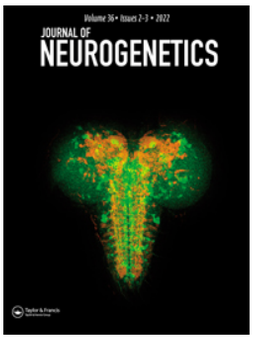

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PERSPECTIVE

The force-from-lipid principle and its origin, a '*what is true for E. coli is true for the elephant*' refrain

Boris Martinac[a,b] and Ching Kung[c]

[a]Molecular Cardiology and Biophysics Division, Victor Chang Cardiac Research Institute, Sydney, Australia; [b]School of Clinical Medicine, UNSW Medicine & Health, St Vincent's Healthcare Clinical Campus, Faculty of Medicine and Health, University of New South Wales, Sydney, Australia; [c]Laboratory of Molecular Biology and the Department of Genetics, University of Wisconsin–Madison, Madison, WI, USA

ABSTRACT

The force-from-lipid (FFL) principle states that it is the lateral stretch force from the lipid membrane that ultimately opens mechanosensitive (MS) channels, not the external tether nor the internal cytoskeleton. Piezo channels for certain touch or proprioception and the hair-cell channels for hearing or balance apparently obey this principle, which is based on the idea that the lipid bilayer is an amphipathic compartment with a distinct internal force-distribution profile. Physical stretch or insertion of chemical impurities alters this profile, driving channel shape change to conform to the new environment. Thus, FFL governs all dynamic proteins embedded in membrane, including Kv's and TRPs. This article retraces the humble origin of the FFL concept. *Paramecium* research first created the mind set and the resources to electrically explore other microbial membranes. Patch clamp revealed MS-channel activities from yeast and *E. coli* spheroplasts. Despite formidable obstacles against interdisciplinary research, the *E. coli* MS-channel protein, MscL, was purified through fractionation by following its activity, much like enzyme purification. Reconstituted into a simple lipid bilayer, pure MscL retains mechanosensitivity, thus firmly establishing the FFL principle in 1994. The relatively simple MscL and its functional cousin MscS soon became ideal models for detailed analyses. Like the DNA-RNA-protein 'central dogma' or ATP synthesis, FFL is a fundamental principle, which appeared early in evolution, retained in all cellular life forms, and is expected to contribute to future molecular research on sensations, homeostasis, and embryonic development.

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KEYWORDS
Mechanosensitive channels; MscL; MscS; piezo1; piezo2; liposomes; lipid force

The Nobel Prize for Physiology or Medicine 2021 was awarded for the discovery of the heat-sensing TrpV1 and for that of the force-sensing Piezo channels (Ernfors, El Manira & Svenningsson, 2021). While there is currently no consensus on the mechanism of temperature sensing, that of force sensing is clear: Piezo1 in membrane blebs from expressing HEK cells devoid of cytoskeleton remains sensitive to tension under patch clamp (Cox et al., 2016) and purified Piezo1 in droplet lipid bilayers remains sensitive to tension (Syeda et al., 2016) leaving no doubt that Piezos receive their gating force from the lipid bilayer (Figure 1). These experiments echo those on the *E. coli* channel MscL, performed over 20 years before (Sukharev, Blount, Martinac, Blattner, & Kung, 1994) (Figure 2) that established the principle of force from lipids (FFL). Below is the singular story on the birth of FFL and tortuous discovery of MscL (*m*echanosensitive *c*hannel of *l*arge conductance).

The Zeitgeist of the Late 1960's

The 1940–60 marks an inflection point of biology, changing it to a molecular science. The revolution has been chronicled repeatedly, most thoroughly in the aptly titled *The Eighth Day of Creation* (Judson, 1979). Briefly, a group of physicists led by Max Delbruck considered that biology was difficult

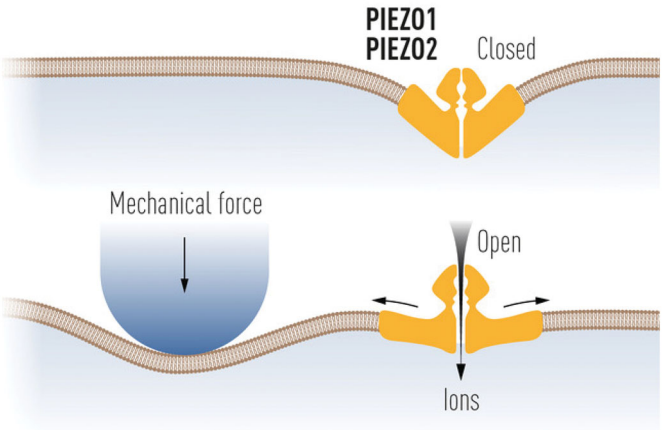

Figure 1. Diagram showing mechanical force is transmitted to piezo channels by stretching the lipid bilayer. (with permission from nobelforum@nobelprize-medicine.org, (Ernfors et al., 2021).

CONTACT Boris Martinac b.martinac@victorchang.edu.au Molecular Cardiology and Biophysics Division, Victor Chang Cardiac Research Institute, Sydney, Australia; Ching Kung ckung @wisc.edu Laboratory of Molecular Biology and the Department of Genetics, University of Wisconsin–Madison, Madison, WI, USA.

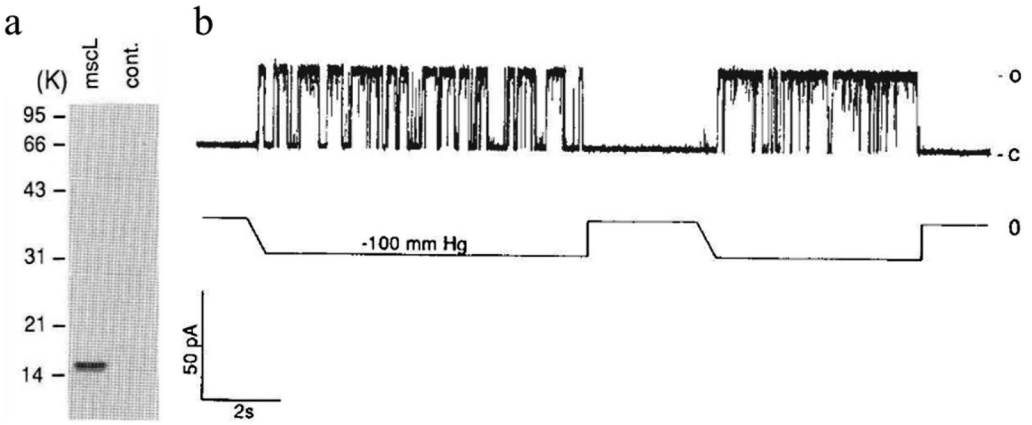

Figure 2. Purified MscL protein is mechanosensitive in lipid bilayer. *a.* Autoradiogram of an SDS-PAGE gel containing cell-free lysate expressing MscL (left lane) or control (right) vector. *b,* Patch-clamp record of MscL activities from liposomes fused with the lysate. (Sukharev et al., 1994)

because it tends to study complex phenomena in complex plants and animals. They chose to focus on the key question of the origin of biological traits using simpler organisms: the bacterium *E. coli* and its enemies, the bacteriophages. At that time, it was known that traits (phenotypes) are determined by genotypes, and 'genes' are particulate (from Mendel) and are aligned along chromosome (from the Morgan school), but the molecular basis of inheritance and the structure of DNA were unknown prior to 1953. History vindicated the reductionistic approach of the 'Phage Group' with the establishment of DNA-RNA-protein informational flow (the 'Central Dogma'). The second author (C. Kung), in graduate school, experienced the excitement of the final resolution of the genetic code in 1966 and can still recall that excitement today. That the Central Dogma covers all organisms vindicated the aphorism 'What is true for *E. coli* is true for the elephant.', which celebrates the success of reductionism.

Buoyed by the success in solving one of the deepest mysteries of life, that of heredity (genetic), the key Phage-Group players then set their aim at another, 'the mind' (neurobiology). For this pursuit, model organisms were needed, and there were many to choose from. Near the end of the 1960s, there was then the 'model organism diaspora' with Delbruck choosing to study *Phycomyces*; Benzer choosing *Drosophila*; Brenner *C. elegans*; Stent leeches; and Streisinger the zebrafish.

Paramecium

By late 1960s, Hodgkin and Huxley's voltage-clamp experiments were already almost 20 years old. The H & H theory fully explained the feedback between the voltage-sensitive Na^+ and K^+ currents underlying action potential. This aspect of neurobiology roughly parallels the status of genetics early on, with known behavior of genes and chromosomes but no DNA. Behind all the sophisticated biophysical analyses of action currents, there were no ion-channel proteins.

To find the relevant proteins or RNAs of a biological machine, biologists often use an approach referred to as 'genetic dissection'. (**Note 1**). Here, this strategy involves finding mutations that block or change the action potential, and this is where *Paramecium* came in. Though not one of the great 'model organisms', it clearly comported with the zeitgeist of the late 1960s. Kung did his graduate work on some paramecium biochemistry but became interested in its behavior. Even as a single-celled animal, *Paramecium* generates a Ca^{++}/K^+ based action potential when stimulated. The entered Ca^{++} causes its cilia to reverse their beat direction and the cell to back away from trouble (Naitoh & Eckert, 1969). Because of its large size, this 'avoiding reaction' was observed early on and its ionic basis was later examined with microelectrodes. In his first postdoc, Kung isolated mutants that 'misbehave' and, in his second, he examined them with intracellular electrodes. They include the 'pawn' mutants that cannot swim backward because the mutations erase the Ca^{++} current as well as the 'paranoiac' mutants that swim backward for long durations because of the loss of the repolarizing K^+ current (Kung, 1971). The *Paramecium* mutations established early the connections between genes and ion currents, linking genetics with electrophysiology. In the dearth of such linkages, these mutants became notable, featured repeatedly in *Science* and *Nature*. The novelty of these mutants also allowed a sizable group of *Paramecium* enthusiasts to gather together. The first author, Boris Martinac, joined the Kung lab in Madison, Wisconsin, during this time, in 1983. After finding the relevant mutations, genetic dissection requires tracing of the mutations to the corresponding genes and proteins. Here, *Paramecium* genetics faltered (**Note 2**), but the approach was successful using *Drosophila*, with the cloning of *Shaker*, *Eag*, *etc*.

Note 1. This is exemplified by the dissection of the biological clock: By finding mutations that specifically wrecked the clock, one can then trace the mutations to the gene products, which would be the equivalents of the springs, the gears, the cogs of a mechanical clock. As is well known, this work eventually solved the puzzle of biological clock and led to the 2017 Nobel prize to three investigators, including Jeff Hall, the founding editor of this very journal.

Note 2. The chief obstacle in cloning a gene based on its mutant phenotype in ciliates is not their altered genetic code (Preer, Jr. et al., 1985) but the polyploidy of the somatic macronuclei, which makes complementation with transgenes very difficult. On the other hand, it is precisely the large number of chromosomes (therefore many ends) that made it possible to isolate telomeres and telomerase in *Tetrahymena*, a *Paramecium* cousin, which won the 2009 Nobel prize.

The *Paramecium* research made other contributions (**Note 3**), but most important, it framed the mindset for microbial electrophysiology. More practically, it made the Kung lab 'rich'. The story of MscL below, from its encounter to the erection of the FFL principle, was funded largely by NIH grants and grants from other sources meant for *Paramecium* research. This research is briefly reviewed here to provide the intellectual backdrop for the electrophysiology of yeast and *E. coli* and finally the establishment of the Force-from-Lipid principle.'

'Microbial Electrophysiology'

What microbial electrophysiology?? There was no such thing 50 years ago and there is still little of it today. The *Paramecium* work might be a bit of historical curiosity, but microbiology and electrophysiology remain two separate disciplines with different subject matter, using different methods, asking different questions. There was also no common language: One may think that '*Bordetella*' is an opera and the other one may think that 'afterhyperpolarization' is a typo. This segregation is regrettable because ion channels are widely used beyond the nervous system. Today, one finds recognizable ion-channel genes in the genomes of all plants, animals, and microbes but we know little the functions these channels serve. E.g. we learn how ions are filtered from the crystal structure of KcsA (Doyle et al., 1998) but don't know (or care to know) what it does for its owner, the Gram-positive *Streptomyces lividans*.

The segregation of micro- and neurobiology has deep subconscious roots. Children and adults alike may admire the big elephants, whales, and dinosaurs, but, in fact, most organisms on earth are microbes, composed of widely different algae, fungi, protozoa, bacteria, and archaea. *Homo sapiens* is not endowed by evolution with microscopic vision and tend to ignore microbes. If it weren't for diseases, we would not care about microbes at all, and even with diseases, the public cannot tell a bacterium from a virus. Further, even professional biologists historically study complex plants and animals, leading to the original complaint by Delbruck *et al.* and their later 'diaspora' back to complex worm, fly, and fish. The other intuition that segregates micro- and neurobiology is, of course, our self-interest and hubris. To understand the mind is to understand the brain and electrophysiology is the study of the nervous system, using complex animals as proxies for human. Yeasts and bacteria are no neurons and were categorically ruled out as subject matter by most electrophysiologists.

Patch Clamp

Beside the intuitive mental barrier, exploring microbes electrically had an added practical hindrance. The three microbes of concern here differ in size. In length, a paramecium is about 100 microns, a yeast cell about 10, and a bacterium is only 1 micron. The conventional glass microelectrodes have a tip diameter of a micron or two. After inserting into a large cell, such as a paramecium, it can measure the sum total of all currents through the entire cell membrane, called the macroscopic current. Yeasts or bacteria are obviously too small for such conventional recording.

Patch clamp was invented in the late 70's and early 80's and soon became popular among electrophysiologists (Hamill et al., 1981). Instead of measuring macroscopic currents, the patch-clamp electrode can measure the currents through individual ion-channel molecules in a small membrane patch, a few microns squared, attached to the inside of a glass pipet electrode near the tip.

The action potential of *Paramecium* was discovered in Japan, where it had a tradition of its study. From that tradition, Yoshiro Saimi of Tokyo University joined the Kung lab in 1979. In 1983, Yoshiro put together the first patch-clamp setup in the lab to take patches from ciliary blisters to scrutinize individual single-channel currents.

Electrophysiology of yeast or *E. coli* was a flight of fantasy initially. *A priori*, there was no reason to think that they have channels. This is like sailing into new continents not knowing if there might be native people or not. This is clearly not the kind of research one can propose to any granting agency. There is curiosity but no hypothesis, no preliminary results and no promise of finding anything. Any study section would just laugh out loud at such an obvious 'fishing expedition'. (**Note 4**)

Yeast Channels: Pulled, Pushed, or Stretched?

Zymolyase strips the cell wall and converts yeast cells into spheroplasts, exposing their plasma membrane for patch-clamp examination. Yoshiro and Boris started generating yeast spheroplasts in 1985 and were soon joined by Mike Gustin to first describe a 20-pS K^+ conductance, showing that even a walled microbe has channel activities similar to those found in nerves (Gustin, Martinac, Saimi, Culbertson, & Kung, 1986). Soon, joined by Xin-Liang Zhou, they also discovered a 36-pS cation-nonspecific mechanosensitive (MS) channel activity in yeast spheroplast membrane and suggested its possible role in osmoregulation (Gustin, Zhou, Martinac, & Kung, 1988).

To this day, there is a confusion that haunts the MS-channel field. For many students or even neurobiologists,

Note 3. Besides setting the stage for the electric study of yeast and *E. coli*, the *Paramecium* work showed that calmodulin can be a detachable subunit to regulate channels (Saimi and Ling, 1990) with a functional bipartition: its N-terminal lobe and its C lobe have different specific effects (Saimi and Kung, 2002). These findings continue to have great implications in Ca-calmodulin regulation of numerous ion channels and enzymes.

Note 4. To this day, Kung cannot articulate why he encouraged the lab to put the patch-clamp electrodes on yeast and *E. coli*, though encourage he did. These microbes, usually grown on petri dishes, were never considered subjects of electric investigation before. Implicit association tests show that humans have subconscious aversion to anything truly novel. Nonetheless, our merging microbiology and electrophysiology was probably inevitable. Novelty comes from "associative memory that works exceptionally well" according to Daniel Kahneman in *Thinking Fast and Slow*, and "Chance favors the one with a prepared mind." according to Louis Pasteur. Indeed, there must have been increased chance of "association" in having patch clamps already "prepared" and having yeast and bacterial laboratories in physical proximity on the Madison campus.

MS-channel is represented by that in the hair cell of the inner ear. In the popular diagram, the channel has a lid, like that on a toilet seat, that is pulled open by an extracellular gating spring with a give. Even though the gating spring corresponds to the visible tip link, this popular diagram was explicitly stated only as a representation of the physics and not the molecular reality. (See below for recent information on that reality.)

Whether MS channels are opened by pull or push (forces perpendicular to the membrane) or by stretch (forces along the membrane plane) is thus a crucial question. This question was answered with the yeast MS channel. Mike examined the activation of MS currents in whole spheroplasts of different sizes and found the activation pressure to be inversely proportional to the diameter of the spheroplasts (Gustin, Zhou, Martinac & Kung, 1988). Converting applied pressure to tension by Laplace's law, the current-activation curves from different spheroplasts, big or small, coincided. Thus, there is no doubt here that the MS channel is gated by membrane stretch and not directly by pressure.

Signal

The lower limit of the patch-clamp pipet opening is about 1 micron and cannot be used directly on individual *E. coli* cells. Fortuitously, the nearby laboratory of Julius Adler of chemotaxis fame was generating giant *E. coli* to further their research in 1986. Cephalexin blocks cell division and thus makes long 'snakes', which can then be treated with lysozyme, converting them to 'giant spheroplasts' some 6 microns in diameter. Boris examined these spheroplasts with the patch clamp. After some experimentation, he was able to form gigaOhm seals on these spheroplasts and encountered clear unitary currents (Martinac, Buechner, Delcour, Adler, & Kung, 1986) in both on-cell or excised-patch mode. The signal was very large, and very consistent, appearing in nearly every patch. Both authors were greatly excited by this astonishing new find from a prokaryotic membrane, as if we had encountered the first native in a new world. Joined by Matthew Buechner and Anne Delcour of the Adler lab, they described that small pipet suctions (tens of mm Hg) cause channel opening, which can last more than a second. The unit conductance is very large, about 1 nS, and has little ion selectivity (Martinac, Buechner, Delcour, Adler, & Kung, 1987). None of these characteristics conform to those of ion channels commonly studied in nerves, which have conductances of only 10 to 100 pS, open briefly, and have keen ion selectivity.

Before the yeast and *E. coli* MS-channel discoveries, much of the early research on mechanosensitive channel currents was carried out with animal cells and the notion that MS channels are pulled open by the cytoskeleton tended to prevail (Sachs, 1997). Because *E. coli* cells are largely shaped by the peptidoglycan and not by cytoskeletons, Boris therefore suspected that these MS channels are pulled by the lipid membrane not by cytoskeleton. Amphipaths such as chlorpromazine or trinitrophenol can wedge into either the outer or the inner leaflet of the lipid bilayer, changing its geometry and internal force distribution (Sheetz and Singer, 1974). These compounds indeed slowly activate the *E. coli* MS channel as they infiltrate into the patch membrane and the activity subsides slowly as the amphipaths are washed out (Martinac, Adler, & Kung, 1990). This was the first evidence indicating that membrane lipid is the medium that transmits the gating force.

Spheroplasts are live cells and can grow normally after the removal from cephalexin and lysozyme. Patch-clamping *E. coli* spheroplasts is therefore an exercise *in vivo*. There were methods to study ion-channel activities in artificial lipid bilayer (the 'black lipid membrane') even before the invention of the patch clamp. Modifying existing methods, Ann and Boris generated *E. coli* liposomes by mixing membrane fractions with exogenous phospholipids (azolectin). They then induced blisters from them and sampled patches from these blisters. There, they encountered at least three types of channel activities, including that of the MS channels (Delcour, Martinac, Adler, & Kung, 1989). Such reconstitutions were also successful in the laboratory of Alexandre Ghazi independently (Berrier, Coulombe, Houssin, & Ghazi, 1989). The fact that these channel activities survive these treatments *in vitro* is key to the eventual biochemical identification of the material behind the activity.

'Noise'

Guharay and Sachs (1984) first reported a 70-pS MS current in cultured chick muscle cell. Owen Hamill and coworkers also reported a 16-pS cationic MS channel current in *Xenopus* oocytes (Hamill & McBride Jr., 1992; Lane, McBride, & Hamill, 1991; Reifarth, Clauss & Weber 1999). To our knowledge, there has not been attempts to find the genes or proteins that correspond to these currents. Molecular studies tended to address mostly voltage-sensitive or neurotransmitter-gated channels. The reports on bacterial MS currents, nS in size and ion nonspecific, though first greeted with interest (Kullberg, 1987) were eventually exposed to a lot of skepticism. Bacteriologists might know about transporters but never thought of ion channels. They never saw a patch-clamp setup and terms like megaOhm and gigaOhm, picoSiemens and nanoSiemens are worse than unfamiliar. Feeling totally alienated, the attitude of some was essentially that of disbelief. The dismissal was basically that 'to a man with a hammer everything is a nail'. More damaging is the skepticism from electrophysiologists. Patch clamp is a delicate operation and is often subjected to noise, especially when the seal between the patch and the glass is not tight. Morris and Horn (1991; Morris & Horn, 1991) patch clamped growth cones and neurons of the snail *Lymnaea*, where they could observe unitary-like currents upon patch suctions but could not elicit macroscopic (whole-cell) currents with a variety of mechanical perturbations. From the latter negative results, they argued that MS currents are likely artefacts. Despite rebuttals (Gustin et al., 1991), this well-publicized paper (in *Science*) created doubts in the mind of non-specialists as to the authenticity of MS channels in general.

Even with this ambient 'noise' in the scientific community, the study of MS-channel activities from *E. coli* stood out. While being neither fish nor fowl, they are nonetheless

prominent (unitary conductance being 10 to 100 times those of eukaryotic channels), robust (encountered all the time), and tell a convincing story of osmotic protection (see below). News on these bacterial MS-channel activities appeared in prominent journals and attracted curiosity and some attention (Kullberg, 1987).

Finding MscL

Goaded by the publicized insinuation that MS currents are artifacts, the two authors decided in 1991 to go beyond the MS currents to get at the substance behind the activity. They chalked down on a blackboard the scheme of stocking starting material, solubilizing the membranes, separating fractions, reconstituting them into liposomes, following the activity with the patch clamp and displaying the proteins by electrophoresis. Knowing that our target is robust and gives huge signals (nanoSiemens), we believed that we could succeed. In late 1991, armed with preliminary results showing that the MS activities survived reconstitution into liposomes after membrane solubilization and crude column fractionation, we wrote a grant proposal to NIH for the identification of the channel protein(s). That proposal was examined by the Physiology Study Section and was firmly rejected! Purifying a channel like that of an enzyme had never been done or even proposed before. This novelty presumably underlies the general skepticism. Concrete criticisms questioned whether the channels could survive the fractionations. Despite the rejection, the two authors were determined to continue the hunt, using *Paramecium* money.

We were fortunate. We were joined in this endeavor by Sergei Sukharev, a biophysicist from the Frumkin Institute in Moscow in 1991 and by Paul Blount, a molecular biologist with extensive experience in acetylcholine-receptor research in 1992. With Boris, Paul, and others, Sergei led the arduous work of solubilizing membranes, fractionating with liquid chromatography, reconstituting by fusing fractions with azolectin liposome, and checking for activities with the patch clamp. This work sorted out the 3-nS MscL from the 1-nS MscS (Levina et al., 1999; Sukharev, Martinac, Arshavsky, & Kung, 1993). Later, by following two series of fractionations, the MscL activity was traced to a 17-kD protein, which was then microsequenced automatically, revealing 37 N-terminal residues. Paul searched the database and matched this partial sequence with some hint in the literature (Hamann, Bossemeyer & Bakker 1987). At that time Frederick Blattner's lab on the UW Madison campus was finishing the *E. coli* genome sequencing. A fragment of a lambda clone from the Blattner collection contains the corresponding gene, which was subcloned and sequenced, revealing the entire nucleotide and amino-acid sequence of MscL (Sukharev et al., 1994; Sukharev, Blount, Martinac, & Kung, 1997). Pure MscL from cell-free expression reconstituted into liposome patches retains MS activities (Figure 2). This was the first MS channel ever been cloned, expressed *in vitro*, and it incontrovertibly showed the force-from-lipid (FFL) principle. A bonus in using *E. coli* is that the starting material is inexpensive and unlimited, requiring no precious tissue culturing or tedious animal dissections. We generated 60 liters of culture and kept the bacteria paste in deep freeze, taking portions for experimentation when needed. This could be one of the reasons why hunting for channel proteins by fractionation described here is unique and has not been reported for any other channels.

Following the discovery of the MscL gene, we carried out a search for mutations after random mutagenesis that stopped growth when expressed. These gain-of-function (GOF) mutations were found to cluster at one facet of the predicted transmembrane helix 1, which we proposed to be key to channel gating (Ou, Blount, Hoffman, & Kung, 1998). The MscL discovery also soon stimulated crystallographers, resulting first in a 15Å -resolution 2D crystal structure with some information (Saint et al., 1998; Blount et al., 1996) followed by a 3D X-ray structure of TbMscL from *Mycobacterium tuberculosis* at 3.5Å resolution (Chang et al., 1998). The latter shows that the channel is a homopentamer with each subunit consisting of two transmemebrane α-helices (TM1 and TM2) and with both the amphipathic N-terminus and α-helical C-terminus facing the cytoplasm (Chang, Spencer, Lee, Barclay MT and Rees, 1998). This pentamer structure was later confirmed as the correct native structure of MscL (Dorwart, Wray, Brautigam, Jiang, & Blount, 2010; Iscla, Wray, & Blount, 2011; Reading et al., 2015) and showed that the facet of TM1 identified by the GOF mutations indeed forms the channel gate. There are currently about 2,300 members of the MscL channel subfamily listed in the UniProt database with homologues found in all three domains of life, Bacteria (Gram-negative and Gram-positive), Archaea (e.g. *Mathanosarcina*) and Eucarya (e.g. *Neurospora*) (Martinac et al., 2014). Interestingly, MscL is absent from many marine bacteria, presumably because of lesser challenges in osmotic fluctuations (Blount & Iscla, 2020).

MscL is half-activated by membrane tension of ~12 mN/m and fully activated by close to lytic tension of a pure lipid bilayer (Sukharev, Sigurdson, Kung, & Sachs, 1999; Nomura et al., 2012). This makes MscL a MS channel requiring the highest membrane tension on the physiological spectrum ranging from 1 to 25 mN/m, which perfectly fits its role of a osmoregulatory emergency nanovalve opening upon extreme changes in turgor pressure during a hypoosmotic shock experienced by bacterial cells (Bialecka-Fornal, Lee, & Phillips, 2015; Levina, Totemeyer, Stokes, Louis, Jones & Booth, 1999). Being relatively simple in structure, MscL has been analyzed extensively on how it opens and closes. Essential in determining its open structure was the activation of MscL by lipid forces, including the insertion of the cone-shaped amphipath lysophosphoshatidylcholine (LPC) into a single leaflet of the lipid bilayer (Perozo, Cortes, et al., 2002). The change from closed to open structure in MscL entails an iris-like expansion resulting in a large pore of 28Å in diameter (Sukharev, Betanzos, Chiang, & Guy, 2001; Betanzos, Chiang, Guy, & Sukharev, 2002; Perozo, Cortes, et al., 2002; Wang et al., 2014). This expansion is driven by 'pulling' the N-terminal helix and tight protein–lipid interactions with TM2 (Bavi, Cortes, et al., 2016; Iscla, Wray, & Blount, 2008).

MscS

MscS was identified several years after MscL as the product of *yggB* of *E. coli* encoding a 286 amino-acid channel protein (Levina et al., 1999). 3D-crystal structure of MscS shows that the channel is assembled as a homoheptamer (Bass, Strop, Barclay & Rees, 2002). Like MscL, MscS can be reconstituted into liposomes and gated by mechanical force according to the FFL principle. It requires about a half the membrane tension that gates MscL (Nomura, Cranfield, Deplazes, Owen, Macmillan, Battle, Constantine & Martinac, 2012).

One can now determine the structural dynamics at near-atomic resolution, such as the movement of alpha helices and the displacement of individual lipids upon opening (Zhang et al., 2021). MscS proteins can be embedded in nanodiscs mimicking membranes under tension and examined with cryo-EM (Reddy et al., 2019). One trick is to use β-cyclodextrin (βCD), which, by removing lipids from the nanodiscs, generates membrane tension without directly applying negative pressure. The changes in fine structure observed were then correlated functionally with patch-clamp recordings from liposome-reconstituted MscS exposed to βCD or other cyclodextrins (Cox, Zhang, Zhou, Walz, & Martinac, 2021). These findings are in accordance with other studies showing that MscS structure contains grooves and pockets allowing lipid molecules to fill them and become interlocked with the channel (Pliotas et al., 2015; Pliotas & Naismith, 2017; Pliotas et al., 2012). Thus, the dynamic structure/function relationship at the finest detail can now be understood for various channels operating under the FFL principle using this βCD approach, combining cryo-EM, patch clamp and molecular dynamics simulations (Zhang, Daday, Gu, Cox, Martinac, de Groot & Walz, 2021).

Piezos

In contrast to the MscL pentamer, which is a small protein of ∼80 kDa m.w., Piezo1, discovered in 2010, is a vastly different protein (Coste et al., 2010; Coste et al., 2012). Piezo1 forms Ca^{2+}-permeable trimeric channels of ∼1.2 MDa m.w., and is thus one of the largest channel proteins known (Wang & Xiao, 2018). It is gated according to the FFL paradigm (Teng, Loukin, Anishkin, & Kung, 2015) (Figure 1) meaning that it is inherently mechanosensitive like MscL (Cox, Bae, Ziegler, Hartley, Nikolova-Krstevski, Rohde, Ng, Sachs, Gottlieb & Martinac, 2016; Syeda et al., 2016). While Piezo1 obeys FFL as the fundamental gating principle of the lipid bilayer (Buyan et al., 2020; Ridone et al., 2020), cell-membrane tension *in vivo* can be modulated by cytoskeletal proteins (Poole, Herget, Lapatsina, Ngo, & Lewin, 2014; Qi et al., 2015; Cox, Bavi, & Martinac, 2019; Young, Lewis, & Grandl, 2022) and interaction with the extracellular matrix (Gaub & Muller, 2017; Li et al., 2021). In its closed state, Piezo1 curves the membrane by adopting a dome-like structure based on its unique propeller blade region resembling a triskelion (Guo & MacKinnon, 2017). When activated by membrane stretch, Piezo1 flattens due to expansion of its tension-sensing blade region as initially indicated by molecular dynamics simulations (De Vecchis, Beech & Kalli, 2021). Single-particle cryo-EM of Piezo1 in liposome confirmed the overall gating mechanism (Yang et al., 2022). The curved or flattened Piezo1 structure could be obtained in the cap-inside or the cap-outside configuration, respectively, in accordance with the FFL activation principle (Cox, Bae, Ziegler, Hartley, Nikolova-Krstevski, Rohde, Ng, Sachs, Gottlieb & Martinac, 2016; Syeda et al., 2016). Either mode can result from curvature mismatch between the Piezo1 protein and surrounding liposome membrane resulting in bilayer tension and bending force that cause the shape change of the channel (Yang, Lin, Chen, Li, Li & Xiao, 2022)

The Force-from-Lipid (FFL) Principle

Besides MscL, MscS, and Piezo1, described above, the two-pore-domain K^+ channels, and TREK (Berrier et al., 2013; Brohawn, Su, & MacKinnon, 2014) as well as OSCA/TMEM63 (Murthy et al., 2018) have also been purified and shown to be mechanosensitive in lipid bilayers. Less rigorously tested are also MS currents observed in various membrane blebs, largely devoid of cytoskeleton. In addition, there are MS channels functionally expressed heterologously, where the channels are alien to the host cytoskeletons and do not likely interact. For example, TRPV4 from rat can be functionally expressed in *Xenopus* oocyte as well as in yeast cells and remains mechanosensitive (Loukin, Su, & Kung, 2009). That MS channels of very different structures, coming from bacteria to human, all draw their gating force from the lipid bilayer indicates that FFL is a general evolutionary conserved principle. In simplest terms, the bilayer is an amphipathic structure with set internal forces. (See below). An embedded channel protein in its closed state has its own amphipathic structure fitting this force environment. Stretching the bilayer generates a mismatch, which drives the protein to a new (open) structure.

As stated above, the hair-cell trapdoor model holds sway among some non-specialists. Most recent findings, however, show that even the hair-cell transduction complex likely also obeys the FFL principle (Jeong et al., 2022). Because mammalian inner ears cannot supply enough material, these authors examined the homologous touch-transduction complex from the nematode *C. elegans*, consisting of two copies of the pore-forming TMC-1 and two additional proteins (CALM-1, TMIE). Single particle cryo-EM of this complex and molecular dynamics simulation show how the complex deforms the bilayer and 'suggest crucial roles for lipid-protein interaction in the mechanism by which mechanical force is transduced to ion-channel gating'. These authors also stated that '… TMIE subunits poised like 'handles' perpendicular to the membrane, and amphipathic TMC-1 H3 helices inserted and parallel to the membrane plane, each providing possible mechanisms for direct or indirect transduction of force to ion channel gating, respectively.' The TMC-1 H3 lateral movement is just like that of the N-terminal helix of MscL upon lateral stretch (Iscla et al., 2012; Bavi, Cortes, et al., 2016). The 'handles' could be analogous to those attached to the cadherin tip link in the hair cell to transmit the vertical pull leading to bilayer stretch that opens the pores (Anishkin & Kung, 2013).

The Bilayer as a Medium

Evolution being opportunistic, FFL does not exclude the possibility that some MS channels, such as the DEG/ENaC family channels, make use of the extracellular tethers or intracellular cytoskeleton, at least as handles, anchors, or modifiers of the membrane properties (Arnadottir & Chalfie, 2010; Chalfie, 2009). Even in these cases, however, the mechanics and energetics of the lipid bilayer cannot be ignored. Early on, the membrane was often seen only as a passive physical barrier or an electric insulator, but the lipid bilayer is in fact a second type of medium besides the cytoplasm, providing an amphipathic compartment with physical and chemical properties entirely different from the cytoplasmic aqueous compartment. The continuum model of the lateral pressure across the membrane bilayer indicates that at the level of the neck of the phospholipids where the hydrophilic heads meet the hydrophobic tails, there is a large tension, akin to surface at the water-oil interphase (Cantor, 1999; Gullingsrud & Schulten, 2004). Because the bilayer is a self-assembled structure at equilibrium, this sharp tension is largely balanced by repulsion among the fatty-acid tails in the interior of the bilayer. This internal force profile is dynamic and can adjust to chemical perturbations such as the monolayer insertion of amphipaths (Martinac, Adler & Kung, 1990), physical perturbations, such as thinning or bending by external forces (Perozo, Kloda, Cortes, & Martinac, 2002) or insertion of membrane proteins (Bavi, Cortes, et al., 2016). The transbilayer pressure profile determined experimentally by NMR spectroscopy confirmed the theoretical and computational models and demonstrated that the internal bilayer pressure varies with the level of saturation of phospholipids that the bilayer is made of (Ridone et al., 2018).

20–30% of all genes in most genomes encode for membrane proteins. At equilibrium, integral membrane proteins embedded in the bilayer have to be themselves amphipathic to fit the hydrophobicity and force profiles. Alteration of the force profile due to stretch-induced thinning leads to lateral pulling forces at the polar/nonpolar membrane interface and a hydrophobic mismatch at the interfaces and can drive the conformational changes of the embedded proteins (Martinac & Hamill, 2002; Perozo, Kloda, et al., 2002). Thus, any dynamic embedded proteins that need to change their footprints inside the membrane to perform protein's function are sensitive to applied stretch force. In other words, FFL is not just about MS channels.

FFL's broad implication is best illustrated by the well-known voltage-dependent K^+ channel, Kv. Kv's opening tendency is much higher in the tensed membrane than it is in membrane constrained by cytoskeleton (Morris, Prikryl, & Joos, 2015; Schmidt, del Marmol, & MacKinnon, 2012). Channel opening by depolarization not only entails work done to move the S1-S4 peripheral domain, detaching it from the S5-S6 core, but also the work done to move the surrounding annular lipids, which is subjected to the innate as well as applied tension. The free-energy change associated with the bilayer deformation is comparable to that of the voltage-dependent part of the total gating energy (Reeves et al., 2008). Thus, Kv is as much a mechanosensitive as a voltage-sensitive channel (Schmidt et al., 2012). Nav, Cav, and TRPs, having the same design as Kv, should behave similarly (Anishkin, Loukin, Teng, & Kung, 2014). The ligand gated NMDA receptor channel also exhibits mechanosensitivity upon reconstitution into liposomes and activation either by membrane tension or arachidonic acid acting as an amphipath (Johnson, Battle, & Martinac, 2019; Kloda, Lua, Hall, Adams, & Martinac, 2007; Maneshi et al., 2017).

In short, the FFL principle should be viewed broadly, addressing not only the externally applied stretch force but also the internal forces that are constitutive to the bilayer, and it governs all embedded material and not just MS channels.

Reductionism Redux

MscL and Piezo both follow the FFL principle (Cox et al., 2019; Young et al., 2022). Once again, 'What is true for *E. coli* is true for the elephant.' Historically, the molecular biology revolution succeeded because of the choice in using simple organisms: bacteria and phages, as reviewed above. The establishment of the FFL principle likewise emphasizes the advantage of using simpler organisms: yeast, bacteria. However, in philosophy, *reductionism* means to analyze a problem not only at a simpler level, but also at a *more fundamental level.*

Not everything that is true for *E. coli* is necessarily true for the elephant. Elephants don't have cell walls; they don't divide; they don't make you sick. On the other hand, the entire catabolic chemistry that entails glycolysis, Krebs cycle, and oxidative phosphorylation, including electron transport and ATP synthesis, is the same in plants, animals, or microbes. Photosynthesis, which converts sunlight to sustain nearly all life forms, is basically the same in plants as in purple- or green-sulphur bacteria and cyanobacteria. It is the *basic and fundamental mechanisms* that are held constant through evolution, not the details that are outfitted for different environments or lifestyles, even though the details may be more eye-catching.

Tiny bacteria in their low Reynolds number world cannot feel any external physical impacts. The mechanical force relevant to their lives (as well as to all lives) is the osmotic force. Osmotic pressure was already thought to be the natural force that opens the yeast and *E. coli* MS channels at their first encounters (Martinac et al., 1987). Previously, diluting the culture medium had long been known to cause the bacteria to release solutes indiscriminately through unknown pathways (Britten & McClure, 1962). *E. coli* has several types of MS channels for this release and when the two major ones, MscL and MscS, were deleted, the bacteria died by cell lysis when the medium was diluted (Levina, Totemeyer, Stokes, Louis, Jones & Booth, 1999). Thus, bacterial MS channels were selected to survive Mother Nature's caprice of rain or shine. In rain, water rushes into the cell, producing a large physical pressure on the cell envelope. MS channels are therefore safety valves, releasing solutes, lowering turgor, preventing rupture.

Elephants did not evolve from *E. coli*. However, mammals are late comers, being only 2×10^8 yr old in the 4×10^9 yr long history of life on Earth. Much of early lives were microbial and the earliest were bacteria-like prokaryotes, in which the 'Central Dogma' and basic metabolism were laid down. Early life forms also had to face the rain-and-shine challenge. It is therefore not difficult to imagine that some primitive devices, not unlike the modern bacterial MS channels, would appear making use of the FFL principle. Thus, like the 'Central Dogma', FFL deals with a *fundamental issue*, that of water. Life is largely aqueous chemistry with water as *the solvent*. Water, 55.5 Molar in concentration, should not be confused with eye-catching signalers (odorants, tastants, hormones, allergens, *etc.*) or other ligands, which are *solutes* working at micro- to miniMolar concentrations. In the long history of evolution, various devices could put FFL to other uses. We speculate that various types of modern force sensing (hearing, proprioception, sensing touch, blood pressure, bone load *etc.*) could come from mechanism that originally gauge the concentration of water, the solvent of Life's aqueous chemistry. We call these the 'Solvent Senses' to distinguish them from the ligand-detecting 'Solute Senses' (smell, taste, hormone, *etc.*) (Kung, 2005).

FFL at Large

The FFL story begins with MscL but will not end with Piezos. Even selfishly considering only the physiology and pathology of humans, many force-driven channels need to be investigated. E.g. TRPV4 apparently measures weight load on developing and mature bones. Mutations results in heritable bone-developmental and other diseases. Heterologously expressed TRPV4 has been shown to respond directly to patch-clamp pipet suctions (Loukin et al., 2009). More sensing mechanisms awaits further investigation; even more force-sensing molecules await discovery. What is the *molecule* in the arterial baroreceptor that measures blood pressure? What is the *molecule* in the circumventricular organ of the hypothalamus that measures blood osmolarity? What *molecules* tell us that our stomachs or our bladders are full? What other *molecules* underlie the large varieties of tactile sensations, from the first kiss to love-making?

TRPs, TMC1, NOMPC and Kv all have the same topology. All are tetramers of subunits, each comprising a S5-S6 core surrounded by four S1-S4 peripheral domains. Recall that even 'Kv channel is as much a mechanosensitive channel as it is a voltage-dependent channel' (Schmidt, del Marmol & MacKinnon, 2012). It would be inconceivable that TRPs are not the same. Although there is no consensus on the detail, it would be difficult to imagine that gating of the heat-sensing TRPV1, which won the other half of the 2021 Nobel, does not involve the lipids that surround it.

As emphasized above, FFL governs all membrane proteins that need to change shape in the bilayer, not just the ion channels. There are *enzymes* such as phospholipase A2 (Lehtonen & Kinnunen, 1992) and *G-protein coupled receptors* such as angiotensin II type 1 (Marullo et al., 2020) that are shown to be sensitive to membrane tension. Moreover, the structural dynamics of prestin, the voltage-dependent motor protein responsible for the electromotive behavior of outer-hair cells (OHCs) has been suggested to follow the changes in deformation of the plasma membrane occurring upon membrane expansion given that prestin conformations in contracted and expanded state changed the membrane bilayer footprint resembling membrane tension (Bavi et al., 2021a, 2021b; Ge et al., 2021). This is analogous to MS channels, which are characterized by occupying larger membrane area in the open state compared to the closed state due to the activation by membrane tension (Hamill & Martinac, 2001) as observed upon activation of the MscL (Perozo, Cortes, et al., 2002) and Piezo1 channels (Yang et al., 2022).

Morphogenesis is also a deep problem. How is it that a lump of amorphous stem cells can develop into an intricately shaped scapula or a different intricately shaped vertebra? It seems intuitive that changing vectoral forces can direct development (Yim & Sheetz, 2012). Marrow-derived mesenchymal stem cells in uniform serum condition develop into neurons on soft gel, into myoblasts on stiffer gel, and into osteoblast on rigid gel (Engler, Sen, Sweeney, & Discher, 2006). Stem-cell MS channels might be involved since substrate rigidity apparently regulates their Ca^{++} oscillation. (Kim et al., 2009). A recent example is TMEM87a/Elkin1, which functions to regulate melanoma cell migration and cell-cell interactions by supporting a Piezo1-independent mechanoelectrical transduction pathway (Patkunarajah et al., 2020).

Force is basic: 4 pN (4×10^{-12} N) breaks a hydrogen bond; 1,600 pN breaks a C-C covalent bond. Chemistry, including biochemistry, can be viewed as mechanics by extension and examined by molecular dynamics/quantum mechanical simulations. Consider that the bilayer not only can be stretched by external forces, but is a compartment with internal forces, in which all membrane proteins operate, many more force-related mechanisms will be revealed in future research. Stay tuned.

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