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The Force-From-Lipid (FFL) principle of mechanosensitivity, at large and in elements

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Abstract

Focus on touch and hearing distracts attention from numerous subconscious force sensors such as the vital control of blood pressure, systemic osmolarity, *etc.* and sensors in non-animals. Multifarious manifestations should not obscure invariant and fundamental physico-chemical principles. We advocate that force-from-lipid (FFL) is one such principle. It is based on the fact that the self-assembled bilayer necessitates inherent forces that are large, and anisotropic, even at life's origin. Functional response of membrane proteins is governed by bilayer-force changes. Added stress can redirect these forces, leading to geometric changes of embedded proteins such as ion channels. The FFL principle was first demonstrated when purified bacterial MscL remained mechanosensitive (MS) after reconstituting into bilayers. This key experiment has recently been unequivocally replicated with two vertebrate MS K_{2p} channels. Even the canonical Kv and the *Drosophila* TRPCs have now been shown to be MS in biophysical and in physiological contexts, supporting the universality of the FFL paradigm. We also review the deterministic role of mechanical force during stem-cell differentiation as well as the cell-cell and cell-matrix tethers that provide force communications. In both the ear hair cell and the worm's touch neuron, deleting the cadherin or microtubule tethers reduces but does not eliminate MS-channel activities. We found no evidence to distinguish whether these tethers directly pulls on the channel protein or a surrounding lipid platform. Regardless of the implementation, pulling tether tenses up the bilayer. Membrane tenting is directly visible at the apexes of the stereocilia.

Keywords

Force-sensing; Mechanosensitivity; Lipid bilayer; K_{2p} ; Touch; Hearing

Introduction

“Never use the words higher or lower.” --- Charles Darwin [20]

For this Special Issue, the Editors’ instruction is as follows: “Encouraged is the presentation of the author’s personal point of view in the context of current literature, controversies *etc.*” We comply. A small portion here covers our recent work, but the bulk deals with broader issues raised by current literature at large and in elements. By “At Large”, we mean to review force sensors beneath human consciousness, in plants and microbes, inside or between cells, and during development. This coverage is intended to balance the emphasis on touch and hearing in current research, reflected in this volume. By “In Elements”, we mean to assert that the force-from-lipid (FFL) paradigm applies to both pro- and eukaryotic ion channels and applies even to channels traditionally classified as voltage-gated or ligand-gated. We argue that FFL is universal. We view the tethers for “higher” mechanosensors as accessories, necessitated by multicellularity. Ultimately, all channels work at a “lower” level obeying the physics of lipid bilayers.

The gears below deck

Being conscious sensations, touch and hearing are near and dear to us. Far more vital, however, are the mechanical force sensors below our consciousness.

Blood pressure is among the first measures taken when one visits a western clinic. Heartbeats and the resulting pressure are too essential to be put under the whim of consciousness. Blood pressure is subjected to baroreflex, a rapid negative feedback that makes short-term adjustments. The sensors, called “baroreceptors”, are not molecules, but are mechanosensitive (MS) nerve endings that are excited by the stretch of major arteries. Despite its importance and the huge medical literature on long-term implications of blood pressure, molecular studies on how stretch excites baroreceptor are rare. ASIC2 of the DEG/ENaC superfamily is expressed in baroreceptors and their ganglia. ASIC2 knockout mice show poor baroreflex and suffer hypertension but live [55]. TRPV1 appears to be expressed in all parts of the baroreceptive neuron and pharmacological blockage of TRPV1 blunts baroreflex [88]. Others found *task-1*^{-/-} mice to have heart symptoms and electrophysiology consistent with a diminished baroreflex [75]. There is no consensus at the moment on which channel(s) conduct the MS receptor current(s) of baroreceptors.

Stretching smooth muscles causes depolarization and contraction, observed in arteries and arterioles that supply skin, lungs, bladder, stomach, intestines, *etc.* This myogenic response, also known as the Bayliss effect, has been examined in assorted arteries, but the force sensor(s) has not been identified. A MS cation conductance on many mammalian cells is well known [26] but is of unknown molecular identity. TRPC1, TRPC6, TRPM5, TRPV4, ENaC, and Cl⁻ channel have all been implicated [6]. See Sharif-Naeini *et al.* (2008) for a critical review [84].

Further below conscious attention is the homeostasis of systemic osmolality. Humans maintain extracellular fluid at 288 mOsm/kg, a set point that is strictly limited to within ±1%. Deviations cause problems from headaches to death. Osmotic homeostasis, found in

all vertebrates and in insects, evolved long before homoeothermy. This early appearance in animals reflects the importance of osmolality and membrane turgor. In vertebrates, plasma osmolality is gauged by “osmoreceptors” in the hypothalamus. Again these are not molecules but neuron clusters. Plasma hyperosmolality depolarizes these neurons to discharge, causing the release of the antidiuretic vasopressin (VP) [83] [9]. Such neurons fire when the bath is made hypertonic or when a deflating suction is applied. Cell-attached patches showed a cation-nonspecific unitary conductance that is stretch-inactivated [70]. Some of these neurons express a shorter N-terminal splice variant of TRPV1. *trpV1*^{-/-} mice show serum hyperosmolality and poor VP responses to osmotic challenge and their neurons respond to hyperosmolality poorly [82]. Nonetheless, these mice survive and mature, showing that TRPV1 cannot be the sole sensor for systemic osmolality. There are evidences including those from *trpV4*^{-/-} mice [48] that indicate the participation of other TRPV channels [83]. Some osmoreceptor neurons also express a variety of two-pore domain K⁺ channels (K_{2p}'s) [9].

VP regulates the function of the kidney, where plasma is filtered into urine across the slit diaphragm between podocytes (glomerular epithelial cells) [78]. Podocytes express components akin to those discovered in the genetic dissection of touch in *C. elegans*. An essential component of the worm MS-channel complex is MEC-2, an SPFH-domain membrane protein that recruits cholesterol [24]. Podocytes expresses its counterpart, podocin, which complex with TRPC6 channel [34]. The latter is activated by hypo-osmolality or by prodding [2]. Cholesterol-rich thickened lipid domain, partly constructed with these SPFH-domain proteins, has been proposed to provide proper platforms to stage MS channels [3]. *SLP3*^{-/-} mice, lacking one of these SPFH proteins show very poor touch sensation [92], much like the *mec2*^{-/-} worm.

It takes an astronaut three years to gain back the bone lost in a three-month space flight. Mature bones constantly measure load to remodel themselves, a remarkable type of mechanical force sensing, though not in the timescale of hearing and touch. Unloading osteoporosis places bed-ridden patients at risk of bone fracture. Unloading-induced bone loss is suppressed in *trpV4*^{-/-} mice [63]. Over 50 alleles of *TRPV4* mutations are now known to cause developmental bone diseases ranging from dwarfism to neo- or prenatal death [67]. We have shown that TRPV4 responds to hypo-osmolality when expressed in yeast [54] and opens directly to stretch force in patches excised from expressing *Xenopus* oocytes [53]. Disease mutations seem to all be gain-of-function alleles, meaning that the mutant channels have higher basal open probability (Po) than that of the wild type. Further, the severity of the bone disease appears to parallel the basal Po, indicating that channel leakage and Ca²⁺ poisoning may be the cause [52]. The mechanism of how bone senses load remains unclear. TRPV4 appears to have a role in the maturation of osteoclasts [60]. A major puzzle in this field is the fact that *trpV4*^{-/-} mice, with a complete loss of TRPV4 function, develop almost normally.

The FFL paradigm

Besides those above, the monitored forces in our body also include shear stress on ciliated epithelia, intraocular pressure, let alone those in the electro-mechanical feedbacks of the

pounding heart, the peristalsis of the digestive tract, and many more. Beyond the obvious, physical forces also play paramount roles during development and cell-cell communication (below). Further, microbes and plants display their own forms of mechanosensitivity (MS) (below). MS is as pervasive as it is mysterious, peculiarly so in comparison to our deep knowledge of ligand- and light sensing. Manifest diversities, however, should not obscure the common fundamental principles behind. All inheritance, from pea-seed color to sickle-cell anemia, traces back to DNA. The H^+ -gradient-based chemiosmotic theory unifies oxidative phosphorylation and photosynthesis, which energize all creatures, pink or green. Occam's razor aside, we believe that there is a fundamental unifying principle for the myriad force sensations.

Soluble proteins are uniformly bombarded by particles all around (Fig. 1a), but proteins embedded in the bilayer are subjected to large anisotropic forces (1b). Akin to surface tension at any water-oil interface, there are large and sharp lateral tensions layers in the membrane. These tensions, peaking at hundreds of mN/m, locate at the two polar-nonpolar interfaces at the level of the lipid neck. Because the bilayer is a self assembled stable structure, this tension is balanced by repulsions between the head groups and between the interior acyl tails (Fig. 1b). This layered internal force distribution has been calculated from thermodynamics [13] and examined by molecular dynamics simulation [50, 27]. Proteins embedded in the bilayer are subjected to these push and pull. See Anishkin *et al.* (2014) [4] for further explanation. Externally added stretch force or chemical modifications of lipids can alter force magnitude and direction, motivating embedded proteins to change shape. An example easy to visualize is bilayer thinning by stretch that leads to hydrophobic mismatch at the protein-lipid interface, driving the protein to a better-matched conformation. The thinning of MscL accompanied by a large area increase (ΔA) when under tension (T) is one such example [73]. $T \Delta A$ describes the energy difference between the closed and the open state and can be deduced from the Boltzmann slope of P_o over T . Bilayer deformation can change protein geometry in different ways. Thinning, for example, can favor and shorten the gramicidin-A hemi-channels to assemble, leading to its well-known MS [56]. Although this review and this volume center on ion channels, the FFL principle applies to all membrane-embedded proteins, including MS enzymes. In a membrane, essentially *any* change in protein shape will exact some energetic price, as some parts will move along or against the strong components of the pressure/tension profile. Therefore, any external condition that change the lipid forces will change the energy cost of the conformational change, rendering membrane proteins mechanosensitive to various extent, physiologically relevant or not.

We have also argued that life's origin requires enclosing RNA *etc.* with an envelope of amphipaths, likely abiotic lipids. Even this primordial bilayer comes with a set of physical properties, including its anisotropic forces described above [4]. In the capricious primordial soup, one of its early functions is likely to deal with the fluctuation in the concentration of water, the solvent of life. Thus stretching the bilayer is likely an "original sense" [4] and a mechanism that has been continuously employed throughout evolution. Perhaps not as erratic, but environmental water concentration still continually changes today. Water concentration is measured by the total particle bombardment on the bilayer, *i.e.* the osmotic force. Water being the universal biochemistry solvent, sensing of osmotic and other forces

can be said to be “solvent senses” as opposed to the “solutes senses” that measures ligands by lock-and-key bindings [44].

The force-from-lipid (FFL) principle was established some 25 years ago. This began in the first patch-clamp survey of *E. coli* membrane, when Boris Martinac encountered large MS unitary conductances [59], and also found them activated by amphipaths [58]. Eventually, purified MscL was reconstituted into lipid bilayers and found to retain its MS [87]. As an exercise of ultimate reductionism, this experiment excludes any tether proteins or other channel subunits, leaving the bilayer as the only source for the forces that gate MscL. In fact, the MscL protein was isolated by tracking the reconstituted MS activity in column fractions starting from *E. coli* lysate, much like enzyme purification [86]. Much crystallographic, biochemical, genetic, and biophysical work on MscL and its analog MscS followed, as reviewed by Martinac, by Rees and their coworkers in this volume and elsewhere. However, there has always been the notion that gating directly by bilayer stretch is restricted to the “lowly” bacteria. A current review [21] states “Two primary models have been proposed for mechano-gating: the lipid bilayer stretch model evidenced by microbial MS channels and the more sophisticated tether model of eukaryotes by which tethers pull open the transduction channel”. Before we deal with the “more sophisticated tether” below, we wish to point out that the FFL principle also applies to animal channels. Membrane blebs, apparently devoid of cytoskeleton, retain the activities of the native MS channels in *Xenopus* oocyte [29], [94]. Doping membrane with various amphipaths activates such MS channels [58] [33]. Lipids with polyunsaturated fatty acids (PUFAs) enhance, and are likely required for the *C. elegans* touch response [91]. Dissociating subcortical cytoskeleton liberates rather than inhibit MS channels (See below). Nevertheless, these experiments were performed on complex cell membranes. It is again the ultimate reductionism of reconstituting purified animal MS channels below that proves the broad, if not universal, application of the FFL principle.

The “proto-behavior” of “eu-channels”

Enriched fractions of NMDA receptor channel [42] or TRPC1 [57] have been reconstituted showing MS. While we await replications and extensions of these works, two recent reports [8, 12] demonstrated incontrovertibly that two-pore-domain K^+ channels (K_{2p} 's) retained their MS upon reconstitution into bilayers, like MscL and MscS. The molecular biology and electrophysiology of the K_{2p} TREK1 (TWIK-related K^+ -1) and its homolog TRAAK (TWIK-related arachidonic acid-stimulated K^+) have been extensively studied by Patel, Honoré, and co-workers [72] [33], laying a solid foundation for further investigation. TREK-1 current is suppressed by hyperosmolarity, and activated by pressure in either direction, and by acidic phospholipids, lysophospholipids, polyunsaturated fatty acids (PUFAs), or volatile general anesthetics [72] [33]. Atomic structures of two K_{2p} 's [62] [11] [10] are now known. TRAAK has features not found in KcsA, Kv, Kir, GIRK, or BK. It has a two-fold symmetry and its second transmembrane helix from each of the two subunits is long, with a kink that makes the inner portion lying almost flat. This portion is amphipathic, placing the two peptides at the membrane-cytoplasm interface of the inner leaflet, rich in acidic lipids *in vivo*. Interestingly, the inner transmembrane half of K_{2p} channels is fenestrated. A sizable portion of TRAAK's inner half is not enclosed by protein, leaving a 5

Å-wide gap extending from the bottom of the filter to the lower edge of the channel, presumably filled with lipids *in vivo* [11].

Berrier *et al.* [8] have recently reconstituted an enriched fraction of the mouse TREK-1 into liposomes and examined patches excised from them. They observed the ~80-pS K⁺-specific outward-rectifying chlorpromazine-sensitive conductance. Surprisingly, these channels displayed spontaneous activities that could not be further increased by pipet suction. Positive pipet pressure pulses, however, could proportionally close the channels. These authors reasoned that Po has been maximized by inherent tension in the patches [8]. Such a tension is likely generated during the gigaOhm-seal formation, bonding the bilayer lipids to the pipet glass surface [71, 80, 79]. See below.

In a recent rigorous study, Brohawn *et al.* [12] purified zebrafish TREK-1 and human TRAAK to homogeneity, as evidenced by the monodispersion of column-elution profiles (Fig. 2a), and also reconstituted them into liposomes made of mixed length phosphatidylcholine. In excised patches, they found the channels to respond instantaneously to applied force (Fig. 2b). These channels are also activated by arachidonic acid, a PUFA. TRAAK responds proportionally to either negative or positive pressures much like the bacterial MscL in a similar setting [74]. This indifference to pressure direction leaves no doubt that it is the added bilayer tension that gates the channels. Beyond demonstrating the FFL principle for animal channels, these authors also expressed TRAAK in cultured mouse Neuro2A cells. Prodding these cells with a probe evokes a K_{2p}-dependent outward current that counteracts the native inward current attributed to Piezo1. Details aside, both sets of reconstitution experiments clearly showed that vertebrate K_{2p}'s, much like MscL and MscS, are themselves sensitive to stretch force from the lipid bilayer, requiring no additional subunits, cytoskeleton, or extracellular tethers (Fig. 1c).

MS everywhere ?

Conventional classification of channels into voltage-gated, ligand-gated, Ca²⁺-activated, *etc.* confines one's imagination. Mechanosensitivity (MS) has been reported for MscL, MscS, Mec-4/10, MCA, TRP, Piezo, K_{2p}, Kv, K_{Ca}, Nav, NMDA receptor, CFTR, AChR, *etc.* by patch clamp, by Ca²⁺ imaging, or by other criteria (see [3] and references therein). Just like voltage or temperature sensitivity, the magnitude and the biological meaning of such MS remains to be explored in each case. Note that these channel families have no sequence homology and one cannot define a "force-sensing" domain. By the FFL principle, force sensing reflects change in channel geometry in the direction of force, not defined by particular amino-acid sequence.

Hypo-osmotic swelling opens the canonical Kv in cultured cells [79]. Kv also behaves very differently in whole cells, in patches, or upon reconstitution into planar bilayers. These differences apparently originate from changes in membrane tension. This tension is low when the membrane is locally constrained by subcortical cytoskeleton, but increases when detached, and further increases when bilayer adheres to the glass [80] [79]. Kinetic analyses show that the tension-sensitive step is the final opening of the gate, after the four voltage sensors have moved, *i.e.* when a lateral expansion (ΔA) takes place. The simplest

interpretation is that tension acts to couple the elevated voltage sensors to the gate through lipids. In excised patches, the tension generated by lipid adhesion to glass is ~ 0.5 to 4 mN/m [71]. As estimated from the changes of the rate of opening step with or without added tension, free-energy changes indicate a ΔA of Kv to be ~ 3 to 4 nm², consistent with structural observation [79]. Because this tension-dependent behavior varies with lipid compositions in the Kv reconstitution [80], the tension must come from the lipids. The effect of this small tension is not trivial. Po rises from 0 to 0.65 between -60 and $+50$ mV, but rises from 0 to 1.0 upon suction at mid-range voltages. Thus, Kv channel is as much a MS channel as it is a voltage-dependent channel [79]. This MS of Kv is exploited by Nature. Hao *et al.* (2013) [30] show that, in DRG neurons, channels with Kv1.1 subunits respond to prodding to pass an outward K⁺ current, acting as a brake to oppose the touch-induced inward current. Mice expressing a *Kv1.1* dominant negative allele suffer severe mechanical allodynia. See the Hao and Delmas review in this volume.

Phototransduction in rods and cones begins with rhodopsin and ends in the closing of a channel gated by cGMP. In *Drosophila*, it ends in the opening of TRP and TRPL, the two canonical TRPC's. One would naturally assume that a second messenger gates these TRPs. Blind-fly analyses implicated lipid metabolism, especially the key role of phospholipase C (PLC), which splits PIP₂ into DAG and IP₃. Yet, much research has failed to show direct activation by lipid ligands. It came as a surprise that a paper entitled "*Photomechanical Responses in Drosophila Photoreceptors*" appears in *Science* in 2012 [31]. Astonishingly, the authors showed that light induces a near-micrometer shrinkage of the ommatidia (units of the compound eye, comprising supporting and photoreceptor cells), which is directly visible under a light microscope or quantified with an atomic force microscope. The contraction precedes the channel current. Deleting the PLC removes the contraction; deleting the channels does not. It is modeled that beheading PIP₂ to make the smaller DAG increases tension in the inner membrane leaflet to pull open channels. Gramicidin A [56], the well-established MS channel, was added to the photoreceptor cell and found to pass current proportional to light intensity. Note that the vertebrate retina also has intrinsically photosensitive ganglion cells that use a homologous pathway for circadian entrainment [35]. The melanopsin there is far more similar to the insect rhodopsin than vertebrate rhodopsin [81]. Melanocyte [7] and keratinocyte [65] of the skin appear to have similar pathways. Small microvilli, cilia, filopodia, or dendritic spines are common, but unlike the ommatidium with thousands of stacked microvilli that house the phototransduction complex, physical movement cannot easily be detected in these isolated small structures. These structures, however, often have signal-transduction pathways with PLC and channels. The current dominant paradigm for these pathways ends in protein phosphorylations due to PLC's activation PKC. The PLC-induced changes in bilayer tension reviewed here may in fact participate in these pathways [49].

The jungle

Our sense of self-importance should not blind us to the vastness and richness of the biological world. Humans, vertebrates, and indeed all animals together are only a minor part of the biosphere. Microbes out-weight animals in number, in variety, and in total mass. *E. coli* is undoubtedly smaller and less complex than *Homo sapiens*, but it is just as modern and

successful. Another common misunderstanding is that microbes are ancient relics and use outmoded machineries. Billion years of divergence have led to different life styles and therefore different implementations of the basic molecular ingredients. Yet, the fundamental machineries necessarily evolved early and remain compulsory for all. Universal are the DNA-RNA-protein information flow, ATP synthesis and hydrolysis, the lipid bilayers wrapping, *etc.*

We have pointed out the need to face up to osmotic changes even for the primordial protocells. Whether it is the osmotic force or a physical impact, the membrane deforms accordingly. A Newton is a Newton. The sense of touch must have evolved early since it is seen in both uni- and multicellular forms. Bacterial flagella respond to the external mechanical force - slowing flagellum rotation is sensed, possibly as the change in the charge flow through the rotor [38]. *Paramecium*, a large eukaryotic unicell, reverses its ciliary beat when impacted at its anterior end to perform an “avoiding reaction” observed in the 19th century [36]. Intracellular recording shows that impact triggers a Ca^{2+} action potential in the cilia [22], which can be erased by deciliation [69] or by genetic means [77] to reveal a cation-nonspecific touch receptor potential originated from the soma. In multicellular animals, primary cilia sense force and are pivotal in development. They also become sensory cilia, including the kinocilia of the inner ear. See review by A. Patel in this volume.

Although some might think that the only reason to study bacteria is to find ways to kill them, microbes in fact contribute to our own survival and to biological research in important ways. Their lifestyle obviates the paraphernalia needed for multicellular existence, which complicate research. For example, in hearing research, various accessories have been identified, but the strenuous and perennial search for the true hair-cell transduction channel remains inconclusive, as is evident from this volume.

The entire Central Dogma was erected from the study of bacteria and bacterial phages, leading to the slogan “What is true to *E. coli* is true to the elephant” during the romantic period of molecular-biology revolution in the 1950’s. We would argue that the revolution is not yet over and some basic principles are yet to be discovered. Microbes are widespread and have explored essentially all organic and inorganic niches on earth where water is available. In so doing, far more molecular fabrications have been explored by microbes than by the animals. Their wide expeditions resulted in materials we can exploit as tools. Recall the restriction endonucleases and taq or pfu polymerase, without which, few of today’s research laboratories or biotech companies can operate. There are still many microbial tricks we have yet to employ. A brand-new tool is called the CRISPR-Cas system (*clustered regulatory interspaced short palindromic repeats - CRISPR-associated*), which is a bacterial and archaeal anti-viral immune system (Yes, they have one). Since 2012 [37] CRISPR-Cas has been advancing research on microbes, worm, fly, fish, mouse, monkey, and plant, *i.e.* revolutionizing the entire fields of genetics and genomics at this very moment [89].

Plants form another sizable part of the biosphere. When touched, *Mimosa pudica* droops within our attention span and therefore draws human attention. This, however, is an exceptional adaptation. Vascular plants detect the forces of rain, wind, predators, *etc.* by thigmomorphogenesis, *i.e.* altering growth pattern “slowly” by human standard. They also

sense gravity to direct the shoots to grow up and the roots to go down (gravitropism). Darwin described in detail these responses in 1881 [19]. Current research showed that these processes employ Ca^{2+} -binding proteins, Ca^{2+} , and therefore Ca^{2+} -passing channels, including MscS homologs [64]. See reviews by Martinac *et al.* in this volume.

From egg to chicken

Eukaryotes include unicellular fungi, algae, flagellates, ciliates, amoebae, diatoms *etc.* Multicellularity recurred in brown-algal, red-algal, animal, fungal, and plant lineages. It could, for example, begin with the failure of daughter cells to separate, a phenotype commonly observed after laboratory mutageneses of unicells. Adhered cells became specialized to gain selective advantage for the clonal colony, a process recapitulated in embryonic differentiation.

Multicellularity presents two challenges. One is the development from the unicellular zygote. The current frantic research on mammalian stem-cell differentiation defies a review here. For our purpose, suffice it to say that embryonic, pluripotent, or adult stem cells all respond strongly to mechanical stimuli [93]. Though small diffusible growth factors or inhibitors are needed, the fate of the stem cells, however, depends on the mechanical properties of the extracellular matrix. There are now chemically defined hydrogels of different stiffness that present peptides to bind the glycosaminoglycans of human embryonic stem (hES) cells. Only stiff gels maintain the proliferation and pluripotency of hES cells [66].

Adult stem cells, such as the marrow-derived mesenchymal stem cells (MSCs) circulate, engraft, and differentiate into different tissues. In identical serum condition, MSCs' fate can be directed by the elasticity of the collagen-containing polyacrylamide surface, controlled by the degree of cross-linking. Matching the stiffness of the target tissue, MSCs become neurons on soft gel, become myoblasts on stiffer gel, and osteoblast on rigid gel [23]. Thus developing cells actively probe their physical environment by an integrin-actomyosin-based force-feedback mechanism. Though some components are identified, the mechanism of how force mediates differentiation as a whole remains obscure [93] and even controversial in details [90]. Multiple K^+ , Ca^{2+} , Cl^- , or TRP channels are found in stem cells and appear needed for proliferation [47]. Lowering the substrate stiffness reduces the frequency and the magnitude of the MSC Ca^{2+} oscillation of unknown significance [40].

Plant growth is ultimately driven by turgor pressure, reminding us again the universal importance of osmotic pressure. See [28] on how forces guide the tissue differentiation at the shoot meristem.

Links and tethers

The second complication of multicellularity is cell-cell communication in the adults. Within animal cells, stresses are borne by the cytoskeleton, which, however, is not a eukaryotic innovation. It presumably existed in the common ancestors, since prokaryotes express FtsZ, MreB and crescentin, the respective homologs of actin, tubulin, and the subunit of

intermediate filament [15]. Prokaryotes use them in growth, morphogenesis, DNA partition, cell division, and motility.

The subcortical cytoskeleton of animal cells refers to the fishnet of actin fibers, which subtends and attaches to the plasma membrane. Rat TRPV4 remains responsive to hypo-osmolarity when expressed in yeast [54] and can be stretched open in *Xenopus* oocytes [53]. It is difficult to see how the rat channel receives force by reconnecting with yeast or toad actins. Cytochalasins or latrunculin, which dissociate actin fibers, enhance rather than disrupt MS-channel activities [26] [45]. Mutating β -spectrin (UNC-70), the link between membrane and cytoskeleton, reduces the force needed to pull membrane tether from the worm's touch-receptor neurons [43]. The canonical Kv is progressively more active in whole cells, in patches, or upon reconstitution into planar bilayers (above) [79]. These and other observations indicate the subcortical actin filaments protect MS channels from opening rather than pulling them open.

Animal cells adhere with neighbors through adherence junctions and to matrix through focal adhesions. Projecting internally from these adhesion foci are stress fibers, each comprising 10–30 actin filaments with attached cross-linkers and myosin motors. The actomyosin produces tensions in the stress fibers between anchors. Stationary focal adhesion sustains a ~2.5 pN stretch force [25]. Stretching actin stress fibers in human umbilical vein endothelial cells with an optical tweezer activates Ca^{2+} -passing channels of unknown identity near focal adhesions. Piezoelectrically moving beads attached to focal adhesion activates current within 10 msec, peaking within 100 msec. [32]. Pulling on magnetic microbeads attached to integrin through an antibody activates a Ca^{2+} influx within 4 msec, attributed to TRPV4 [61]. Even though it is far from the speed of the hair-cell transduction channels [17], there is little doubt that stress fibers can relay force to open channels. However, it is not known whether the channel is attached to the fiber directly, through linker proteins, or through a lipid raft (see below and Fig. 1d,e).

Accessory structures besides the MS channel are found in the two key animal models: the vertebrate hair cells for hearing and the *C. elegans* touch cells. In both cases, removing these structures reduces but does not eliminate the MS-channel responses. Engineered mutations into protocadherin-15 or cadherin-23 remove the tip link, but transduction currents can still be recorded from hair cells with reduced amplitude and, surprisingly, responding to the negative phase of the sinusoidal force stimulus, in the direction of moving the hair bundle away, instead of toward, the kinocilium [1]. Mutants lacking Tmc1 and Tmc2 also display reduced and reverse-phase transduction currents [39], as do nascent hair cells in fish lateral-line organs before tip-link formation [41]. Though there are other explanations, it seems possible that such currents are through MS channels in untethered membrane being flexed by vibrations. Prodding the touch neuron in the dissected worm generates transient inward currents. As expected, mutants lacking channel pore-forming subunits (MEC-4, MEC-10) or the crucial cholesterol-gathering protein (MEC-2) do not show such currents. However, the mutant without the long microtubule due to a null mutation in *mec-7*, which encodes β tubulin, still displays such currents, albeit smaller [68]. It seems possible that tethering the MS channels to the tip link or the microtubule relay, orient, or amplify the force signal, but the channels ultimately respond to membrane deformations by the incoming mechanical

force (Fig. 1d,e), though the response would be greatly reduced without these accessories. See other reviews in this volume.

The lipid bilayer

Voltage, heat, and force are universal physical parameters. The sensing of all three might all have originated from and still requires the lipid bilayer. It is certainly this insulator that erects any electrochemical gradients, including the all-important H^+ gradient. Live cells, from human to bacteria, all maintain a resting potential across this insulator. S4's arginines dominate current thinking in channel voltage sensitivity. However, all α helices are dipoles, often even decorated with polar or charged residues, making essentially all membrane protein voltage-sensitive to differing degrees. Electrophysiological protocols are applied to many ion channels not classified as being voltage-gated, such as Cl^- channels, TRPs, *etc.* Heat governs all molecules and reactions. Though there are thermodynamic arguments [18], we currently find no convincing molecular mechanisms that explain the unusual heat or cold sensitivity of certain TRP channels. Involvement of surrounding and bound lipids seems inevitable. In the recent atomic structure, a density, likely from an endogenous lipid, occupies the capsaicin-binding site of the closed TRPV1 [14].

Parallel to the “RNA-world” theory for the origin of life, there is a “lipid-world” theory that argues for the need and the early appearance of an amphipathic envelope that partitions the cell from the primordial sea [16]. Such abiotic amphipaths include fatty acids, which could have arrived from meteorites and can be generated from reactions simulated in the Miller-Urey experiments. Key to the lipid-world notion is that these amphipaths, on their own, assemble into micelles, monolayers, bilayer fragments, or vesicles, through entropy-driven hydrophobic interactions, requiring no genetic or divine guidance. Note that anisotropic forces within even a naked lipid bilayer can respond to deformation, heat, pH, ions, or heat-induced chemical modifications (See [4] for references). These primitive senses afford a base for improvement by the inclusion of embedded impurities as arsenal in evolutionary competitions.

The ever-increasing number of atomic structures of ion channels greatly enhances our knowledge. Visualizing the amino-acid arrangements, however, often led us to forget the surrounding lipids. When included in the crystallization process of Kv, adjacent lipids can clearly be imaged. They are an integral part of the channel structure, tightly bound to both the peripheral and the pore domains of Kv. These lipids retain an overall semblance of a bilayer, but some are clearly distorted by specific binding to amino acids [51]. The surrounding lipids, often called annular lipids, are continuous with the bilayer, while those bound specifically to certain amino-acid pockets can be regarded as channel co-factors [46]. The force distribution calculated from the pure lipid bilayer reviewed above and elsewhere [44, 4] will certainly be distorted at the lipid-protein interface. When an embedded protein changes conformation, the associated lipids will have to reconfigure as well. The free-energy change associated with the bilayer deformation is estimated to be comparable to that of the voltage-dependent part of the total gating energy of voltage-gated channels [76].

Anishkin and Kung (2013)[3] reviewed evidences showing that, in animal cells, foci that are subjected to external forces are membrane rafts enriched with cholesterol and sphingolipids. Such domains are found at the apex of hair cell [95], at the *C. elegans* touch receptor complex, and at ordinary adherens junctions between cells as well as the cell-matrix focal adhesions. These more ordered lipid platforms function to suppress mechanical noise, which is liable to cause leakage through sensitive MS channels. Such platforms also confine and/or redirect incoming force. These authors even speculate that such lipid platforms can be dynamic and rearrange in response to the external force. Regardless of such lipid platforms, an unanswered question for animal force sensors is whether the tether force is pulling the channel protein directly or pulling the surrounding lipids (Fig. 1d, e). Proteins function in aggregates: ribosome, lysosomes, chromosomes, endosomes, spliceosomes, *etc. etc.* Ion channels can certainly be associated with various tethers, but there is no clear evidence that these associations transmit force directly. The possibility of the tether pulling on the lipid platform remains open.

Assuming that the ion channel is directly pulled by the tether, we still need to consider the fact that the channel is intimately glued to the annular lipids and beyond. As cited above the free-energy change in deforming the bilayer is comparable to that associated with voltage gating of the Kv protein [76]. In other words, when such a tether displaces and deforms a MS channel, it necessarily deforms the surrounding lipids. This is even directly visible, as tip-link leaning causes tenting of the apical membrane of stereocilia [5] (Fig. 1d, e, left vs. right).

Retrospect and prospect

Mechanical force sensing is pervasive: above and below our awareness, during development and in adult, in organs and in cells, in bacteria, archaea, and eukaryotes (green or otherwise). ***Such extraordinarily broad manifestations should not blind us from basic molecular principles.*** We advocate that force-from-lipid (FFL) is the unifying paradigm. Due to entropy-driven hydrophobic interactions, lipid bilayer self-assembles at the life origin to enclose the first cytoplasm. It continues to be crucial, although often regarded as a simple barrier, cartooned as two straight lines. Historically, tremendous efforts had been wasted in the 1950's and 60's hunting for "the" elusive high-energy intermediate for ATP synthesis, while the answer lay in the H^+ gradient sustained by the bilayer. In recent years, voltage sensing is gradually, if only grudgingly, accepted to involve bilayer lipids. We believe that the bilayer holds other secrets, some relate to its mechanical properties as described here and elsewhere [3] [4].

We all begin our scientific education with the 17th century formula $F = m \cdot a$. Thus, it is a great irony that, in this 21st century, we still don't have a clear molecular explanation for how we, ourselves, feel F . Don't ask your physicians what measures blood pressure inside your body. It will only serve to embarrass them. Current research in the vast field of mechano-sensations is peculiarly retarded, compared to photo- or chemo-sensations. FFL could provide a paradigm for future research, but research will remain challenging. Lipids are numerous and mixed. Membrane is a protein-rich mosaic and not a lipid sea with protein icebergs. Lipid chemistry is unfamiliar, especially difficult *in situ*. Some current biophysical

tools, *e.g.* patch clamp, have inherent artifacts and complexities [85]. New tools, such as fluorescent dyes that gauge bilayer deformations, will be greatly welcome. Meanwhile, anthropocentricity and the drumbeat for translational research aside, we encourage broader thinking to avoid the risk of degrading biology into a drug-discovery-fest.

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Fig. 1.

a and **b** contrast forces acting on soluble vs. membrane proteins. While soluble protein is isotropically pressed by the bombardment of surrounding molecules, membrane-embedded proteins are subjected to large and anisotropic pulls and pushes originating from the lipid bilayer. See text and [4]. **c–e** illustrate the force-from-lipid (FFL) principle applied to different arrangements. **c** diagrams that a stretch of the bilayer opens purified channels reconstituted in lipid bilayer, including the bacterial MscL, MscS, but also the vertebrate TREK-1, TRAAK and others. **d** and **e** are two possible models of how a tether gates an MS

channel, indistinguishable by current data. The tether either pulls on the channel protein directly (**d**) or on the surrounding lipids, likely a cholesterol-rich raft (orange) [3] (**e**). Regardless of the actual arrangement, tether pulling deforms and tenses up the bilayer around the channel, shown here as tenting, (**d**, **e** right), which has been directly observed in the apexes of stereocilia [5].

Fig. 2. Purified human TRAAK reconstituted into lipid bilayers retains mechanosensitivity. **a.** Monodispersion of column eluate indicates the purity of TRAAK protein used. **b.** Such protein is reconstituted into liposomes. TRAAK channels in a patch excised from such a proteoliposome are activated (upper traces) in proportion to applied suction (lower traces). From [12].