Stiffened lipid platforms at molecular force foci

Andriy Anishkin[a] and Ching Kung[b,1]

[a]Department of Biochemistry and Center for Computational Proteomics at the Huck Institute of Life Sciences, Pennsylvania State University, University Park, PA 16802; and [b]Department of Genetics, Laboratory of Cell and Molecular Biology, University of Wisconsin, Madison, WI 53706

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2011.

Contributed by Ching Kung, February 5, 2013 (sent for review January 11, 2013)

How mechanical forces are sensed remains largely mysterious. The forces that gate prokaryotic and several eukaryotic channels were found to come from the lipid membrane. Our survey of animal cells found that membrane force foci all have cholesterol-gathering proteins and are reinforced with cholesterol. This result is evident in overt force sensors at the tips of stereocilia for vertebrate hearing and the touch receptor of *Caenorhabditis elegans* and mammalian neurons. For less specialized cells, cadherins sustain the force between neighboring cells and integrins between cells and matrix. These tension bearers also pass through and bind to a cholesterol-enriched platform before anchoring to cytoskeleton through other proteins. Cholesterol, in alliance with sphingomyelin and specialized proteins, enforces a more ordered structure in the bilayer. Such a stiffened platform can suppress mechanical noise, redirect, rescale, and confine force. We speculate that such platforms may be dynamic. The applied force may allow disordered-phase lipids to enter the platform-staging channel opening in the thinner mobile neighborhood. The platform may also contain specialized protein/lipid subdomains enclosing mechanosensitive channels to open with localized tension. Such a dynamic stage can mechanically operate structurally disparate channels or enzymes without having to tie them directly to cadherin, integrin, or other protein tethers.

force sensing | lipid bilayer | lipid rafts | mechanosensitivity

We rely on things "tangible," but are "touched" by things that are not. When "stressed," we are "tense" or even "depressed." We wish to "hang loose" and not be "uptight." These at times conflicting terms are not just quirks of the English language. "Feelings" in Chinese can be a disyllabic compound of "sense," 感, and "touch," 触. Mechanosensation is clearly deep in the human psyche and it can surface as ecstasy or agony, from the first kiss to childbirth, including all of the mechanics in between.

Besides hearing, touch, and other overt senses, such as balance, proprioception, and organ extension, there are key force-sensing processes that do not rise to our consciousness. These processes include the myogenic tone adjustment (the Bayliss effect), the regulation of blood pressure, and the less discussed but even more crucial homeostasis of systemic osmolarity. Embryonic development entails local and global migration of cells that gauge and respond to traction, and unwanted migrations lead to tumorigenesis and metastasis. In the broad sphere of biology, nearly all animals, including unicellular paramecia and amoebae, display a sense of touch. Those that fly respond to wind and gravity; those that swim respond to current, waves, and tides. Plants use gravity to orient the growth of their roots and shoots: they proportion their growth in girth and in height according to how much they are jostled by wind and rain. Less obvious is the sensing and responses to osmotic pressure, which is the fundamental way for organisms to measure water concentration. Despite its universality and importance, how force is sensed remains poorly understood. This ignorance is glaring compared with our deep knowledge of how light and ligands are detected. We know that stereocilia detect sound, Merkel cells sense touch, and specialized neurons (baroreceptors) gauge blood pressure, and so forth, but the frontline molecules that receive and transduce force are unknown. We seem to have fewer tools or clear concepts to study, such molecules. Among these molecules, mechanosensitive (MS) channels allowed the deepest investigation at the moment.

This article is not a comprehensive review of mechanosensation or even MS channels. Rather, we shall first briefly recall the study of the bacterial MS channels, MscL and MscS, of which there is a better understanding. These channels reveal a basic principle: the mechanical force that operates these channels comes from the lipid bilayer and not any putative associated proteins. We will then assert that all channels, and indeed all membrane-embedded proteins, are mechanosensitive, at least in principle. The remainder of this article reviews a sizable literature from diverse sources, never previously brought together. It appears that the bilayer is locally modified at points of force reception. Where an animal cell is pulled by a molecular tether, the bilayer is apparently more ordered and stiffened by the concentration of cholesterol and sphingolipids. This is not only true to specialized force foci for hearing and touch but also to the more general cadherin foci and integrin foci. Although the cell-biology literature emphasizes the role of lipid rafts in membrane traffic and signal transduction, we herein address the mechanical aspects of the cholesterol-rich platforms. We end by proposing models, speculating that such ordered bilayer platforms may be dynamic or may harbor specialized subdomains enclosing the channel. In such arrangements, channels or enzymes therein can experience and adapt to the force without having to be directly attached to the force-transmitting tether.

Force from Lipid Bilayer

The nanoSiemens (nS = 1,000 pS) unitary conductances of MscL and MscS stood out in the first patch-clamp survey of the surface of contrived giant *Escherichia coli*. Pipet suctions readily activated these channels (1). Amphipaths, expected to alter the geometry and therefore the internal force distribution, were soon found to activate these channels (2). These unitary conductances and their mechanosensitivity were found to survive reconstitution of material from the bacterial membrane lysate into artificial liposome membranes (3). This crucial observation, together with the large electric signal and the unlimited starting material, which is cheap and does not arouse ethical concerns, set the stage for purifying the channel proteins (4). Purifying a channel like an enzyme by following its activity has never been done before or since. Partial sequence of the protein reveal the small MscL gene (4). The electrophysiological, crystallographic, genetic, biochemical, EPR spectroscopic, and molecular dynamics simulation studies that follow are frequently reviewed (3, 5–7) and not detailed here. In short, MscL is a homopentamer of subunits, each with only 136 residues forming two transmembrane α–helices (TMs). The five TM1s converge to close the channel. Upon a bilayer stretch they lean down and move outward, flattening the entire

Author contributions: A.A. and C.K. analyzed data and wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: ckung@wisc.edu.

protein. This conformational change opens a 25 Å aperture that allows the passage of particles up to some 1,000 in molecular weight and accounts for the 3-nS conductance. Comparable advances were made in the study of the 1-nS MscS (8). That wild-type bacteria, but not the *mscL*$^{-}$*mscS*$^{-}$ double mutant, that survive dilution of the medium showed that these channels act as safety valves upon osmotic down shock, releasing osmotica to avoid lysis (8). This observation not only explains the selective pressure leading to the evolution of such MS channels in nearly all free-living prokaryotes, but also explain why near membrane lytic tension is required to open some of these channels such as MscL (3).

That MscL or MscS, purified to homogeneity, can be reconstituted into bilayers of defined lipids and remain mechanosensitive, leaves no doubt that the gating force comes from lipids. The work that drives channel opening is equal to the force times the changes in geometry, in this case, tΔA (i.e., membrane tension times the increase in the footprint of the channel protein and the immediate surrounding lipids). The lipid-embedded TM helices form the gate and need to expand or contract within the lipid bilayer. As described above, MscL flattens to open, as if to meet a thinner bilayer (5–7, 9, 10). The ΔA is large for these channels, being ~20 nm^2 in the case of MscL (3). We also have some knowledge on how the gating of such channels is actuated beyond this general formulation. Because lipids are amphipaths, they self-organize to face water, leading to a reduction the free energy at the interface and generating a sizable lateral tension (hundreds of milliNewtons per meter) therein. This stretch is balanced by the push in the bilayer's interior hydrocarbon (11). All materials embedded in the bilayer are subjected to the constitutive push and pull of this highly anisotropic force environment. Added stretch forces thin and bend the bilayer, changing the magnitude and direction of these internal forces at the channel–lipid interface (12). The antibiotic gramicidin A, a popular biophysical model channel, responds to stretch force differently depending on the bilayer thickness, pointing to the importance of lipid microdomains (13).

Forcing All Channels

Ion channels are the direct stimulus-to-flux transducers. By their physiological stimuli, channels are traditionally classified as voltage-gated, ligand-gated, Ca^{2+}-activated, and so forth. The better advertised force-gated channels are members of the MscL, MscS, K_{2p}, acid-sensing ion channel (ASIC)/epithelial Na^+ channel (ENaC), transient receptor potential (TRP), or piezo families (14). However, many voltage- or ligand-gated channels also respond directly to force under patch clamp. Channels in the plasma membrane are usually multimers of subunits, each having several TM α-helices. Reported so far, channels that respond to stretch force include MscL (2TM × 5) (15), MscS (3TM × 7) (16), MEC-4/-10 (2TM × 3) (17), TRP (6TM × 4), Piezo (>24 TM × 4) (18), K_{2p} (4TM × 2) (19) (20), Kv (6TM × 4) (21) (22), K_{Ca} (6TM × 4) (23), Nav, (24TM × 1) (24, 25), NMDA receptor (3TM × 4) (26), CFTR (12TM × 1) (27), AChR (4TM × 5) (28), and others. There is no sequence similarity among these disparate structures. There are also no common recognizable force-sensing domains comparable to the voltage-sensor motif or ligand-binding pockets.

No surprise should come with the universality of mechanosensetivity, which as stated above is nothing more than the geometric change in the direction of applied force. Geometric changes such as the pore expansion upon opening, are expected. Furthermore, by definition all channels live in the lipid bilayer, which is an anisotropic force environment (11). In channels like the voltage-gated K^+ channels (Kvs), which show a less dramatic movement upon opening than MscL, their behavior is nonetheless strongly dependent on the composition of the surrounding lipids as well as applied force (21). Recent analyses (29) showed that the MS step is at the point when the protein expands, transitioning to the open state from a closed state after all four voltage sensors have already moved. Although Kvs have smaller ΔA (about 3–4 nm^2), their lower energy cost for opening allows them to have the smaller estimated gating tension, which makes Kvs responding to far lower tensions than MscL. Because Kvs are the canonical structural model for many cation channels, this work implies that these other channels are also sensitive to membrane force, probably in a similar manner. Although we do not know whether the membrane forces ever change where some of these channels operate, force-sensing of voltage-gated channels is clearly important in the electro-mechanical coupling of the heart (22, 24, 25). An unexpected physiological relevance of channel mechanosensitivity recently showed up in the *Drosophila* compound eye. The photon-rhodopsin-G protein-activated phospholipase C apparently beheads the rod-shaped phosphatidylinositol 4,5-bisphosphate (PIP_2) into the cone shaped diacylglycerol (DAG), changing the geometry of the bilayer to mechanically open TRP and TRP-like in the rhabdomere (30). DAG is commonly known to activate protein kinase C to phosphorylate target proteins. Thus, this work not only adds to the list of reported mechanosensitive TRP channels but also makes us wonder how much of the previously reported DAG effects are mechanical instead of—or in addition to—chemical.

UFO No More

The cytoplasm is not a solution of enzymes. Proteins and RNAs are functionally organized into a hundred kinds of assemblies: ribosomes, nucleosomes, centrosomes, lysosomes, primosome, spliceosomes, among others. Similarly, cell membrane is not a uniform lipid sheet with lone protein icebergs drifting about. Once labeled the "unidentified floating objects" (31), lipid rafts are no longer controversial (32). Originally defined as neutral detergent-insoluble fractions of membrane, recent advances in microscopy and spectroscopy in natural membranes confirmed the existence of these dynamic 10-nm size microdomains enriched in cholesterol, sphingolipid, and certain proteins. The planar and rigid cholesterol associates with saturated fatty acids, enforcing a more ordered region thicker and stiffer than ordinary bilayer. Sphingolipids are found in such regions because they tend to have longer and saturated hydrocarbon tails, which also leads to tail intercalation between leaflets, thus increasing their cohesion and force transmission between them. The small rafts can coalesce, and, together with agreeable proteins, form larger platforms for distinct biological functions, such as membrane traffic. Signal transduction can initiate from such platforms using various kinases, G proteins, GTPases, and so forth. The events after a signal is received are not to be confused with how the signals are received. Here, we address how a crucial signal, namely the physical force, is received and transduced.

Hearing and Touch

Although the inner-ear transduction channel remains to be identified, the force that gates them clearly comes from the stereocilia tip link, which comprises a cadhedrin-23 and a protocadhedrin-15 homodimer. Such a tip link is apparently too stiff to account for the mechanical compliance associated with channel activation (33, 34). Thus, this compliance arises either from the channel's own anchor to cytoskeleton or the channel's surrounding membrane (35, 36), which may also be anchored. Lipids are not evenly distributed in the stereociliary membrane (37). Filipin, a cholesterol-binding fluorescent antibiotic, stains the very tip of the stereocilia, where the transduction channels reside (37) (Fig. 1*A*). A recent simulation evaluated the source of the gating spring for a conventional membrane, but has not incorporated the membrane stiffening by cholesterol or the likely connections to cytoskeleton (36).

In the touch receptor of *C. elegans*, genetic interactions and coimmunoprecipitation showed a complex (38) that includes an

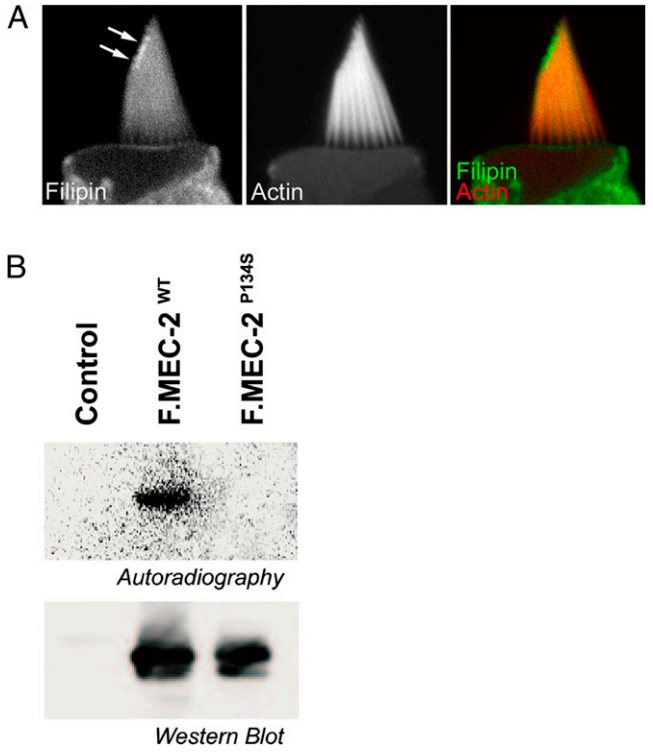

Fig. 1. Enrichment of cholesterol at foci of force perception. (*A*) Filipin, a fluorescent antibiotic that binds cholesterol, preferentially stains the tips of bullfrog stereocilia. (Reproduced with permission from ref. 37.) (*B*) MEC-2, which complexes with the ion channel in *C. elegans* touch receptor, binds cholesterol. [^{3}H]photocholesterol-treated MEC-2s from expressing HEK-cell lysate were immunoprecipitated and electrophoresed. For similar protein amount (*Lower*) wild-type but not MEC-2 from a touch-blind mutant captures cholesterol (*Upper*). (Reproduced from ref. 42.)

ENaC-ASIC–type ion channel (Mec-4, Mec-10) together with MEC-2 (39, 40) and its homolog UNC-24 (41). MEC-2 is a necessary component for channel function (38). MEC-2 and UNC-24 are stomatin/prohibitin/flotillin/HflK/HflC (SPFH)-domain proteins, which recruit cholesterol (Fig. 1*B*) (40, 42, 43) (see below). This worm receptor has an equivalent in the slit diaphragm between glomerular epithelial cells (podocytes) that filters plasma into urine (44). Here the MEC-2 counterpart is podocin, which associates with the TRPC6 channel (42). Just as *mec-2* mutations can make the worm blind to touch, knocking out the SPFH protein SPL3 greatly reduces touch sensation in *SPL3*$^{-/-}$ mice (45). Judging by the known components and evolutionary conservation, these specialized protein ensembles for touch and hearing are likely derived from generic force sensors reviewed below.

Two General Types of Force Foci

Cells glue together to form an animal. This glue resists forces that tend to rip the neighbors apart. Although there are junctions for other purposes, this physical glue is comprised of two types of molecular devices: type 1, typified by adherens junction, links the neighboring cells through cadherins; type 2, typified by focal adhesion, links the cells to organic matrix with integrins. Although not homologous, the two types of linkages are analogous. Fig. 2*A* is a generic diagram of these molecular ensembles that bear force. The key molecule has an extracellular holdfast, a single-pass TM helix per molecule, and an intracellular region that binds other proteins, which indirectly attach to a cytoskeletal filament. In type 1, neighboring cells extend their cadherins outward and shake hands with their cadherin-domain repeats. Each cadherin passes through the membrane and its internal region binds other proteins (catenins, and so forth), which anchor cadherin indirectly to actin or other fibers. Similarly, in type 2, the heterodimer of α- and β-integrin externally grips fibronectin, collagen, among others, in the matrix secreted by other cells. Each integrin makes a single pass through the membrane,

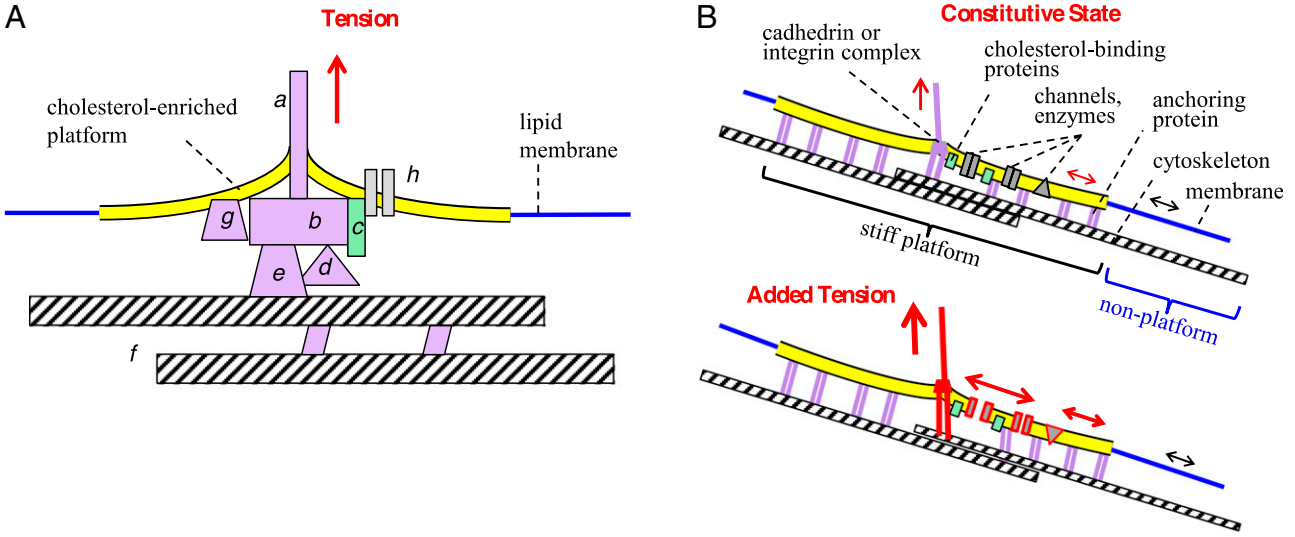

Fig. 2. A generic diagram of a force-bearing focus in animals. (*A*) Representation of the components of such a focal ensemble with protein *a*, which has an extracellular domain that anchors outward. It makes a single pass per protein through the membrane and binds protein *b,* which then binds other proteins, including those (*c*) that recruit cholesterol and those (*e*) that bind the cytoskeleton (*f*) as well as enzymes (*g*) or channels (*h*). In type 1, the cell–cell junctions, *a* is cadherin, *b* is catenin, *c* is an SPFH-domain protein such as stomatin, and *e* can be vinculin that binds F-actin (*f*). In type 2, the cell-matrix junctions, *a* is the $\alpha\beta$ integrin dimer, *b* includes talin, *c* is an SPFH protein such as flotillin, and *e* includes α-actinin and filamin that binds F-actin (*f*). The SPFH proteins gather cholesterol to produc-the ordered lipid platform (yellow) distinct from the disordered lipid membrane (blue). The entire ensemble bears a constitutive ~picoNewton stretch (red arrow) at rest. Not drawn to scale. (*B*) Representation of such a focus at its constitutive state (*Upper*) and under added tension (*Lower*). The tension on the tether (upward arrow) is redirected into membrane stretch (two-headed arrows) by and confined within the ordered lipid platform (yellow).

and internally binds other proteins, including those that anchor onto cytoskeleton, such as vinculin. By using a FRET-based tension sensor engineered into vinculin, Grashoff et al. (46) found that the stationary focal adhesion bears a stretch force of ~2.5 pN. This stretch increases at the advancing end of migrating cells where traction is needed and decreases at the retracting end. Using a similar method, Borghi et al. (47) showed that cadherin in kidney epithelial cells is under picoNewton (pN) tension, which increases when the cell-cell contact is stretched. Interestingly, a tension seems to be required to assemble (48) and maintain these force-bearing complexes. Weighing anchor causes cholesterol to disperse and some focal-adhesion components to enter endocytosis (49, 50). The resting tension on the gating spring of the sterocilia channel is estimated as ~10 pN (35). Tensions on early lateral and tip links also seem to be required for stereocilia cohesion and proper orientation (51). In short, carefully studied molecular force foci all sustain picoNewton constitutive forces, presumably internally generated. These forces can be adjusted by the dynamic cytoskeleton (e.g., in the "slow adaptation" process of the hair cell) (35).

Among the internal clusters of proteins there are always those with the SPFH domain, such as stomatin, flotillin, mec-2, that recruit cholesterol (see below). Thus, the membranes at both types of junctions are reinforced with a cholesterol-rich platform. Cultured myoblasts make cadherin junctions (type 1) with neighbors. Triton X-100–resistant fractions of these cells are enriched with junction components, which are seen to colocalize at the junctions with cholesterol when stained by filipin (48, 52). Using the fluorescent Laurdan agent, which aligns parallel to the lipids and shifts to longer peak emission wavelength in more fluid membrane, Gaus et al. (49) showed focal adhesions (type 2) to be highly ordered, even more ordered than the well-known caveolae. However, extracting cholesterol does not disassemble the complex, indicating that the complex is assembled through protein-protein binding (49).

Players in downstream signal transductions cluster around these force foci and can use the constitutive and applied forces as signal. Src kinase binds integrins but is not activated until integrins are attached to a fibronectin-coated surface (53). Pulling on beads attached through fibronectin-integrin link with a laser-tweezer activates Src kinase in minutes (54). Pulling an integrin in a focal-adhesion assembly with an attached magnetic bead activates TRPV4 channels within 4 ms (55). Ion channels or receptors, such as certain Kvs (56), K_{Ca}s (57), TRPs (58), K_{2p}s, Kirs (59), and muscarinic acetylcholine receptors (60) are in such cholesterol-enriched foci. However, no ion channels are attached directly to cadherins or integrins to our knowledge.

Cholesterol-Gathering Proteins

MEC-2 and stomatin belong to a large family of proteins that bear the SPFH domain. First studied as a band-7 protein in the red-cell membrane, stomatin is widely expressed. It has an ~30-residue hydrophobic hairpin, which enters the inner leaflet of the bilayer, followed by an ~240-residue stomatin domain. Stomatin exists as oligomers of some 9–12 monomers in the plasma membrane of epithelial cells (61). Ring-like oligomers of stomatin relatives have been observed by electron microscopy (62, 63) (Fig. 3*A*). A small C-terminal region of stomatin is apparently needed for the multimerization as well as association with cholesterol-rich lipid domains (64). Crystal structures of stomatin-domain dimer, trimer, and a tubular multimer have been solved (65, 66). An interaction of the N-terminal hydrophobic pocket interacting with the C terminus of ASIC subunit was shown to be involved in the inhibition of channel activity (45). The banana-shaped dimer, the smallest functional building block, appears to be a functional unit (Fig. 3*B*). This dimerization is required to inhibit ASIC (66). How SPFH proteins recognize

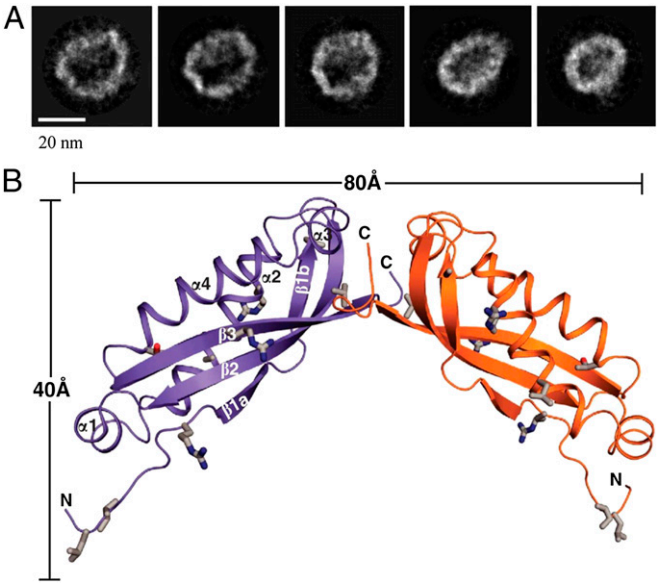

Fig. 3. Structures of Stomatin-family proteins. (*A*) Averaged images of some ring complexes of purified yeast prohibitins observed by single particle electron microscopy. (Reproduced from ref. 63.) (*B*) Crystal-structure diagram of the mouse stomatin dimer, the smallest functional unit. [Reprinted by permission from Macmillan Publishers Ltd: *The EMBO Journal* (ref. 66), copyright (2012).]

cholesterol is poorly understood (67). Mutating the conserved proline of MEC-2 removes cholesterol association (Fig. 1*B*), apparently not because this proline binds cholesterol but because the mutation rewires the protein conformation completely (68). SPFH proteins do not usually traverse the bilayer. Their N-terminal domain reaches only the inner leaflet of the plasma membrane. Overexpressed stomatin concentrates in membrane-protruding folds (69), which may generate additional asymmetry in the shape and force distribution of the platform of interest here. That these proteins and possibly the cholesterol they gathered are mainly in the inner leaflet may have special relevance to cation channels structurally of Kvs ilk, because their gates are located at the inner half of the proteins.

Mechanical Roles of Cholesterol-Rich Platform

A key role of the platform is likely to suppress mechanical noise. There are multiple sources of noise that are likely to affect channel gating. Some noise may come from the thermally driven conformational changes of the channel itself. As often observed in molecular dynamics simulations, even under equilibrium conditions conformation of a protein (including MS channels) might fluctuate on a scale of tens of nanoseconds with dimensions up to 5–10% of the protein size (70, 71). These fluctuations would be suppressed by more ordered and stiff lipids of the platform, which is more resistant to tilting of the α-helices of the protein frame. In a general case, lipid bilayer can also be a potent source of noise as it exhibits local variations of density and thickness on the scale of nanosecond and nanometer and beyond, originating from coherent movement of lipid neighbors as well as individual lipid protrusions (72–74). This result can be observed in simulations (75). The rigidity of the platform should reduce such variations by increasing the elastic moduli of lipids and thereby suppressing bilayer geometry fluctuations (76–79). The third potential source of noise for a channel comes from encounters with other proteins in the bilayer; this can be effectively reduced by the stiffened platform because not all membrane proteins are allowed in. Proteins within appear to either be

interconnected or associated with cholesterol and therefore much less free to collide, further reducing the noise.

The anchored platform stiffened by proteins also redistributes external forces. First, a normal (relative to the membrane plane) pulling force in an anchored tether can redirect into a lateral stretch of the platform (Fig. 2*B*). This finding means that a channel that needs to be stretched open laterally can exploit the normal force. Second, the cholesterol-rich platform is likely to be heterogeneous, consisting of protein-stiffened "mesh" and regions similar to pure lipid rafts, which are liquid, albeit more ordered than a regular membrane (80). Therefore, regardless of the source of the impact, force is distributed isotropically within the lipid domains of the platform. Thus, the enzymes or channels that make use of the force can be located anywhere within the liquid-ordered domains and need not be directly attached to the tether. Third, the protein-reinforced rigid part of the platform, especially with multiple anchors to cytoskeleton, can confine the force within, making it unnecessary to deform the entire plasma membrane of the cell. Finally, the size and therefore the physical properties of the platform can be scaled according to functions they serve from the sizable desmosomes or hemidesosomes to the possible single-channel ensemble for hearing and touch.

The SPFH protein SLP3 colocalizes and coimmunoprecipiates with ASIC channels (45, 81). As argued above and as observed in the suppression of ASIC activity by stomatin, the cholesterol-rich environment inhibits channel opening. This finding raises the question of how force-induced protein-conformation changes are implemented in a rigid platform. We note that the force-sensing protein scaffolds do not depend on the cholesterol for its integrity. Removing cholesterol does not disassemble the scaffold, which suggests strong protein–protein interactions and preferential distribution of the load through the protein-stiffened scaffold. Stretch forces can reshape the protein scaffold (e.g., the conformation of integrins) (82).

Speculative Models on Platform Dynamics

If the scaffold is indeed dynamic; one can envision various ways in which it actuates channel opening by force. For example, upon an added stretch force, the SPFH-domain proteins may spread out, thus allowing ordinary phospholipids to enter the platform and dilute the cholesterol-rich regions between the proteins. This action should make the membrane near the channel thinner and release the inhibitory effect of the rigid raft-like surround, allowing the channel to open. An extreme form of this model can be a ring of SPFH protein (Fig. 3) completely surrounding a channel within, with lipid exchange allowed when the ring is under stretch (Fig. 4*A*). On the release of the external force the system might exclude the regular lipids back to the bulk membrane and return to the original state of confined raft-like lipids, assuming that, at rest, contact of raft lipids with channel is more favorable than their contact with regular lipids.

It is not necessary for the channel to be in direct contact with cholesterol to stay closed. It is possible that the channel is in a small puddle of ordinary lipids in a protein corral—surrounded by cholesterol outside—and remains closed in the absence of applied tension. Close proximity of the outside raft might affect the confined ordinary lipids, making them more rigid, ordered, and tall. Softening the lipid raft surrounding or stretching the protein-reinforced frame can in turn stretch the confined lipids in the puddle and open the channel therein (Fig. 4*B*). The effects of adding or removal cholesterol vary among types of ion channels (83). Some ENaC or TRP channels are inhibited by the removal of cholesterol (83). Cholesterol-rich surroundings might be necessary for maintaining the protein-stiffened frame around the channel, tension transmission to lipids, and confining the stretch within the puddle. Dissolving the platform may require stretching the entire plasma membrane to activate mechanosensitive channels. Native to most cultured mammalian cells is a ~20 pS stretch-activated conductance (SAC) of unknown molecular nature. Cholesterol depletion decreases its stretch-induced opening probability, which can be restored by actin disassembly. Here, the actin network is thought to inhibit SAC activity by preventing membrane deformation (84).

Versatile and dynamic nature of lipid-protein arrangements, lateral phase separation and engagement with cytoskeleton, and external connection provide rich basis for force redistribution and control. There are possibilities for many particular implementations. Such models remain to be tested by experimentations and molecular dynamics simulations. Given the universal concentrations of cholesterol in force foci, the known physical property of cholesterol-rich domains, and importance and mystery of animal force sensations, we feel that such tests are worthwhile.

Unification and Ramification

The bacterial MscL and MscS are of known crystal structures; they can also be purified and reconstituted into defined lipid bilayers

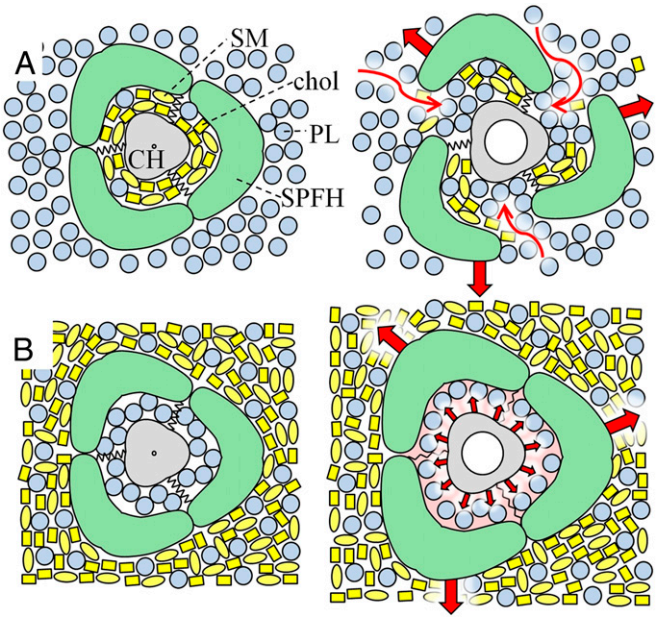

Fig. 4. Speculative dynamic staging for channel activation by cholesterol-enriched platforms. (*A*) Such a nano-platform comprises lipids enriched with cholesterol and sphingolipids outwardly enclosed by multimers of SPFH proteins (e.g., stomatins) and inwardly surround the channel (e.g., ASIC). (*Left*) Such enrichment enforces order in the stiff platform, suppressing mechanical noises and making the membrane thicker to ensure channel closure. (*Right*) Upon a stretch force transmitted through the bilayer or the direct connections of the frame to the external proteins, the SPFH proteins are displaced, allowing common phospholipids to enter the platform. The thinner and more liquid bilayer and possibly the stretch, reaching the channel through the disordered lipids, open the channel. On tension release the system might return to the closed arrangement because of propensity of the raft lipids to phase separation and selective contacts with the channel. (*B*) An alternative scheme has the ordered lipids (the entire square) outside the SPFH-protein ring, which corrals a puddle of ordinary lipids surrounding the channel. (*Left*) Noise is suppressed and channel closed by the indirectly ordered lipids in the absence of added tension. (*Right*) Under external forces the stiffened frame expands, produces local stretch in the confined puddle, and opens the channel without lipid diffusion. The SPFH protein may be also attached to the channel through linkers as implied by the zigzag line. Diagrams here are representatives of many possible variations (e.g., direct and completely surrounding SPFH-channel binding without a lipid puddle inbetween). In cases where membrane stretch originates from a protein tether (Fig. 2), the stiffened platform also serves to redirect and confine the stretch force. See *Mechanical Roles of Cholesterol-rich Platform*. CH, channel; chol, cholessterol; PL, phospholipids; SM, sphingomyelin.

and retain mechanosensitivity. It is therefore incontrovertible that the gating force comes from the lipid bilayer (5–7). Purified eukaryotic NMDA receptor channel and Kv have also been reconstituted and showed mechanosensitivity (21, 26). Even if there are other relevant forces or gating stimuli, all channels are governed by the push and pull within the bilayer, as described above. The bilayer membrane with its internal forces presumably evolved in early cells some 4 billion years ago. The continued use of lipid forces to shape proteins simply reflects the parsimony of evolution in deploying basic physico-chemical principles. Lipids do not all mix equally well. Phase separation can further be affected by temperature, bilayer hydration, as well as the ionic strength and pH of the medium, and presumably by added stretch force. What we have reviewed here seems to be one form of elaboration by nature to modify the local mechanical environment by tinkering with lipid compositions. By stiffening a patch, it builds a platform that dampens mechanical noise and, at the same time, confines, amplifies, and redirects the mechanical signals. By attaching the platform to cytoskeleton, it anchors the platform at the force focus, provides the counterforce, and protects the platform from being torn off. We envision here the platform to be dynamic and even heterogeneous (Fig. 4). Varieties of enzymes and channels of disparate structures can exploit such a platform without having to be directly tied to a force-transmitting protein string.

Nothing is truly new under the sun. Two decades of genomics showed, across the three domains of life, impressive conservation of enzymes and channels, including voltage- or neurotransmitter-gated channels, which can be found in prokaryotes. The flat, ring-structured hopanoids, the sterol counterparts in bacteria, similarly order local lipid domains in vitro (85) and in *Cyanobacteria* (86). Hopanoids are synthesized like sterols with similar enzymes but require no molecular oxygen and could have been manufactured before the earth's oxygen atmosphere (85).

Compared with those for chemical events, we have fewer tools appropriate to apply and measure the small forces and their effects on biological molecules. The dearth of tools contrasts the surfeit of challenges facing biology. Building an animal from an egg is far more than transcriptional control. Forces govern global and local developmental events (e.g., Wnt signals through β-catenin, a component of adherens junction) (87). The molecular bases of hearing and touch (Fig. 1), as well as those maintaining blood pressure or plasma osmolarity, remain obscure. The universal presence of cholesterol-enriched platforms in force foci (Figs. 1 and 2) hints at its general relevance in physiology and pathology (88). Physiologically active PIP_2, DAG, and polyunsaturated fatty acids, as well as various anesthetics should interact with these platforms. Integrins play key roles in anchorage dependence, defending against tumor formation and metastasis (89). Pulling at integrin activates the proto-oncogene–encoded tyrosine kinase c-Src (54). The challenges are immense. We need concentrated studies on how mechanical force works on membrane proteins.

ACKNOWLEDGMENTS. Work in our laboratories is supported by the Huck Institute of Life Sciences (A.A.), and National Institutes of Health Grant GM096088 and the Vilas Trust of the University of Wisconsin-Madison (to C.K.).

1. Martinac B, Buechner M, Delcour AH, Adler J, Kung C (1987) Pressure-sensitive ion channel in *Escherichia coli*. *Proc Natl Acad Sci USA* 84(8):2297–2301.
2. Martinac B, Adler J, Kung C (1990) Mechanosensitive ion channels of *E. coli* activated by amphipaths. *Nature* 348(6298):261–263.
3. Sukharev SI, Blount P, Martinac B, Kung C (1997) Mechanosensitive channels of *Escherichia coli*: The MscL gene, protein, and activities. *Annu Rev Physiol* 59:633–657.
4. Sukharev SI, Blount P, Martinac B, Blattner FR, Kung C (1994) A large-conductance mechanosensitive channel in *E. coli* encoded by *mscL* alone. *Nature* 368(6468): 265–268.
5. Anishkin A, Kung C (2005) Microbial mechanosensation. *Curr Opin Neurobiol* 15(4): 397–405.
6. Kung C, Martinac B, Sukharev S (2010) Mechanosensitive channels in microbes. *Annu Rev Microbiol* 64:313–329.
7. Martinac B (2011) Bacterial mechanosensitive channels as a paradigm for mechanosensory transduction. *Cell Physiol Biochem* 28(6):1051–1060.
8. Levina N, et al. (1999) Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: Identification of genes required for MscS activity. *EMBO J* 18(7):1730–1737.
9. Perozo E, Cortes DM, Sompornpisut P, Kloda A, Martinac B (2002) Open channel structure of MscL and the gating mechanism of mechanosensitive channels. *Nature* 418(6901):942–948.
10. Perozo E, Kloda A, Cortes DM, Martinac B (2002) Physical principles underlying the transduction of bilayer deformation forces during mechanosensitive channel gating. *Nat Struct Biol* 9(9):696–703.
11. Cantor RS (1997) Lateral pressures in cell membranes: A mechanism for modulation of protein function. *J Phys Chem B* 101:1723–1725.
12. Phillips R, Ursell T, Wiggins P, Sens P (2009) Emerging roles for lipids in shaping membrane-protein function. *Nature* 459(7245):379–385.
13. Martinac B, Hamill OP (2002) Gramicidin A channels switch between stretch activation and stretch inactivation depending on bilayer thickness. *Proc Natl Acad Sci USA* 99(7): 4308–4312.
14. Nilius B, Honoré E (2012) Sensing pressure with ion channels. *Trends Neurosci* 35(8): 477–486.
15. Chang G, Spencer RH, Lee AT, Barclay MT, Rees DC (1998) Structure of the MscL homolog from *Mycobacterium tuberculosis*: A gated mechanosensitive ion channel. *Science* 282(5397):2220–2226.
16. Bass RB, Strop P, Barclay M, Rees DC (2002) Crystal structure of *Escherichia coli* MscS, a voltage-modulated and mechanosensitive channel. *Science* 298(5598):1582–1587.
17. Jasti J, Furukawa H, Gonzales EB, Gouaux E (2007) Structure of acid-sensing ion channel 1 at 1.9 A resolution and low pH. *Nature* 449(7160):316–323.
18. Coste B, et al. (2010) Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels. *Science* 330(6000):55–60.
19. Patel AJ, et al. (1998) A mammalian two pore domain mechano-gated S-like K^+ channel. *EMBO J* 17(15):4283–4290.
20. Brohawn SG, del Mármol J, MacKinnon R (2012) Crystal structure of the human K2P TRAAK, a lipid- and mechano-sensitive K^+ ion channel. *Science* 335(6067):436–441.
21. Schmidt D, MacKinnon R (2008) Voltage-dependent K^+ channel gating and voltage sensor toxin sensitivity depend on the mechanical state of the lipid membrane. *Proc Natl Acad Sci USA* 105(49):19276–19281.
22. Morris CE (2011) Voltage-gated channel mechanosensitivity: Fact or friction? *Front Physiol* 2:25.
23. Takahashi K, Naruse K (2012) Stretch-activated BK channel and heart function. *Prog Biophys Mol Biol* 110(2-3):239–244.
24. Shcherbatko A, Ono F, Mandel G, Brehm P (1999) Voltage-dependent sodium channel function is regulated through membrane mechanics. *Biophys J* 77(4):1945–1959.
25. Beyder A, et al. (2010) Mechanosensitivity of Nav1.5, a voltage-sensitive sodium channel. *J Physiol* 588(Pt 24):4969–4985.
26. Kloda A, Lua L, Hall R, Adams DJ, Martinac B (2007) Liposome reconstitution and modulation of recombinant N-methyl-D-aspartate receptor channels by membrane stretch. *Proc Natl Acad Sci USA* 104(5):1540–1545.
27. Zhang WK, et al. (2010) Mechanosensitive gating of CFTR. *Nat Cell Biol* 12(5):507–512.
28. Pan NC, Ma JJ, Peng HB (2012) Mechanosensitivity of nicotinic receptors. *Pflugers Arch* 464(2):193–203.
29. Schmidt D, del Mármol J, MacKinnon R (2012) Mechanistic basis for low threshold mechanosensitivity in voltage-dependent K^+ channels. *Proc Natl Acad Sci USA* 109(26): 10352–10357.
30. Hardie RC, Franze K (2012) Photomechanical responses in *Drosophila* photoreceptors. *Science* 338(6104):260–263.
31. Lai EC (2003) Lipid rafts make for slippery platforms. *J Cell Biol* 162(3):365–370.
32. Lingwood D, Simons K (2010) Lipid rafts as a membrane-organizing principle. *Science* 327(5961):46–50.
33. Kachar B, Parakkal M, Kurc M, Zhao Y, Gillespie PG (2000) High-resolution structure of hair-cell tip links. *Proc Natl Acad Sci USA* 97(24):13336–13341.
34. Sotomayor M, Weihofen WA, Gaudet R, Corey DP (2010) Structural determinants of cadherin-23 function in hearing and deafness. *Neuron* 66(1):85–100.
35. Gillespie PG, Müller U (2009) Mechanotransduction by hair cells: Models, molecules, and mechanisms. *Cell* 139(1):33–44.
36. Powers RJ, et al. (2012) Stereocilia membrane deformation: Implications for the gating spring and mechanotransduction channel. *Biophys J* 102(2):201–210.
37. Zhao H, Williams DE, Shin JB, Brügger B, Gillespie PG (2012) Large membrane domains in hair bundles specify spatially constricted radixin activation. *J Neurosci* 32(13): 4600–4609.
38. O'Hagan R, Chalfie M, Goodman MB (2005) The MEC-4 DEG/ENaC channel of *Caenorhabditis elegans* touch receptor neurons transduces mechanical signals. *Nat Neurosci* 8(1):43–50.
39. Huang M, Chalfie M (1994) Gene interactions affecting mechanosensory transduction in *Caenorhabditis elegans*. *Nature* 367(6462):467–470.
40. Goodman MB, et al. (2002) MEC-2 regulates *C. elegans* DEG/ENaC channels needed for mechanosensation. *Nature* 415(6875):1039–1042.
41. Zhang S, et al. (2004) MAC-2 is recruited to the putative mechanosensory complex in *C. elegans* touch receptor neurons through its stomatic-like domain. *Curr Biol* 14: 1888—1896.
42. Huber TB, et al. (2006) Podocin and MEC-2 bind cholesterol to regulate the activity of associated ion channels. *Proc Natl Acad Sci USA* 103(46):17079–17086.

43. Huang M, Gu G, Ferguson EL, Chalfie M (1995) A stomatin-like protein necessary for mechanosensation in *C. elegans*. *Nature* 378(6554):292–295.
44. Schermer B, Benzing T (2009) Lipid-protein interactions along the slit diaphragm of podocytes. *J Am Soc Nephrol* 20(3):473–478.
45. Wetzel C, et al. (2007) A stomatin-domain protein essential for touch sensation in the mouse. *Nature* 445(7124):206–209.
46. Grashoff C, et al. (2010) Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature* 466(7303):263–266.
47. Borghi N, et al. (2012) E-cadherin is under constitutive actomyosin-generated tension that is increased at cell-cell contacts upon externally applied stretch. *Proc Natl Acad Sci USA* 109(31):12568–12573.
48. Taulet N, et al. (2009) N-cadherin/p120 catenin association at cell-cell contacts occurs in cholesterol-rich membrane domains and is required for RhoA activation and myogenesis. *J Biol Chem* 284(34):23137–23145.
49. Gaus K, Le Lay S, Balasubramanian N, Schwartz MA (2006) Integrin-mediated adhesion regulates membrane order. *J Cell Biol* 174(5):725–734.
50. Del Pozo MA, Schwartz MA (2007) Rac, membrane heterogeneity, caveolin and regulation of growth by integrins. *Trends Cell Biol* 17(5):246–250.
51. Lefèvre G, et al. (2008) A core cochlear phenotype in USH1 mouse mutants implicates fibrous links of the hair bundle in its cohesion, orientation and differential growth. *Development* 135(8):1427–1437.
52. Causeret M, Taulet N, Comunale F, Favard C, Gauthier-Rouvière C (2005) N-cadherin association with lipid rafts regulates its dynamic assembly at cell-cell junctions in C2C12 myoblasts. *Mol Biol Cell* 16(5):2168–2180.
53. Arias-Salgado EG, et al. (2003) Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci USA* 100(23):13298–13302.
54. Wang Y, et al. (2005) Visualizing the mechanical activation of Src. *Nature* 434(7036): 1040–1045.
55. Matthews BD, et al. (2010) Ultra-rapid activation of TRPV4 ion channels by mechanical forces applied to cell surface beta1 integrins. *Integr Biol (Camb)* 2(9):435–442.
56. Martens JR, et al. (2000) Differential targeting of Shaker-like potassium channels to lipid rafts. *J Biol Chem* 275(11):7443–7446.
57. Bravo-Zehnder M, et al. (2000) Apical sorting of a voltage- and Ca^{2+}-activated K+ channel alpha -subunit in Madin-Darby canine kidney cells is independent of N-glycosylation. *Proc Natl Acad Sci USA* 97(24):13114–13119.
58. Lockwich TP, et al. (2000) Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J Biol Chem* 275(16):11934–11942.
59. Riquelme G, de Gregorio N, Vallejos C, Berrios M, Morales B (2012) Differential expression of potassium channels in placentas from normal and pathological pregnancies: Targeting of the K(ir) 2.1 channel to lipid rafts. *J Membr Biol* 245(3):141–150.
60. Feron O, Smith TW, Michel T, Kelly RA (1997) Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J Biol Chem* 272(28):17744–17748.
61. Snyers L, Umlauf E, Prohaska R (1998) Oligomeric nature of the integral membrane protein stomatin. *J Biol Chem* 273(27):17221–17226.
62. Boehm M, et al. (2009) Structural and mutational analysis of band 7 proteins in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* 191(20):6425–6435.
63. Tatsuta T, Model K, Langer T (2005) Formation of membrane-bound ring complexes by prohibitins in mitochondria. *Mol Biol Cell* 16(1):248–259.
64. Umlauf E, Mairhofer M, Prohaska R (2006) Characterization of the stomatin domain involved in homo-oligomerization and lipid raft association. *J Biol Chem* 281(33): 23349–23356.
65. Yokoyama H, Fujii S, Matsui I (2008) Crystal structure of a core domain of stomatin from *Pyrococcus horikoshii* illustrates a novel trimeric and coiled-coil fold. *J Mol Biol* 376(3):868–878.
66. Brand J, et al. (2012) A stomatin dimer modulates the activity of acid-sensing ion channels. *EMBO J* 31(17):3635–3646.
67. Epand RM (2008) Proteins and cholesterol-rich domains. *Biochim Biophys Acta* 1778(7–8):1576–1582.
68. Kadurin I, Huber S, Gründer S (2009) A single conserved proline residue determines the membrane topology of stomatin. *Biochem J* 418(3):587–594.
69. Snyers L, Thinès-Sempoux D, Prohaska R (1997) Colocalization of stomatin (band 7.2b) and actin microfilaments in UAC epithelial cells. *Eur J Cell Biol* 73(3):281–285.
70. Jeon J, Voth GA (2008) Gating of the mechanosensitive channel protein MscL: The interplay of membrane and protein. *Biophys J* 94(9):3497–3511.
71. Sotomayor M, Schulten K (2004) Molecular dynamics study of gating in the mechanosensitive channel of small conductance MscS. *Biophys J* 87(5):3050–3065.
72. Rawicz W, Olbrich KC, McIntosh T, Needham D, Evans E (2000) Effect of chain length and unsaturation on elasticity of lipid bilayers. *Biophys J* 79(1):328–339.
73. Henriksen JR, Ipsen JH (2004) Measurement of membrane elasticity by micro-pipette aspiration. *Eur Phys J E Soft Matter* 14(2):149–167.
74. Watson MC, Penev ES, Welch PM, Brown FL (2011) Thermal fluctuations in shape, thickness, and molecular orientation in lipid bilayers. *J Chem Phys* 135(24):244701.
75. Lindahl E, Edholm O (2000) Mesoscopic undulations and thickness fluctuations in lipid bilayers from molecular dynamics simulations. *Biophys J* 79(1):426–433.
76. Sullan RM, Li JK, Hao C, Walker GC, Zou S (2010) Cholesterol-dependent nanomechanical stability of phase-segregated multicomponent lipid bilayers. *Biophys J* 99(2):507–516.
77. Berkowitz ML (2009) Detailed molecular dynamics simulations of model biological membranes containing cholesterol. *Biochim Biophys Acta* 1788(1):86–96.
78. Chiu SW, Vasudevan S, Jakobsson E, Mashl RJ, Scott HL (2003) Structure of sphingomyelin bilayers: A simulation study. *Biophys J* 85(6):3624–3635.
79. Niemelä PS, Hyvönen MT, Vattulainen I (2009) Atom-scale molecular interactions in lipid raft mixtures. *Biochim Biophys Acta* 1788(1):122–135.
80. He HT, Marguet D (2011) Detecting nanodomains in living cell membrane by fluorescence correlation spectroscopy. *Annu Rev Phys Chem* 62:417–436.
81. Lapatsina L, et al. (2012) Regulation of ASIC channels by a stomatin/STOML3 complex located in a mobile vesicle pool in sensory neurons. *Open Biol* 2(6):120096.
82. Campbell ID, Humphries MJ (2011) Integrin structure, activation, and interactions. *Cold Spring Harb Perspect Biol* 3(3): pii a004994.
83. Levitan I, Fang Y, Rosenhouse-Dantsker A, Romanenko V (2010) Cholesterol and ion channels. *Subcell Biochem* 51:509–549.
84. Chubinskiy-Nadezhdin VI, Negulyaev YA, Morachevskaya EA (2011) Cholesterol depletion-induced inhibition of stretch-activated channels is mediated via actin rearrangement. *Biochem Biophys Res Commun* 412(1):80–85.
85. Sáenz JP, Sezgin E, Schwille P, Simons K (2012) Functional convergence of hopanoids and sterols in membrane ordering. *Proc Natl Acad Sci USA* 109(35):14236–14240.
86. Rexroth S, et al. (2011) The plasma membrane of the cyanobacterium *Gloeobacter violaceus* contains segregated bioenergetic domains. *Plant Cell* 23(6):2379–2390.
87. Nelson WJ, Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 303(5663):1483–1487.
88. Staubach S, Hanisch FG (2011) Lipid rafts: Aignaling and sorting platforms of cells and their roles in cancer. *Expert Rev Proteomics* 8(2):263–277.
89. Schwartz MA (1997) Integrins, oncogenes, and anchorage independence. *J Cell Biol* 139(3):575–578.