 (2019).

51. Bernardi, P. Why F-ATP synthase remains a strong candidate as the mitochondrial permeability transition pore. *Front. Physiol.* 9, 1543 (2018).

52. Carraro, M., Checchetto, V., Scabio, I. & Bernardi, P. F-ATP synthase and the permeability transition pore: fewer doubts, more certainties. *FEBS Lett.* 593, 1515–1533 (2019).

53. Neginsky, M. A. et al. ATP synthase C-subunit-deficient mitochondria have a small cyclosporine a-sensitive channel, but lack the permeability transition pore. *Cell Rep.* 26, 11–17 (2019).

54. Shinzawa-Itoh, K. et al. Bovine heart NADH-ubiquinone oxidoreductase contains one molecule of ubiquinone with ten isoprene units as one of the cofactors. *Biochemistry* 49, 487–492 (2010).

55. Pullman, M. E., Penefsky, H. S., Datta, A. & Racker, E. Partial resolution of the permeability transition in human mitochondria by Ca^{2+}-dependent channel formed by recombinant ADP/ATP carrier from *Neurospora crassa* resembles the mitochondrial permeability transition pore. *Biochemistry* 41, 11804–11811 (2002).

56. McMillan, D. G., Watanabe, R., Ueno, H., Cook, G. M. & Noji, H. Biophysical characterization of the thermoalkaliphilic molecular motor with a high stepping torque gives insight into evolutionary ATP synthase adaptation. *J. Biol. Chem.* 291, 23965–23977 (2016).

57. McMillan, D. G. et al. A1AO-ATP synthase of Methanobrevibacter ruminantium couples sodium ions for ATP synthase under physiological conditions. *J. Biol. Chem.* 286, 39882–39892 (2011).

58. Hauer, F. & Gerle, C. www.grader-protocol.com.

59. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion data. *Biophys. J.* 108, 1143–1147 (2015).

60. McMillan, D. G. et al. A1AO-ATP synthase of Methanobrevibacter ruminantium couples sodium ions for ATP synthase under physiological conditions. *J. Biol. Chem.* 286, 39882–39892 (2011).

61. Wisniewski, J. R., Zougman, A., Nagaraj, N. & Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* 6, 359–362 (2009).

62. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372 (2008).

63. Cox, J. et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 10, 1794–1805 (2011).

64. Yifrach, O. Hill coefficient for estimating the magnitude of cooperativity in gating transitions of voltage-dependent ion channels. *Biophys. J.* 87, 822–830 (2004).

65. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucl. Acids Res.* 47, D442–D450 (2019).

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Competing interests

The authors declare no competing interests.

Additional information

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