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Dynamic bonds and their roles in mechanosensing

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Mechanical forces are ubiquitous in a cell's internal structure and external environment. Mechanosensing is the process that the cell employs to sense its mechanical environment. In receptor-mediated mechanosensing, cell surface receptors interact with immobilized ligands to provide a specific way to receive extracellular force signals to targeted force-transmitting, force-transducing and force-supporting structures inside the cell. Conversely, forces generated endogenously by the cell can be transmitted via cytoplasmic protein–protein interactions and regulate cell surface receptor activities in an 'inside–out' manner. Dynamic force spectroscopy analyzes these interactions on and inside cells to reveal various dynamic bonds. What is more, by integrating analysis of molecular interactions with that of cell signaling events involved in force-sensing and force-responding processes, one can investigate how dynamic bonds regulate the reception, transmission and transduction of mechanical signals.

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Introduction

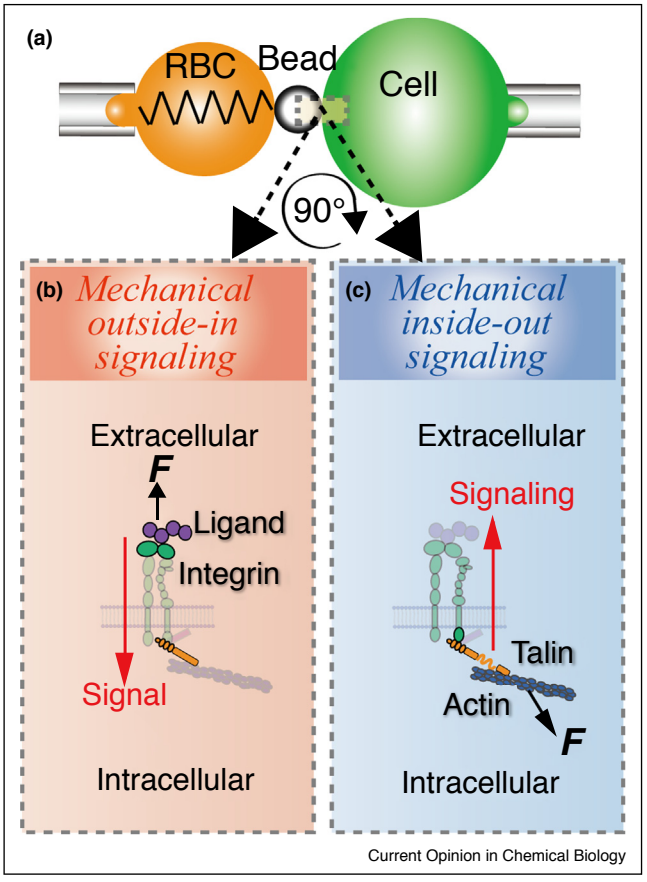
As the building blocks of life that live in a stressful mechanical milieu, cells are subjected to a myriad of forces, including tension, compression, fluid shear, and hydrostatic pressure. Mechanobiology studies how a cell senses, processes, responds, and adapts to mechanical cues [1–3]. A crucial element of cell mechanobiology is mechanosensing, by which the information encoded in the force waveform exerted from outside or within the cell is transduced biochemically into biological signals [3]. At the molecular level, mechanosensing involves force-modulated interactions of and conformational changes in proteins where dynamic bonds play crucial roles.

Cell surface receptors mediate mechanosensing by receiving force-modulated signals that come through specific ligands, providing an opportunity for researchers to use dynamic force spectroscopy (DFS) to study cell mechanosensing. To take this opportunity requires upgrading the DFS techniques that were used to mainly study force-dependent ligand binding and conformational change of purified receptors to allow experiments on live cells and to enable concurrent examination of molecular dynamics and cellular response to biomechanical stimulations (Figure 1a) [4]. These technological advances include: (1) Combining DFS with simultaneous imaging of intracellular signaling to directly correlate molecular processes with the triggered cellular responses on a single cell [5,6,7•,8,9,10••,11••,12]; (2) Expanding the list of live-cell molecular processes for analysis from receptor–ligand binding to include integrin (un)bending [13,14], protein unfolding [11••,15–17], uncoupling [18–20], cleavage [21–23], molecular stiffness [14,24,25•,26••,27], changes in the receptor's intracellular interaction [7•,25•], affinity maturation [26••], and binding reinforcement [27,28]; (3) dual DFS systems for analysis of dual receptor crosstalk by quantifying the spatiotemporal requirements and functional consequences of the upstream and downstream signaling events [29]. Here we review recent findings enabled by DFS on cells. We will focus on 'outside–in' (Figure 1b) and 'inside–out' (Figure 1c) signaling processes as modulated by mechanical forces via formation of receptor–ligand dynamic bonds and their relevance to cell mechanosensing.

Various receptor–ligand dynamic bonds and their biological roles

In DFS experiments, three types of tensile force waveforms have been employed to examine force modulation on receptor–ligand dissociation: ramped force, clamped force, and cyclic force (Figure 2a,b), resulting in multiple bond types with different responses to force, collectively referred to as dynamic bonds. The simplest and most widely used DFS assay is pulling the bond until rupture, corresponding to a ramping force waveform (Figure 2a). Analysis of rupture force distributions measured using a range of ramping rates (Figure 2c) usually results in slip bonds where the average bond lifetime decreases (multi-)exponentially with force [30–36] (Figure 2e). Using force-clamp DFS

Figure 1



Applying dynamic force spectroscopy (DFS) to study mechanical outside–in and inside–out signaling.
(a) An example DFS setup: Biomembrane Force Probe. A red blood cell (RBC) is pressurized by a micropipette with a glass bead glued to the apex to act as the force transducer. The bead is functionalized by the ligand of interest. On the opposing side, a cell expressing the receptor of interest is held by a second micropipette, and brought into repeated contact with the bead to measure various kinetic and mechanical properties of the receptor as it interacts with the ligand under force. **(b, c)** Mechanical outside–in (b) and inside–out (c) signaling exemplified using the integrin system. Binding of a ligand to the integrin headpiece under tension can trigger outside–in mechano-signaling across the membrane to regulate intracellular processes. *Vice versa*, intracellular force transmitted from the cytoskeleton can allosterically regulate ligand binding via the extracellular region of the integrin, causing inside–out signaling.

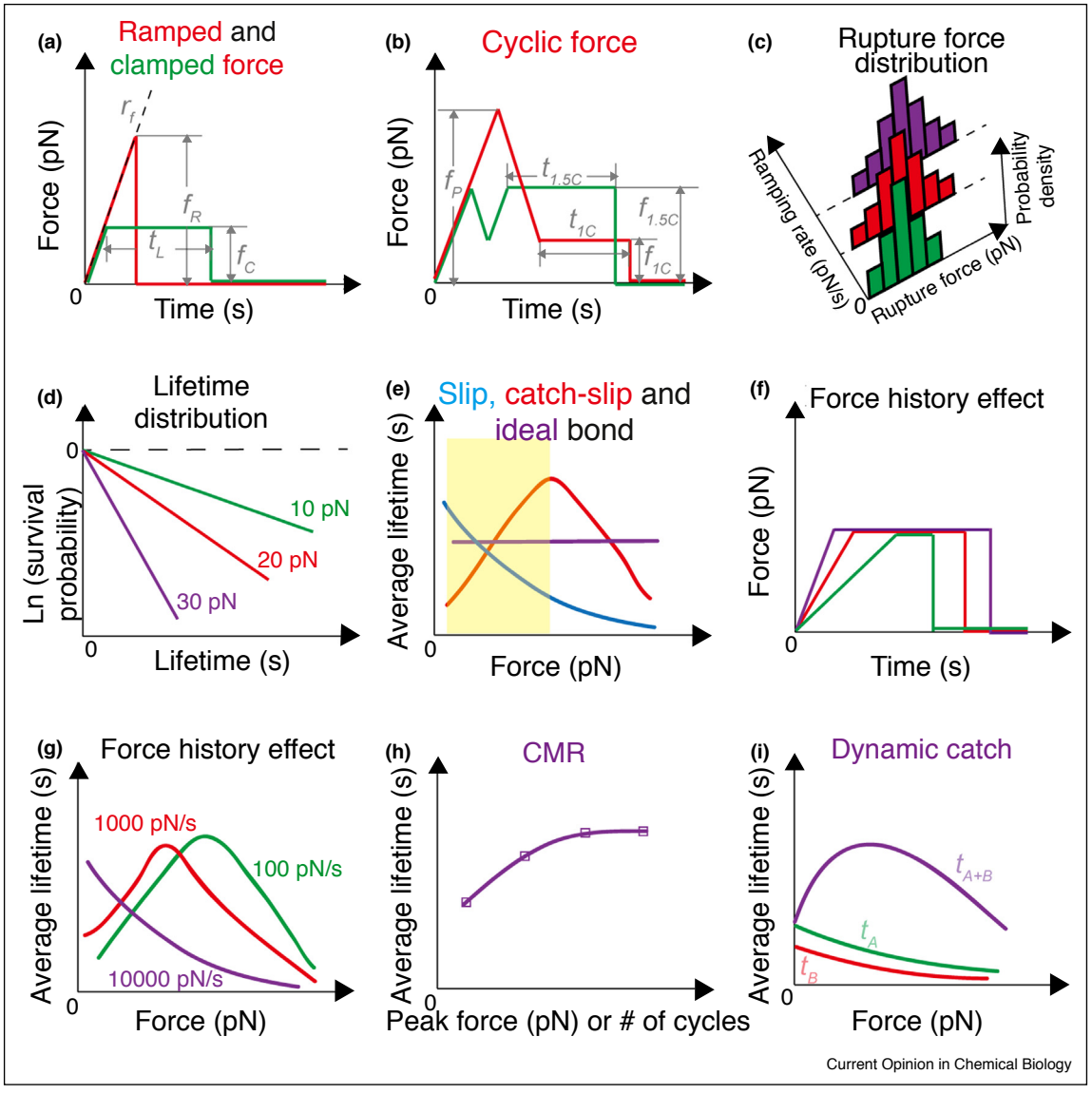
assay (Figure 2a) to measure bond survival probabilities over a range of forces (Figure 2d), however, often observed catch bonds for the same interactions [35,37–42], where the average bond lifetime increases with force in a certain regime before they turn into slip bonds at high forces (Figure 2e). In addition, ideal bonds were occasionally observed, where the lifetime is insensitive to force change [43] (Figure 2e).

The distinctive bond types observed from testing the same receptor–ligand pair with different force waveforms may be attributed to the different force application histories experienced by the bonds, which has been shown to affect their dissociation rate [35,44–46] (Figure 2f,g). Agreeing with this concept, the integrin $\alpha_5\beta_1$–fibronectin [28] and actin–actin [47] interactions manifests a 'cyclic mechanical reinforcement (CMR) behavior where ramping the force to a high level or loading the bond cyclically before measuring bond lifetimes at a lower clamped force (Figure 2b) substantially prolongs the lifetime (Figure 2h). How and why different applied force waveforms can elicit distinct dynamic bond types have yet to be fully elucidated. It may be related to the force-dependent conformation/extension fluctuations of the receptor and/or ligand, which can affect the formation of catch or slip bonds [48–50]. More importantly, it may be connected to the observation that distinct signals can be induced by different applied force waveforms (see below).

Using the same force-clamp assay, whether a receptor–ligand interaction behaves as catch or slip bond may still depend on other factors. It may depend on the ligand species and on the receptor's activation state, as in the case where resting $\alpha_{IIb}\beta_3$ forms a catch bond with fibrinogen but slip bond with fibronectin, which both become more pronounced catch bonds when $\alpha_{IIb}\beta_3$ is activated [26••]. The same ligand may form different bond types with cell surface and purified receptors, as in the case where fibrinogen forms slip bond with purified soluble $\alpha_{IIb}\beta_3$ (containing only the ectodomain) [51,52] but catch bond with platelet membrane $\alpha_{IIb}\beta_3$ [26••]. In addition, force loading direction was also shown to affect the catch bond formation in an intracellular protein–protein interaction: the vinculin–actin catch bond required force to apply toward the pointed (–) end of the actin filament, which resulted in a 10-times long bond lifetime compared with when force was applied toward the barbed (+) end [53•]. This polarity, or dependency of a dynamic bond property on the F-actin end, has also been observed in the CMR of G-actin–F-actin interactions where CMR is more effective at the pointed end compared to that at the barbed end [47]. It is worth noting that such a polarity was not observed in the catch bonds of G-actin–F-actin interactions [54], indicating catch bonds and CMR are distinct kinetic bond types independent from each other.

DFS complements other biophysical techniques in reporting cell adhesion and cell signaling induced by mechanical stimulations. The tension force waveforms employed by DFS need to be carefully selected to best approximate the mechanical environment that cells experience *in vivo*. On the cellular level, two-dimensional (2D) traction force mapping techniques are capable of reporting force tangential to the contact interface only [55]. However, at the molecular scale, tension along the long axis of the receptor–ligand bond should be the dominant force component [56•], largely owing to the much less

Figure 2



Various force waveforms employed by DFS and the resulting protein–protein dynamic bonds.
(a) Superimposed 'force versus time' traces of the commonly used ramped (*red*) and clamped (*green*) force waveforms. **(b)** Single-cycled (*red*) and multiple-cycled (*green*) cyclic force waveforms with the respectively lifetime (t_{1C} and $t_{1.5C}$), clamped force (f_{1C} and $f_{1.5C}$) and the peak force (f_P) of the single-cycled case indicated. **(c, d)** Distributions of rupture forces (f_R, c) for three ramping rates (r_f) obtained from experiment using ramp force waveform and of lifetimes (t_L, d) for three clamped forces (f_C) obtained from experiment using clamp force waveform, respectively. **(e)** Average lifetime versus force curves of slip (*blue*), catch-slip (*red*) and ideal (*purple*) bonds. The 'catch' regime is highlighted in yellow background as this bond eventually will be overpowered by force to turn into a slip bond. **(f, g)** Example force waveforms used to study the force history effect (f), where the initial force ramping rate directly affects the subsequent bond lifetime measured under a clamped-force (g). **(h)** Post cycling (reinforced) average lifetime measured at a low clamped force increases with the peak force (single-cycled CMR) and # of force cycles (multi-cycled CMR). **(i)** Dynamic catch, where a ligand forms slip bonds with two receptors separately (t_A and t_B), but a catch bond with both receptors cooperatively (t_{A+B}).

ability for the linear molecular structure to support bending moment than tensile force at the receptor–cell membrane interface. Studies using a laminar flow chamber have provided evidence for catch bonds and mechanosensing under physiological flow conditions. Adhesions of leukocytes and platelets, respectively mediated by selectins and GPIb, were found to be reinforced by an increase in the wall shear stress [57–59], which supports the observation of catch bonds by these two receptors in DFS [40–42,60]. In addition, recent DFS experiments on live platelets showed that GPIb-induced Ca^{2+} signaling was triggered by durable bonds [11••], which is also in

agreement with flow chamber observations that platelets fluxed intracellular Ca^{2+} while translocating on von Willebrand factor (VWF) surface, where the translocation velocity negatively correlated with Ca^{2+} signal intensity [61,62]. Furthermore, molecular tension probes measured the endogenous forces of cells applied on single integrins [63–65], which fell into the same range (0–>50 pN) as the forces usually applied by DFS experiments [3,14,26••,27,37,66]. The time course of force application in integrin-based adhesion events at the cellular level also seems to resemble the forceramp and slowly loaded force clamp waveforms used in DFS experiments. These results support the relevance of using DFS to study cell mechanosensing.

Receptor–ligand dynamic bonds play important biological roles, many of which are related to cell mechanosensing. The catch bond of integrin $\alpha_M\beta_2$ with ligands is suppressed by a systemic lupus erythematosus-associated single-residue replacement R77H, which impairs $\alpha_M\beta_2$'s negative regulation of autoimmune responses [66]. The binding of sema3E to plexinD1 weakens the $\alpha_4\beta_1$–VCMA-1 catch bond, which allows thymocyte movement toward the medulla, thus enforcing the orchestrated lymphoid trafficking required for effective immune repertoire selection [38]. Suppressing the T cell antigen receptor (TCR) catch bonds with ligands by using cancer-associated somatic mutations in peptide-major histocompatibility complex (pMHC) ligands [19] or altered peptide ligands (APLs) [7•,10••,67] correlates with TCR's reduced ability to signal T cell activation. Suppressing the selectin–ligand catch bonds reduces β_2 integrin activation and focal adhesion assembly on neutrophils [68•]. The Jagged1 ligand undergoes conformational changes upon binding to the Notch receptor, and exhibits catch bond that prolongs lifetime in the force range required for Notch activation [69]. GPIb mechano-signaling induces the intermediate state activation of $\alpha_{IIb}\beta_3$ to mediate biomechanical platelets aggregation [26••], while eliminating the GPIb catch bond with VWF by type 2B von Willebrand disease (VWD) mutations suppresses GPIb mechano-signaling [11••], supporting a role of GPIb mechano-signaling in VWD.

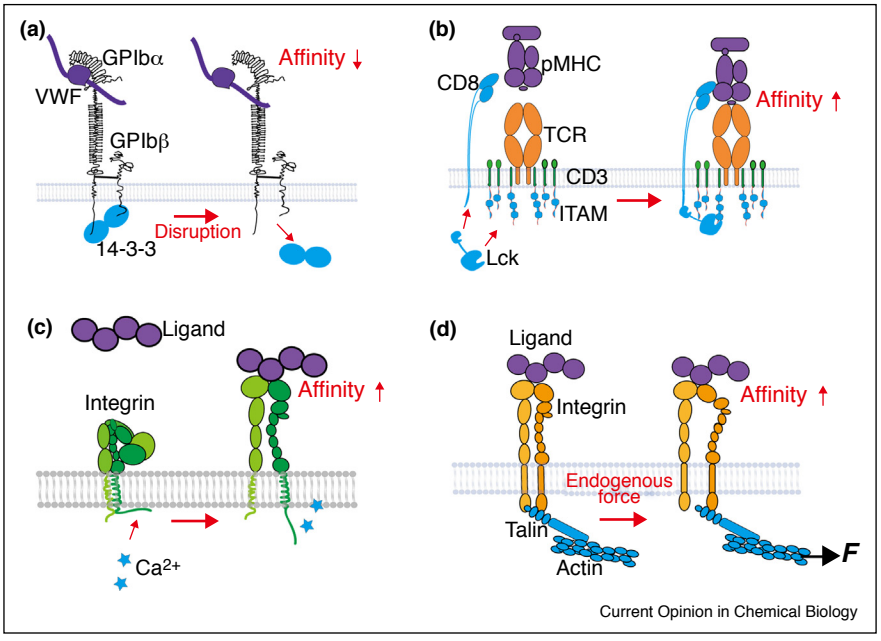
Some ligands can bind two receptors cooperatively, creating a new bond type called dynamic catch (Figure 2i). This was first demonstrated in the endothelial ligand Thy-1, which forms bimolecular slip bonds with integrin $\alpha_5\beta_1$ or syndecan-4 separately, but a trimolecular catch bond with both receptors concurrently and synergistically [27]. Inhibition of the cooperative binding suppresses the FAK-mediated and myosin II-mediated cell signaling at focal adhesions, indicating the relevance of this dynamic catch with cell mechanosensing. Another dynamic catch shows an interesting ligand-dependency: negative-selection pMHCs form trimolecular catch bonds with the TCR and the coreceptor CD8, whereas positive-selection pMHCs do not, suggesting a critical role of dynamic catch in thymocyte selection [25•].

Since different force waveforms may result in different dynamic bonds and induce distinct biological responses, a natural question is what force waveforms cells experience through their surface receptor *in vivo*. Although no current technique allows direct measurement to answer this question, many lines of evidence suggest that many cell surface receptors likely experience forces *in vivo* [70]. The force externally applied to the cell surface receptor in a DFS experiment may be resisted passively (i.e. independent of ATP on short time scales) or reacted more actively in a time-dependent fashion by forces generated by cytoskeleton. An example for the latter case has been reported by Feng *et al.* who found that the force on the TCR–pMHC bond sometimes was rapidly relaxed by cytoskeletal trafficking of the TCR [7•]. In their experiment, force was applied via a step movement tangential to the interface between T cell and the pMHC-bearing bead trapped by optical tweezers. Force was relaxed in a step-wise fashion with a step size corresponding to a ~8-nm lateral movement of the TCR. Treatment with cytoskeletal disrupting drugs suggests that the force relaxation depends on the actomyosin contractile apparatus but not microtubules. Similarly, in the adhesion mediated by the trimolecular binding of Thy-1, integrin $\alpha_5\beta_1$ and syndecan-4, myosin inhibition disrupted adhesion maturation and promoted nascent adhesions, indicating the requirement of actively generated (ATP-dependent) intracellular force in mechano-signaling [27]. The on-rate (and binding affinity), but not force-dependent off-rate (and dynamic bonds) of TCR–pMHC interactions was also found to be substantially suppressed by cytoskeletal and membrane microdomain disrupting drugs [71].

Intracellular regulation of extracellular dynamic bonds and inside–out mechano-signaling

In contrast to the traditional view of 'outside–in' receptor-mediated mechanosensing, increasing lines of evidence suggest new 'inside–out' regulatory mechanisms driven by the interplay between the receptor's cytoplasmic domain and the transmembrane and/or intracellular structures, which can allosterically affect the receptor's extracellular binding behavior. For example, disrupting the linkage of platelet receptor GPIb with its cytoplasmic adaptor protein 14-3-3 downregulates its extracellular binding to VWF (Figure 3a) [72]. Another example comes from the abovementioned dynamic catch of TCR and CD8 with pMHC, which can be regulated intracellularly by the kinase activity, signaling capacity, co-receptor association, and/or substrate quantity of the protein tyrosine kinase Lck. Thus, the *trans*-junctional (cross-junctional) interactions of the ectodomains of TCR and CD8 can be reinforced by the *cis*-(lateral) interactions among the cytoplasmic domains of CD8 and CD3 with the intracellular kinase Lck either directly or via additional scaffolding proteins

Figure 3



Intracellular regulation of extracellular dynamic bonds.
(a) Disruption of the intracellular linkage between the GPIb receptor and the adaptor protein 14-3-3 downregulates the affinity of GPIbα binding to its extracellular ligand VWF. **(b)** The intracellular association of CD8, Lck, and CD3 can induce the dynamic catch of the TCR and CD8 binding to their extracellular ligand pMHC. **(c)** Intracellular Ca^{2+} disrupts the ionic interaction between integrin $\alpha_L\beta_2$ cytoplasmic tail and the cell membrane, resulting in the separation of the αβ transmembrane domains to upregulate the integrin binding affinity for extracellular ligand. **(d)** Endogenous force transmitted through the actin cytoskeleton to talin associated with the integrin cytoplasmic tail separates the αβ tails and stabilizes the integrin in the extended-open conformation for high-affinity ligand binding.

in a manner reminiscent of 'inside–out' signaling for the TCR that was previously only known for its inside–out signaling role (Figure 3b) [25•]. Yet another example is the recent report on intramembrane ionic protein–lipid interaction of integrin $\alpha_L\beta_2$, which suggests a novel 'inside–out' modulation mechanism: intracellular Ca^{2+} uses its charge to disrupt this ionic interaction, leading to a separation in the integrin's αβ transmembrane segments and the subsequent extracellular domain extension to increase adhesion activity [73•] (Figure 3c).

Intracellular forces are known to exist on cytoplasmic proteins that have scaffolding, supporting, and motion-generating roles, which can result from transmission of extracellular forces through connected molecular assemblies across the cell membrane, and can also be extensional or contractile forces endogenously generated by the cell itself via myosin-dependent contraction [74], actin polymerization and retrograde flow of the actin cytoskeleton through cell spreading, motility, and formation of focal adhesion and immunological synapse [7•,25•,75]. Intracellular forces have been shown to modulate the cytoplasmic proteins' conformation and interaction with cell surface receptors, in turn regulating the receptors' activity in an 'inside–out' manner. A good example is the recently proposed integrin 'cytoskeletal force model' where lateral force pulling on the β cytoplasmic tail via talin/kindlin bound to actin retrograde flow could regulate the orientation and alignment of integrins and stabilize their extended-open conformation for high-affinity ligand binding (Figure 3d) [76•,77•,78•]. Similarly, membrane tethering and restructuring have been shown to associate with significant integrin $\alpha_{IIb}\beta_3$-dependent platelet adhesive function [79]. The importance of intracellular force modulation of extracellular integrin dynamic bonds has also been supported by structural analysis and physical modeling, which demonstrated that single-integrin activation requires the balance between the cytoskeletal force transmitted from talin-integrin β cytoplasmic tail axis (inside–out signal) and the ligand binding force (outside–in signal) [76•,80,81]. Similar to integrins, E-cadherin mediated cell–cell junction formation is also subjected to mechanical inside–out regulation through different cytoplasmic adaptors [82,83]. In this context, the recent discovery that the actin dynamic bond type can be switched by the RhoA-formin module suggests a mechanism for intracellular biochemical signals to modulate the force-dependency of actin dynamics, in turn affecting the inside–out regulation [84]. Considering that formin senses both force and torque to regulate F-actin filament polymerization [85], these data altogether suggest a 'crosstalk' between intracellular biochemical and

mechanical signaling pathways during the transmission of cytoskeletal forces.

Extra-cellular and intra-cellular protein conformational changes and their modulation by dynamic bonds

Many proteins can undergo conformational changes, which is often regulated biochemically, for example, upon their (de)phosphorylation, but may also be modulated mechanically, for example, force-induced unfolding of globular domains and uncoupling of interdomain connections. An example for the latter is bending and unbending of the integrin ectodomains, which is generally thought to be regulated by inside–out and outside–in biochemical signaling. Real-time observations have revealed that single integrin $\alpha_L\beta_2$ [13] and $\alpha_V\beta_3$ [14] on live cells undergo spontaneously bending and unbending conformational changes, where force on integrin–ligand bonds tilts the conformational equilibrium to favor unbending over bending. As another example, force on GPIb unfolds the N-terminal leucine-rich repeat domain to prolong the bond lifetime [17], and unfolds the juxtamembrane mechanosensitive domain [15], which triggers intraplatelet calcium signaling [11••] and leads to platelet clearance *in vivo* [86]. Also, force on a TCR–pMHC or pre-TCR–pMHC bond can induce a sudden increase in its length, which can decrease back to its original length in a repeated cycle, and the length change correlates with the biological activity of the ligand [18,87]. A majority of this length increase is attributed to the conformational change in pMHC and a minor fraction to the TCR [19]. Interestingly, the length increase occurs mainly at the force range where the TCR–pMHC catch bond transitions to slip bond with the longest lifetime. Intracellularly, talin rod domains unfold and refold stochastically in a force-dependent fashion, making talin an effective force buffer [88–90]. α-Catenin unfolds under force to trigger vinculin binding with nanomolar affinity, which prevents domain refolding after force is released [91].

From dynamic bonds to cell mechanosensing

As biophysical properties, dynamic bonds impact mechanosensing partly because they modify the upper limit of the 'dose' of mechanical signals that a receptor can receive from its ligand, that is, modulating the 'energy' intake if we regard a molecular mechanosensing system as a machine. Here 'mechanical dose' refers to the strength/intensity of the mechanical stimulation as gauged by quantifiable parameters [26••]. Thus, different dynamic bonds are expected to generate distinct mechanical dose-response curves, which, in turn, modulates the conformational changes of interacting members of a protein assembly, providing the opportunity for conversion between mechanical and biochemical signals [3]. For example, 'molecular clutch' is a key concept for the regulation of cell migration and rigidity sensing [92]. Integrin and talin are key components of the molecular clutch where talin may be unfolded by force to recruit vinculin and regulate focal adhesion formation [93]. However, the unfolding of talin rod domains requires integrin binding events under a narrow force range where catch bond is elicited [88], which can only be achieved on stiff substrates, providing a delicate mechanism for cell rigidity sensing [89].

Just like a machine that can run only when supplied with certain energy forms, a force signal may have to adopt specific waveforms to be recognizable for mechanosensing. This may be due to the force-modulated conformational changes of certain molecules/domains which exhibit different sensitivities to different types of force waveforms. For example, the leucine rich repeat domain of GPIbα can be unfolded by ramping forces but not clamping forces [11••]. Such ability to discriminate different force waveforms may subsequently translate into differential signaling responses, explaining the selective sensitivity to the force waveform of receptor-mediated mechanotransduction. Repeated intermittent ramping forces on integrin $\alpha_{IIb}\beta_3$ can trigger extracellular Ca^{2+} influx, integrin activation and coagulation activities in platelets [26••,94]; in contrast, the same force waveform on GPIb failed to triggered mechano-signals in platelets. Instead, a single clamped force at the optimal level with above-threshold duration on GPIb triggers mechano-signals in platelets [11••]. Whereas force on a single TCR–pMHC bond is incapable of inducing signaling, accumulation of lifetimes from multiple bonds to exceed a threshold within an initial period better correlates intracellular calcium in T cells [10••]. Interestingly, permitting CD8 to bind pMHC together with TCR changes the force waveform requirement, enabling repeated intermittent ramping forces to trigger Ca^{2+} in T cells as well [95]. Alternatively, a rapidly stepping-down force on the TCR–pMHC bond along a specific direction can also induce Ca^{2+} in T cells [7•]. These observations demonstrate that single receptor–ligand bonds can trigger cell mechanosensing without receptor clustering [3,26••,81], suggesting that clustering in focal adhesion is not the only way of triggering outside–in mechano-signaling [3,96].

Concluding remarks

Powered by recent technological advances, significant progress has been made in single-molecule DFS studies. Analysis on live cells has connected various dynamic bonds and protein conformational changes to the inner workings of molecular assemblies that play key roles in the presentation, reception, transmission, transduction, and/or response to mechanical signals into and out of the cell. Dynamic bonds formed via these receptors have been found to be mechanically modulated in both 'outside–in' and 'inside–out' manners. Specific protein conformational changes have been shown as working principles of mechanosensing apparatus, pushing the mechanical analysis of receptor-mediated cell mechanosensing to a submolecular scale. Future studies will

combine these experimental observations with structural analysis and computational modeling to further elucidate these principles in a smaller scale. For example, with the recently determined crystal structures of full-length integrin [97], talin [98,99], kindlin–integrin complex [100], it becomes possible to perform molecular dynamics simulations on the integrin-talin/kindlin complex to simulate the detailed process of integrin conformational change as modulated by cytoskeletal forces, providing atomic-resolution insights on integrin activation and function under force. Future studies will also integrate the recent DFS findings with cell biology and genetic manipulation to study the whole cell scale mechanobiology such as rigidity sensing. With future technical advancements, DFS should also allow the comprehensive investigation of the multi-step cell mechanosensing [3] by monitoring the whole process of signal transduction from the extracellular environment all the way to the cytoskeleton and/or into the nucleus. Ultimately, increasing understanding of the inner working principles of nature's nano-machines will allow us to design better therapeutics and engineer new mechanosensors with synthetic biology approaches to harness the cell's mechanosensory functions.

Conflict of interest statement

Nothing declared.

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