Receptor-mediated cell mechanosensing

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ABSTRACT Mechanosensing describes the ability of a cell to sense mechanical cues of its microenvironment, including not only all components of force, stress, and strain but also substrate rigidity, topology, and adhesiveness. This ability is crucial for the cell to respond to the surrounding mechanical cues and adapt to the changing environment. Examples of responses and adaptation include (de)activation, proliferation/apoptosis, and (de)differentiation. Receptor-mediated cell mechanosensing is a multistep process that is initiated by binding of cell surface receptors to their ligands on the extracellular matrix or the surface of adjacent cells. Mechanical cues are presented by the ligand and received by the receptor at the binding interface; but their transmission over space and time and their conversion into biochemical signals may involve other domains and additional molecules. In this review, a four-step model is described for the receptor-mediated cell mechanosensing process. Platelet glycoprotein Ib, T-cell receptor, and integrins are used as examples to illustrate the key concepts and players in this process.

INTRODUCTION

In all forms of life, survival is based upon the ability to adapt to environmental pressures, including diverse sets of mechanical forces. Force therefore plays an important role in the shaping, development, and maintenance of tissues and organs. Virtually all organisms have evolved structures from the macroscale (organs, tissues) to the microscale (cells) and nanoscale (molecular assemblies, single proteins)

that are sensitive and responsive to myriad forces, including compressive, tensile, shear stress, and hydrostatic pressure. At the cellular level, mechanobiology is concerned with how the cell detects, interprets, responds, and adapts to the mechanical environment. At the molecular level, mechanobiology includes not only enlisting the molecular players and elucidating their interconnections, but also understanding the design and working principles of various mechanosensing machineries so as to re-engineer them for specific applications.

Mechanobiology includes the long history of investigations on mechanosensation, referred to as an organism's active response to environmental mechanical stimuli, such as the functioning of the auditory and haptic system (Gillespie and Walker, 2001; Ingber, 2006). The received signals travel across multicellular tissues/organs to the central nervous system (along the route of a reflex arc), so as to trigger the awareness of the organism and its response. The initial reception of the mechanical stimulations, although presented in a macroscopic scale, is via somatic cells. Certain membrane proteins are found to convert extracellularly applied mechanical stimuli into intracellular chemical signals by opening/closing channels formed by their transmembrane domains (TMDs) to enable/disable movement of substances across the cell membrane (Ingber, 2006).

Mechanobiology is much broader than mechanosensation that can be initiated only by limited types of neurological cells using "professional" components for reception of highly specific types of mechanical signals. By comparison, a wide variety of other cells in all tissues and organs are endowed with machineries that allow them to sense and respond to mechanical cues in their microenvironment,

which are also subjects of mechanobiology research. In these cases, the reception and processing of, and the response to the mechanical signals are all accomplished in a single cell. Receptor–ligand engagement is absent in the initiation of mechanosensation but is required in such important type of mechanosensing—the receptor-mediated cell mechanosensing. In this review, we will focus on receptor-mediated mechanosensing by cells, discuss its requirements and steps, and study how a cell can use such an elegant process to sense and respond to the mechanical environment.

Cells can support mechanical loads via specific or nonspecific structures. As an example of the latter, pressure is borne by the entire cell surface. By comparison, targeted mechanical stimulations are usually applied to specific receptors on cells in direct physical contact with the extracellular matrix (ECM) or adjacent cells through ligand engagement, resulting in receptor-mediated cell mechanosensing. Receptor-mediated cell mechanosensing is of physiological importance, because it plays a crucial role in cell (de)activation, (de)differentiation, proliferation/apoptosis, and many other cellular processes (Orr et al., 2006; Vogel and Sheetz, 2006). For example, focal adhesion assembly enables reinforcement of cells' attachment to the ECM, which requires initial adhesion to transduce force signals to the cytoskeleton, which then regulates actin (de)polymerization and integrin convergence (Wozniak et al., 2004; Roca-Cusachs et al., 2012). Cell spreading (Qiu et al., 2014), contraction (Lam et al., 2011; Sheehy et al., 2012; Garcia and Garcia, 2014; Iskratsch et al., 2014; Myers et al., 2016), migration (Pathak and Kumar, 2012; Schaefer and Hordijk, 2015; Paluch et al., 2016), and differentiation (Engler et al., 2006; Moore and Sheetz, 2011; Wen et al., 2014) also depend on adhesion receptor–mediated mechanosensing, as evidenced by their sensitivity to substrate stiffness (Elosegui-Artola et al., 2016), which in population can even fulfill sophisticated tissue- and organ-level tasks like ECM remodeling (Cox and Erler, 2011).

In these examples of receptor-mediated mechanosensing, adhesion molecules (e.g., cadherins and integrins) have been suggested as, at least parts of, the mechanosensing machineries, for their perturbation alters whether and how a cell responds to the changing mechanical environment. Using the same strategy, many intracellular proteins have also been identified as candidate molecules that play a role in mechanosensing, including adaptor and scaffolding proteins (e.g., talin and vinculin), kinases and phosphatases (e.g., focal adhesion kinase, FAK), and even cytoskeletal components (i.e., actin filament and microtubule). Unlike cell surface receptors that are at the first-line of mechanosensing, these molecules are further downstream and play different roles, for example, transmitting the mechanical signals into the cell, converting them into biochemical signals, integrating and interpreting these signals for decision making, and dispatching the decisions to the appropriate cellular organelles for actions. There have been a number of excellent reviews for these aspects of mechanobiology (Dahl et al., 2008; Kuo, 2013; Romet-Lemonne and Jegou, 2013; Seong et al., 2013; Haining et al., 2016), and they will not be the focus here.

In addition to responding to externally applied forces, cells can internally generate and exert forces on cell surface receptors. For example, forces from actin polymerization, retrograde flow of the actin cytoskeleton, and myosin II–dependent contraction may be transmitted to T- or B-cell receptors (TCR or BCR) bound to peptide–major histocompatibility complex (pMHC) molecules or antibodies anchored to a surface during signaling activation (Hu and Butte, 2016; Murugesan et al., 2016; Hong et al., 2017), cell motility (Mempel et al., 2004; Kim et al., 2009), and formation of immunological synapses and kinapses (Mossman et al., 2005; Sims et al.,

2007; Dustin, 2008; Ilani et al., 2009; Hammer and Burkhardt, 2013). Indeed, internal forces generated by T- and B-cells have been observed being exerted via respectively engaged pMHCs and antigens on the TCR (Bashour et al., 2014a,b; Liu et al., 2016; Ma et al., 2016) and BCR (Wan et al., 2015), respectively. External force applied to the TCR has been shown to induce intracellular calcium flux (Kim et al., 2009; Li et al., 2010; Liu et al., 2014a; Pryshchep et al., 2014; Feng et al., 2017), regulate pMHC dissociation kinetics from the TCR (Liu et al., 2014a; Das et al., 2015) and pre-TCR (Mallis et al., 2015; Das et al., 2016), and potentiate cytotoxic T-cell killing of target cells (Basu et al., 2016). Both T-cells (Axmann et al., 2012; Liu et al., 2014a; Das et al., 2015, 2016; Hong et al., 2015) and B-cells (Natkanski et al., 2013) are able to use mechanical forces to amplify antigen discrimination. In these examples of the emerging field of mechanoimmunology, TCR and BCR represent signal receptors. Unlike receptors for soluble agonists, these receptors bind immobilized ligands and, as such, are subject to mechanical forces. Thus, signaling via these receptors can be modulated or even triggered by force (Upadhyaya, 2017).

Receptor-mediated mechanosensing of a cell is hardly a onetime deal in physiology. Rather, it is usually a complex process composed of many concurrent, sequential, and coordinated signaling events triggered by the same and/or different types of receptors throughout the cell surface (e.g., in focal adhesions, clusters of integrins receive signals simultaneously) over a period of time. Moreover, the ability of cells to provide feedback to the sensed mechanical stimulations and to adjust the force and adhesion strength accordingly (via deformation and modifying cytoskeletal support) enables constant cross-talk between the cell and the environment (Roca-Cusachs et al., 2017). Nonetheless, focusing on the cell sensing of a single wave of unidirectional mechanical signal may simplify the problem and provide a useful angle to begin the mechanistic investigation. In this review, a four-step model is described for the receptor-mediated cell mechanosensing process, covering the presentation, reception, transmission, and transduction of a single wave of mechanical signal. Kinetics of receptor–ligand interaction and mechanics of the molecular players are discussed. Biophysical nanotools commonly used to study receptor-medicated mechanosensing on cells are summarized. Platelet glycoprotein Ib-IX, T-cell receptor, and integrins are used as examples to illustrate the key players in this process.

FOUR-STEP MODEL FOR RECEPTOR-MEDIATED CELL MECHANOSENSING

Upon ligand engagement, cell surface receptors can transduce signals across the membrane. When force is exerted upon receptor–ligand bond formation, this can initiate cell mechanosensing. As demonstrated in Figure 1A, in many cases, such a mechanosensing process may be broken down into four steps carried out by distinct structural components of the receptor–ligand axis:

Step 1. Mechanopresentation: In this step, mechanical cues are presented for the cell to sense. If the force to be sensed is exerted to a receptor, mechanopresentation requires a ligand that is anchored on a surface (mechanopresenter) to support the force upon its exertion (Figure 1A, step 1, purple). In contrast, soluble ligands do not sustain force, and therefore cannot present mechanical cues.

Step 2. Mechanoreception: This is the step during which the mechanopresenting ligand is engaged with a cell surface receptor onto which force is exerted (Figure 1A, step 2, orange). The receptor is termed a mechanoreceptor, because it is the molecule that receives the mechanical signal, which may induce
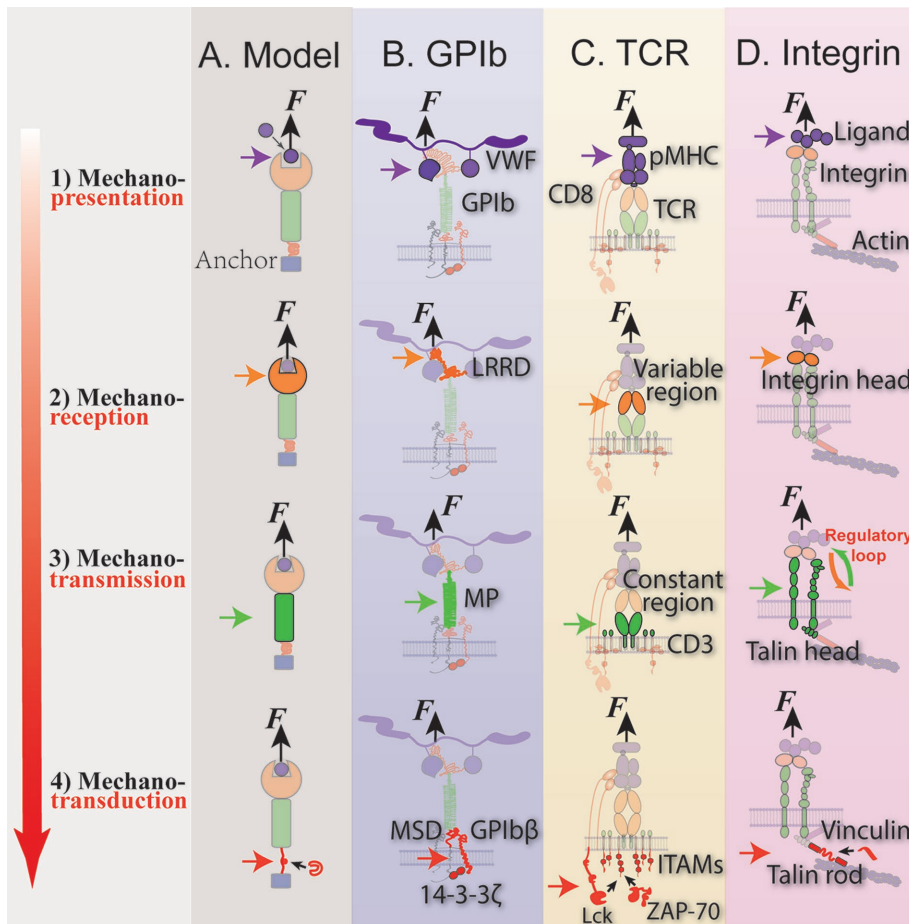
FIGURE 1: Model of mechanosensing with three exemplary systems (GPIb, TCR, and integrin), which is broken into four steps: 1) mechanopresentation; 2) mechanoreception; 3) mechanotransmission; and 4) mechanotransduction. Purple, orange, green, and red arrows indicate the location of each step carried out by a molecule or molecular assembly: the mechanopresenter, mechanoreceptor, mechanotransmitter, and mechanotransducer, respectively. Black arrows indicates external force, *F*. Steps of mechanosensing of a generic model (A) and three model systems, GPIb (B), TCR (C), and integrin (D), were depicted in correspondence to the proposed model. Due to the existence of multiple mechanosensing mechanisms in integrins, the talin unfolding mechanism is selected as representative in D.

contains the structure(s) undergoing mechanical changes, together with other participants of the chemical event, is termed the mechanotransducer (Figure 1A, step 4, red).

Importantly, a key property of mechanotransduction is the capability to distinguish different mechanical stimulations via either a digital or analogous mechanism. In other words, it may react to certain stimulations only, remaining inert to others (digital), or the intensity of the triggered biochemical signal may correlate with the strength of the mechanical stimulation (analogous) (Ju *et al.*, 2016). Mechanotransmission and mechanotransduction are similar in some aspects. However, the central distinction between these two steps is that the latter translates mechanical signals into chemical signals, but the former does not. It is also important to note that a molecule may play more than one role in the mechanosensing process. For example, platelet glycoprotein (GP) Ibα acts as the mechanoreceptor, mechanotransmitter, and also part of mechanotransducer in GPIb-mediated cell mechanosensing, which will be demonstrated below in the section that discusses *Platelet mechanosensing via single GPIb*.

KINETIC AND MECHANICAL ASPECTS
Kinetic constraints of mechanopresentation and mechanoreception

The first two steps of mechanosensing require binding of a mechanoreceptor to an immobilized ligand to allow force to be exerted on the receptor–ligand bond. The receptor–ligand binding kinetics that governs how fast the bond associates and dissociates, quantified by an on-rate and a force-dependent off-rate, are critical to mechanosensing, because they determine the magnitude, duration, and frequency of force application. In other words, kinetics places a constraint on mechanopresentation and mechanoreception, because it limits the frequency, magnitude, and duration of the force that can be presented by a mechanopresenter to a mechanoreceptor. For example, the large volumetric fraction of red blood cells in the bloodstream creates a margination effect that pushes platelets toward the vascular wall (Vahidkhah *et al.*, 2014). This may decrease the gap distance between the von Willebrand factor (VWF)-bearing vascular bed surface and the GPIb-bearing platelet surface, thereby increasing the apparent on-rate of their association (Ju *et al.*, 2015c) and in turn enabling more frequent mechanopresentation and mechanoreception. Even in cases of signal receptors that can be triggered in the absence of force, force may still modulate the signal initiation by impacting the durability of ligand engagement.

At the single-bond level, bond lifetime, that is, the duration of a bond (which is inversely related to off-rate) can be prolonged, unaffected, or shortened by force, defining the catch, ideal, or slip

conformational changes in the binding site of the receptor or the ligand to alter the bond properties.

Step 3. Mechanotransmission: This is the step performed by the mechanotransmitter, whereby the mechanical signal propagates away from the ligand binding site (the business end of the mechanoreceptor) toward the cell interior (Figure 1A, step 3, green). Note that the propagating mechanical signal is not limited to the mechanical force only. As we will show later in the examples of TCR- and integrin-mediated mechanosensing, propagation of force-induced molecular conformational changes should also be counted as part of mechanotransmission, notwithstanding that it may also facilitate mechanotransduction.

Step 4. Mechanotransduction: This is the step when the mechanical cue is translated into a biochemical signal. Note that this definition is different from that used in some other articles, wherein "mechanotransduction" is referred to as the entire "cell mechanosensing" process. Usually, a certain region of the receptor or its linked subunit(s) undergo(es) conformational changes in response to the force waveform (cf. Figure 2), enabling a biochemical event to occur in the cytoplasm. The molecule that
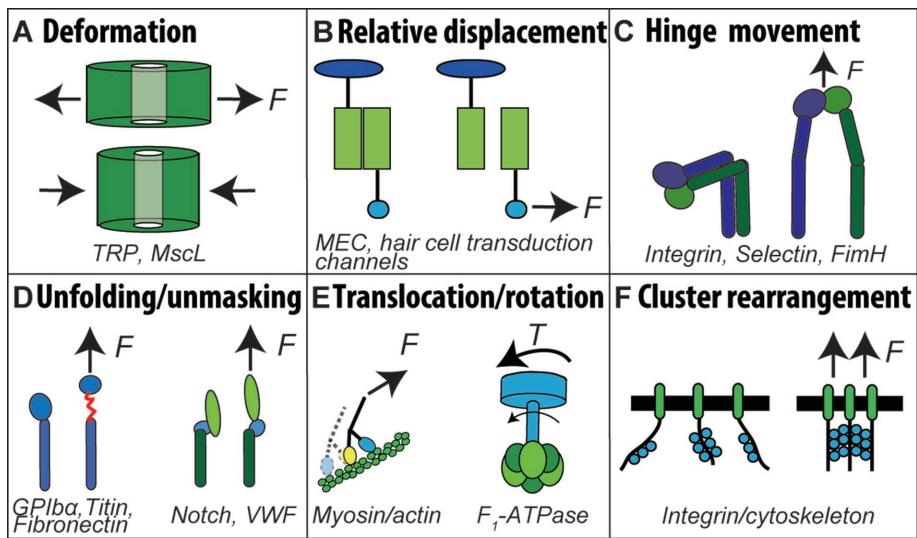
FIGURE 2: Mechanisms of protein mechanosensitivity. Mechanosensitive proteins contain a motif or motifs that can change structure in response to mechanical forces, giving rise to (A) deformation, (B) relative displacement, (C) hinge movement, (D) unfolding and unmasking, (E) translocation and rotation, and (F) cluster rearrangement.

1. Deformation (Figure 2A): Changes in the environment impose mechanical forces and/or hydrophobic effects onto the mechanosensitive proteins and cause deformation of their global shape. By adopting this mechanism, pore-forming membrane ion channels like transient receptor potential channels and bacterial large-conductance mechanosensitive channels can deform in response to membrane tension change and control the channel opening/closing (Sukharev et al., 1999; Orr et al., 2006).

2. Relative displacement (Figure 2B): Two subunits of a transmembrane channel are respectively linked with two external structures. Deflections of these structures cause their relative displacement, which mediates the opening/closing of the channel. This mechanism is mostly studied under mechanosensation, such as in deformation of the skin, vibration of a fly's bristle, and oscillation of a hair cell's hair bundle (Gillespie and Walker, 2001).

3. Hinge movement (Figure 2C): For proteins or their subunits that consist of two or more distinct globular domains connected by a hinge region, such as integrin (Luo and Springer, 2006), selectin (Somers et al., 2000), and FimH (Le Trong et al., 2010), force can relieve the conformational constraints and allosterically promote hinge opening. Take integrin as an example: many studies have demonstrated that force applied through ligand binding facilitates the conformational switch from a bent to an extended conformation (Chen et al., 2012, 2016; Springer and Dustin, 2012).

4. Unfolding and unmasking (Figure 2D): Force can unfold and unmask a specific protein domain to expose cryptic cleaving, binding, or enzymatic sites. For example, several recent studies have demonstrated that force can unfold distinct domains of GPIbα to mediate signal transduction (Zhang et al., 2015; Deng et al., 2016; Ju et al., 2016). Force-induced exposure of a binding site has been shown for intracellular proteins such as talin and vinculin, as well as extracellular proteins such as fibronectin and VWF (Smith et al., 2007; Sing and Alexander-Katz, 2010; Yao et al., 2014; Elosegui-Artola et al., 2016). Force-induced exposure of an enzymatic cleavage site has also been shown for Notch activation (Stephenson and Avis, 2012) and VWF proteolysis (Zhang et al., 2009; Wu et al., 2010).

5. Translocation and rotation (Figure 2E): External force applied to a protein noncovalently complexed with a subunit or subunits of a polymeric filament can drag the protein to translocate along the filament, repeatedly breaking old bonds with the subunit(s) from one side and forming new bonds with the subunit(s) from the other side. This mode of relative movement has been observed in the traveling of linear motors (myosins, kinesins, and dyneins) on their respective filamentous tracks (F-actin and microtubules) (Cross, 2006; Gebhardt et al., 2006; Kodera et al., 2010; Cross and McAinsh, 2014). Myosins are important in adhesion-mediated cell cytoskeletal rearrangement and deformation, and have been postulated to play a critical role in substrate rigidity sensing of cells (Holle and Engler, 2011). Kinesins mediate chromosome segregation during anaphase, a key step in cell mitosis. Application of an external force can cause backward

bond behavior, respectively (Dembo et al., 1988). The physics of the catch bond has been an intriguing problem for biophysicists ever since it was first demonstrated in biological experiments (Marshall et al., 2003). Interested readers are referred to reviews that summarized current models of the catch bond and the underlying mechanisms (Rakshit and Sivasankar, 2014; Liu et al., 2015a). Catch and slip bond characteristics can be important for cell mechanosensing via regulation of bond strength or durability. As demonstrated in the TCR (Liu et al., 2014a) and GPIb (Ju et al., 2016) systems, the levels of intracellular calcium triggered by the receptor–ligand bonds display a similar force dependency to their corresponding lifetimes. Interactions of TCR with agonist pMHC and of GPIb with wild-type (WT) VWF form catch-to-slip bonds, and the levels of Ca^{2+} induced by exerting force on the two receptors show the same pattern, first increasing and then decreasing with increasing force (Liu et al., 2014a; Ju et al., 2016). Furthermore, the force at which the bond lifetime becomes the longest coincides with the force at which the Ca^{2+} level become the highest for both interactions. Moreover, changing the respective ligands to antagonist pMHC and type 2B von Willebrand disease (VWD) mutant VWF converts their respective interactions with TCR and GPIb to slip-only bonds, and the induced Ca^{2+} level also changes its pattern, monotonically decreasing with force in both cases (Liu et al., 2014a; Ju et al., 2016).

Mechanical changes in mechanosensing molecules

Like any materials, biomolecules undergo mechanical changes in response to mechanical loads. Molecules of mechanosensing machineries usually possess certain motifs that are sensitive to mechanical perturbations (termed mechanosensitive domains, or MSDs). Mechanical changes in a MSD may carry out certain mechanosensing function by coupling directly or allosterically to a biochemical event, such as binding to, proteolysis by, or enzymatic modification by another molecule (Ingber, 2006). In this process, the mechanical signals are converted into biochemical signals to propagate further downstream inside the cell, fulfilling mechanotransduction. To date, the most-studied mechanisms of mechanical changes can be classified into six categories:

stepping of a myosin-V on actin, opposite to the direction of ATP-consuming walking (Gebhardt *et al.*, 2006). Rotational movement, on the other hand, occurs in rotary motors, for example, ATPases, where a rod-shaped subunit inserted into the center of a circular complex of subunits can rotate relative to the circular complex (Noji *et al.*, 1997; Nakanishi-Matsui *et al.*, 2010). For example, the F_1 portion of ATP synthase, F_1-ATPase, hydrolyzes ATP to create a torque that rotates its γ subunit relative to α3β3 subunits; reversely, an externally applied torque can rotate the γ subunit to result in ATP synthesis (Itoh *et al.*, 2004; Rondelez *et al.*, 2005).

6. Cluster rearrangement (Figure 2F): The external force is dispersed on a group of clustered proteins, which, through their mutual interactions, act on the adjacent structures and change their arrangement. In focal adhesion, a large number of high-affinity integrins are recruited at the dorsal side of the cell, which affects the dynamic structural rearrangement of the cytoskeleton via linkage between the integrin tail and actin network (Schwartz and DeSimone, 2008). This and the "translocation/rotation" model both achieve mechanosensitivity via cooperation within a group of molecules, in contrast to the other "single-molecule" models.

Depending on the specific physiological function of the mechanotransducer, the type of conformational change (Figure 2, A–F) that it adopts may vary. A specific type of conformational change induces a biochemical signal and generates a functional output, yet it also puts forth limitations. For example, the cluster rearrangement mechanism (Figure 2F) found in mechanosensing of large clusters of integrins in focal adhesions is able to facilitate the rearrangement of the actin skeleton. By comparison, on a platelet, the unfolding of the MSD on a single GPIb is apparently not sufficient to mediate the actin network rearrangement. Instead, its intraplatelet calcium flux triggering has to be via a pathway that can be fulfilled by a single GPIb (Ju *et al.*, 2016).

Besides performing mechanotransduction on cell surface receptors and intracellular molecules, mechanical changes can also occur and be important to other steps of mechanosensing. Taking GPIb mechanosensing as an example, in normal blood circulation, VWF multimer, a mechanopresenter in GPIb mechanosensing, adopts a folded, globular conformation that shields the GPIbα binding sites in its A1 domain and the ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) cleavage site in its A2 domain (Mendolicchio and Ruggeri, 2005). Only when it experiences sufficient fluid shear will its globular structure be straightened. The exposure of the A1 functional site makes it available for GPIbα binding, whereas A2 unfolding allows its cleavage by ADAMTS-13 to reduce its size, which becomes less adhesive. Therefore, the mechanosensitivity of VWF allows its mechanopresentation to be modulated by force before engaging the GPIbα (Fu *et al.*, 2017).

While the two previous subsections discuss kinetics and mechanics separately, the two can be coupled at the level of single receptor–ligand pair. For example, conformational changes can be induced in the receptor or ligand by the bonding force, which in turn modulates their bond strength. Indeed, force has been suggested to expose the cryptic sites of fibronectin, which modifies its binding preference against different integrin species (Vogel, 2006). The leucine-rich repeat domain (LRRD) in GPIbα is unfolded by the force on the VWF–GPIbα bond, which in turn strengthens this interaction. The prolonged bond lifetime in turn increases the unfolding likelihood of the MSD in GPIbα (Ju *et al.*, 2015b), thus enhancing calcium triggering in platelets (Ju *et al.*, 2016). Furthermore, the waveform of the receptor–ligand binding can also affect the occurrence of mechanical events. For example, only abruptly increasing, but not constant, forces are able to unfold the LRRD of GPIbα, whereas both modes of force application are able to unfold the MSD of GPIbα (Ju *et al.*, 2016).

NANOTOOLS FOR STUDYING RECEPTOR-MEDIATED CELL MECHANOSENSING

Dynamic force spectroscopy

Electron microscopy, crystallography, and antibody mapping are the most commonly used approaches to visualize conformations and characterize behaviors of purified proteins (Springer and Dustin, 2012). However, these approaches only take snapshots of proteins' stable states and lack real-time details of transient processes. With the exception of antibody mapping, these methods cannot be used to investigate the coupling of protein conformational changes with subsequent signaling events on live cells. Over the past two decades, dynamic force spectroscopy (DFS) (Neuman and Nagy, 2008; Dulin *et al.*, 2013; Liu *et al.*, 2015a) has provided various biomechanical approaches for manipulation, characterization, and visualization of single receptor–ligand interaction and conformational change with tunable force (Liu *et al.*, 2015a). DFS analyses are usually performed using ultrasensitive force probes (Figure 3A) such as atomic force microscopy (AFM; Figure 3B), optical tweezers (Figure 3C), magnetic tweezers (Figure 3D), and biomembrane force probes (BFPs; Figure 3E), but recently they have been conducted using a novel centrifuge force microscope that enables high-throughput measurements (Halvorsen and Wong, 2010; Yang *et al.*, 2016). With nanometer spatial, submillisecond temporal, and piconewton force resolutions, these techniques can be used to induce, follow, and analyze single-molecule mechanical events in real time, thus revealing individual molecular details inaccessible by conventional methods.

In a DFS experiment, automated precise movement brings together ligands and receptors on the respective force probe (i.e., an AFM cantilever in Figure 3B or a bead in Figure 3, C–E) and target (i.e., a bead in Figure 3A, right, a cell, or a molecule-functionalized surface) with controlled contact time, area, and force. The following separation of the two surfaces applies a piconewton-level force to the receptor–ligand bond to induce mechanical changes in the molecules and modulate bond dissociation. Usually, a DFS experiment is set to perform in either the force-ramp or force-clamp mode. For force-ramp DFS, the force is continuously ramped until the bond ruptures (Figure 4C). For force-clamp DFS, the force is ramped to and clamped at a preset level until the bond ruptures. The bond lifetime is the clamp period (Figure 4E). The rupture forces measured in force-ramp DFS and bond lifetimes measured in force-clamp DFS are used to characterize the force-dependent dissociation (off-rate) of receptor–ligand bonds (Liu *et al.*, 2015a). In addition, multiplexed modes of operation can also be used to exert different force waveforms on the molecular bond, for example, jump-and-ramp DFS (Evans *et al.*, 2004) and cyclic-force DFS (Kong *et al.*, 2013; Li *et al.*, 2016a). In general, DFS experiments have been done to analyze force-dependent receptor–ligand (un)binding and protein domain unfolding/refolding. In the context of receptor-mediated cell mechanosensing, the force-dependent receptor–ligand (un)binding may be involved in mechanopresentation and mechanoreception, whereas protein domain unfolding/refolding may be involved in mechanotransmission and/or mechanotransduction. Thus, DFS analysis provides a tool to dissect the steps of a receptor-mediated cell mechanosensing process and examine them one at a time.

However, DFS alone is not sufficient to fully characterize cell mechanosensing without readout of the intracellular signaling induced by
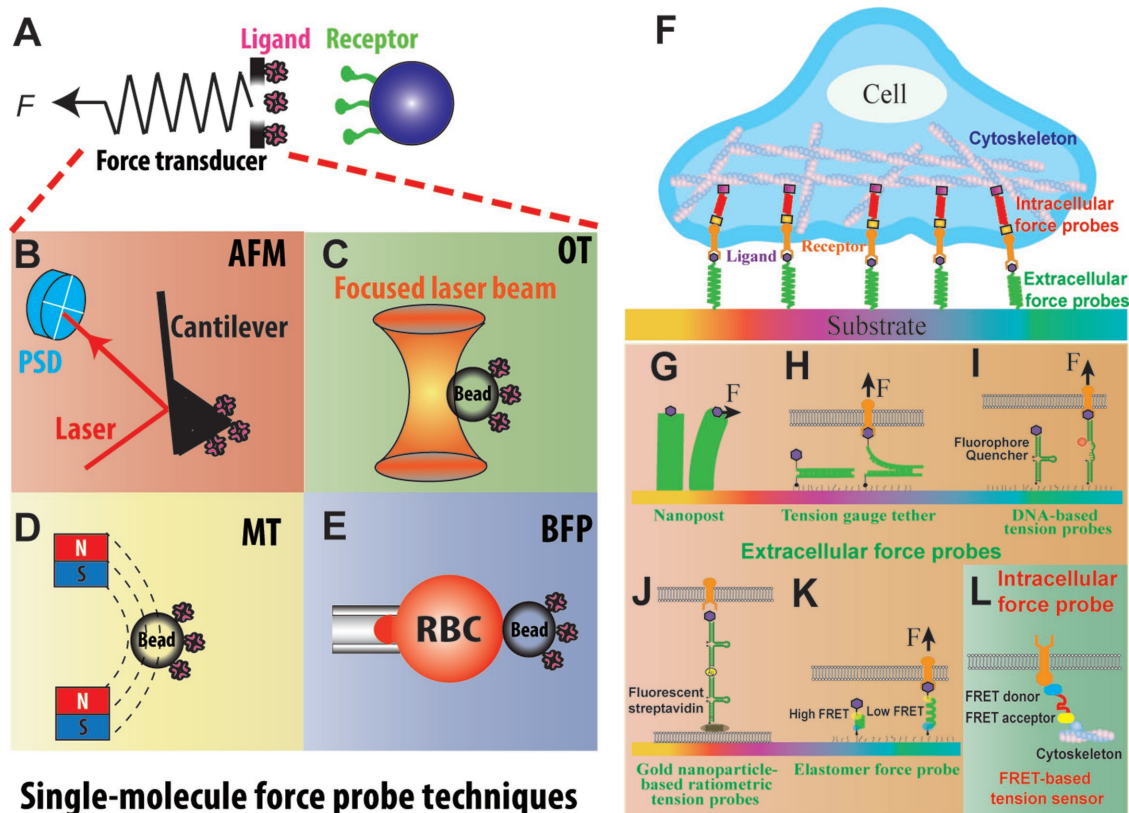
FIGURE 3: Single-molecule force probe techniques (A–E) and microscopic probes for cell tractions and intracellular forces (F–L) techniques. (A) A generic force probe that applies forces (*F*) to the receptor–ligand bond spanning a surface and a force transducer. (B) Atomic force microscopy (AFM): force is applied to individual molecules tethered between a functionalized cantilever and a surface. (C) Optical tweezers (OT): a protein-coated bead is held by a laser beam. (D) Magnetic tweezers (MT): permanent/electrical magnets are used to manipulate a protein-coated magnetic bead. (E) Biomembrane force probe (BFP): the protein-coated bead is attached to the apex of a micropipette-aspirated red blood cell (RBC). (B–E) Force is determined respectively by cantilever deflection (B), bead displacement (C), gradient of the magnetic field (D), and RBC deformation (E). (F) Extracellular and intracellular tension sensors allow microscopic observation of endogenous forces experienced by different proteins. (G) Nanopost: the deflections of the polydimethylsiloxane posts reflect the lateral components of tractions exerted by adhered cells. (H) Tension gauge tethers (TGTs): DNA strands with defined tension tolerances are repurposed to test the tension required to activate cell adhesion. (I) Molecular tension-based fluorescence microscopic probe. The fluorophore and quencher are coupled to report the force-induced unfolding of the DNA hairpin, thereby unquenching the fluorescence to report the molecular forces. (J) Gold nanoparticle-based ratiometric tension probes on supported lipid bilayer monitor hairpin opening due to force while controlling for clustering of mobile ligands using a secondary fluorescent readout. (K) Elastomer force probes report drop in FRET signal due to the elongation of the nanospring domain upon force application. (L) Intracellular force probes genetically inserted into domains of intracellular proteins allow force measurement of key domains involved in mechanotransduction inside the cell.

the mechanical cues. To directly observe single receptor-mediated cell mechanosensing events on a living cell requires performing DFS with concurrent imaging of intracellular signaling (e.g., calcium flux), which was recently achieved by us with fluorescence BFP (Liu *et al.*, 2014a; Chen *et al.*, 2015; Ju *et al.*, 2016) and others using fluorescence optical tweezers (Kim *et al.*, 2009; Feng *et al.*, 2017). This allows one to apply a controlled force waveform and concurrently analyze the resulting receptor–ligand unbinding, receptor mechanical events, and intracellular signaling. In the context of investigating receptor-mediated cell mechanosensing, different force waveforms created by the different DFS modes can respectively mimic the abrupt, sustained, and even more complex mechanical cues a cell may receive physiologically. As demonstrated in recent studies, only durable forces applied via force-clamp, but not transient forces applied by force-ramp, on the respective TCR–pMHC and GPIbα–VWF bonds are able to effectively trigger calcium signaling in T-cells and platelets, respec-

tively (Liu *et al.*, 2014a; Ju *et al.*, 2016). This indicates that cells can distinguish different force waveforms and respond by eliciting different calcium signals. More recently, the dual biomembrane force probe (dBFP) has been developed, which allows examination of the signal reception, initiation, and transduction from one receptor to another on a single cell step by step in space and time. It has demonstrated that the mechano-signals induced by TCR–pMHC and GPIbα–VWF lead to the up-regulation of integrin functions on a T-cell and a platelet, respectively (Ju *et al.*, 2017).

Magnetic twisting cytometry
Another powerful technique that has been extensively used in studies of mechanobiology is magnetic twisting cytometry (MTC) (Wang *et al.*, 1993; Kasza *et al.*, 2011). Instead of applying force, as in DFS experiments, MTC applies a twisting torque to the cell surface via an adherent ligand-functionalized magnetic bead
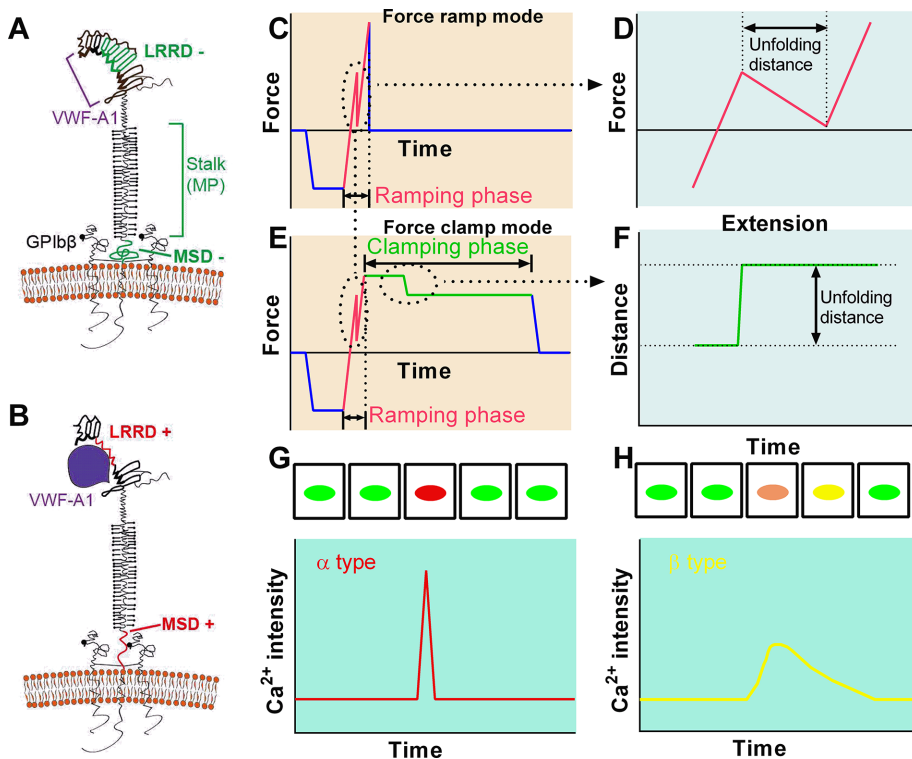
FIGURE 4: Identification and characterization of GPIb mechanosensing mechanism. (A, B) Schematics of GPIb on the platelet membrane, highlighting the folded (−) and unfolded (+) LRRD and MSD. Ligand binding domain for VWF-A1 and other regions are indicated. (C–F) Illustrative BFP force traces showing zoom-in views of unfolding signatures in both ramping and clamping phases. (G, H) Illustrative analysis of GPIb-mediated single-platelet Ca²⁺ flux. Top, pseudo-colored images of intracellular Ca²⁺ in platelets in time sequences. Bottom, time courses of normalized intracellular Ca²⁺ intensity of the α (G) and β (H) types.

specialized cell types (Vogel and Sheetz, 2006; DuFort *et al.*, 2011). A major limitation of the ultrasensitive force techniques depicted in Figure 3, A–E, is that they externally apply forces to the cell but provide no information on whether, and if so, how, the cell actively exerts force, and what cytoplasmic structures support such force intracellularly. To overcome such limitations, a variety of microscopic probes have been developed in recent years to visualize cell tractions and internal forces (Figure 3F). The limitation, however, is that, unlike DFS, which can control the amount of mechanical stimulation fed to the cell, experiments using microscopic probes are incapable of observing cell mechanosensing of a single wave of mechanical signal, but rather provide a summed result of all mechanosensing events that occurred over the whole cell–substrate interface. Moreover, they cannot dissect the individual steps of mechanosensing independently, again unlike DFS.

The first class of the microscopic probes, traction force microscopy, measures bulk traction forces generated by adherent cells. A cell is seeded onto a ligand-coated surface of an elastic substrate, the deformation of which is measured microscopically. The substrate is either an elastic hydrogel containing fluorescent fiducial beads (Plotnikov *et al.*, 2014; Muhamed *et al.*, 2016) or arrays of flexible micro/nanoposts (Figure 3G) (Tan *et al.*, 2003; Fu *et al.*, 2010). The substrate

(Andresen Eguiluz *et al.*, 2017). It is a high-throughput method, as more than one bead is often placed on the surface of a cell, and the responses of many cells are measured spontaneously. This method often involves the engagement of multiple receptors to the bead surface ligands, because a single receptor–ligand bond cannot bear a torque about long axis of the bond. Yet, from the perspective of each single bond, the load still takes the form of mechanical force. A direct readout of an MTC experiment is the rotational angle of the bead in response to the applied torque. Apparent cell stiffness can be calculated as the ratio of the twisting torque to the rotational angle of the bead. MTC experiments generated the first evidence of integrin-mediated mechanosensing by observing stiffening of the cell body in response to a torque (Wang *et al.*, 1993). Moreover, when MTC was combined with fluorescence microscopy (Zhang *et al.*, 2017), various cell signaling events following the application of torque on cadherin-expressing cells (le Duc *et al.*, 2010; Barry *et al.*, 2014; Kim *et al.*, 2015; Muhamed *et al.*, 2016) and up-regulation of dihydrofolate reductase transcription induced by force-regulated integrin ligation (Tajik *et al.*, 2016) were observed.

Microscopic probes for cell traction and internal force
Mechanosensing includes two aspects: the cell senses mechanical forces exerted upon it and the cell actively reaches out to extract mechanical information embedded in its environment (e.g., stiffness, texture, and microtopology). The latter is important, because probing the mechanical properties of the surroundings can influence a cell's behavior, for example, leading cancer cells to become metastatic and mesenchymal stem cells to differentiate into other

stiffness in both cases is measurable, so that deconvolution of the fiducial beads' displacements or deflections of the micro/nanoposts can generate a map of traction force (Sabass *et al.*, 2008; Jin *et al.*, 2017). A three-dimensional version of this technique has also been developed by seeding cells within a cubical hydrogel/biopolymer matrix. Fluorescently tagged beads, again, were used as indicators of local deformation (Legant *et al.*, 2010; Toyjanova *et al.*, 2014; Steinwachs *et al.*, 2016). In the context of cell mechanosensing, traction force microscopy has been instrumental in understanding focal adhesion-related cell mechanosensing (Legant *et al.*, 2010) and the adhesion activity of T-cells (Basu and Huse, 2016). Working with MTC in parallel, traction force microscopy has been used to study how force priming on cadherin cooperated with cell rigidity sensing to induce intracellular signaling (Andresen Eguiluz *et al.*, 2017). Combining with other live-cell imaging methods, traction force microscopy allows one to concurrently visualize the distribution and dynamics of traction forces and relevant molecular players during cellular processes (Feghhi *et al.*, 2016). Furthermore, using the basic setup of traction force microscopy, a technique called monolayer stress microscopy has been developed, in which a cluster or monolayer of cells instead of a single cell is seeded. The tension on the junction of adjacent cells and the internal pressure of the cells are calculated using Newton's laws, given that force balance is achieved on each individual cell (Trepat and Fredberg, 2011). This technique is capable of capturing the mechanical interaction between the cells; however, it cannot provide accurate absolute values for the force parameters, because the mechanical properties of cells are still unknown in such settings (Roca-Cusachs *et al.*, 2017).

The second class of microscopic force probes inserts a polymer (e.g., a double-stranded DNA or a DNA hairpin) between the ligand and the substrate anchor, which extends when the tension applied via engaged cell receptors exceeds a threshold. Two types of DNA-based force probes have been developed, one to limit and the other to report tension above a designed threshold on a ligand: 1) tension gauge tethers (TGTs), for which the idea is to tether ligands to a substrate with a double-stranded DNA that has a defined tension tolerance for rupture (Wang and Ha, 2013; Luca *et al.*, 2017) (Figure 3H). When force generated by the cell and applied through the receptor–ligand bonds exceeds the tolerance force, the TGT irreversibly ruptures and reduces the availability of the tethered ligands, thereby inhibiting force-dependent cellular functions such as spreading, migration, or signaling. By observing whether such functions are inhibited in cells seeded on ligands tagged to TGTs with a range of tolerance forces (from 12 to 56 pN), one can define the force required by such functions. 2) Molecular tension-based fluorescence microscopy probes, in which a DNA hairpin is used that unfolds under a threshold force (ranging from 4 to 16 pN) but refolds upon force removal. The two feet of the hairpin are conjugated with a fluorophore–quencher pair that prevents/allows fluorescent signal when the hairpin is folded/unfolded, thereby reporting whether the force exceeds the unfolding threshold (Blakely *et al.*, 2014; Zhang *et al.*, 2014; Liu *et al.*, 2016) (Figure 3I). Because unfolding is reversible, this design should not affect cell functions, which allows molecular tension-based fluorescence microscopy probes to visualize the spatiotemporal distributions of tensions on the receptor–ligand bonds under more physiological conditions. The sensitivity of these DNA-based force probes can be enhanced by immobilizing DNA hairpins on gold nanoparticles, because the fluorophore will be dual-quenched by both the molecular quencher and gold nanoparticle to yield ~100-fold higher signal-to-noise ratio (Liu *et al.*, 2016). In addition, the immobility of the probes can be overcome by anchoring on supported lipid bilayers (Figure 3J) (Ma *et al.*, 2016), allowing the probes to provide diffusible ligands for mechanosensing by cells. Taking advantage of the fluorescence reporting method, DNA-based force probes also afford the possibilities of dual/multicolor imaging of multiple receptor–ligand species to study their interplay in complex systems.

The third class of microscopic force probes is similar to DNA-based force probes, only with the DNA strands replaced by elastic polypeptides. It can report the dynamics and distribution of forces not only extracellularly (Morimatsu *et al.*, 2013) (Figure 3K) but also intracellularly when the polypeptides and the reporter (e.g., fluorescence resonance energy transfer [FRET] pair or an organic fluorophore) are genetically encoded into an intracellular protein (Grashoff *et al.*, 2010) (Figure 3L). A well-known example of polypeptide-based force probes is the tension sensor module (TSMod), which consists of an elastic domain flanked by two fluorophores whose fluorescence intensity is most sensitive to force in the regime of 1–6 pN. TSMod was originally encoded into vinculin to measure force applied on intracellular vinculin molecules during their recruitment to developing focal adhesions (Grashoff *et al.*, 2010). It has also been inserted along the cytoplasmic tails of the integrin α_L and β_2 subunits to show that migrating cells exert force through the β but not the α subunit (Figure 3L) (Nordenfelt *et al.*, 2016). Incorporation of these intracellular tension probes into various key players in the cell will allow researchers to monitor tensions inside the cell in response to specific mechanical perturbations, which is especially advantageous for studying mechanotransduction.

The various tension-sensing probes have provided researchers with a set of tools to measure force requirement of cellular behaviors and observe force loading and relaxation on a cell. Although the current force ranges of these probes cannot provide a "one-size-fits-all" solution to all receptor types, the iteration of new probe designs continues to push boundaries on the capabilities of these probes. Currently, single-molecule imaging of the probes has not been attained due to limitations in signal detection. Nevertheless, single molecule–level sensitivity and receptor force monitoring using current probe designs may become a reality in the near future as better imaging methods continue to mature.

PLATELET MECHANOSENSING VIA SINGLE GPIB

Next, we will use three systems to illustrate the concepts of receptor-mediated cell mechanosensing outlined earlier. The first example to be looked at in this section is platelet mechanosensing via GPIb receptor. Platelets play a central role in hemostasis and thrombosis. Being anuclear cells with simplified signaling machinery and rapid responses to highly variable mechanical environments, platelets represent a natural model for studying mechanosensing. The subunit of GPIb (GPIbα) contains an N-terminal LRRD that interacts with the VWF-A1, a highly glycosylated long stalk region (macroglycopeptide region, MP) (Fox *et al.*, 1988), a juxtamembrane MSD (Zhang *et al.*, 2015), a single-span TMD, and a short cytoplasmic tail (CT) (Figure 4A). GPIbα is covalently linked to GPIbβ through disulfides, and together they associate tightly with GPIX to form the GPIb–IX complex (referred as GPIbβ from here below) (Luo *et al.*, 2007b; McEwan *et al.*, 2011) and interact with cytoplasmic adaptor proteins such as 14-3-3ζ (Figure 1B). The plasma protein VWF has a limited binding potential for platelet GPIb in circulation due to autoinhibitory mechanisms that mask the GPIbα binding site on VWF-A1 by the adjacent D'D3 domain (Ulrichts *et al.*, 2006), the N-terminal flanking region of the A1 domain (residues 1238–1260) (Auton *et al.*, 2012; Ju *et al.*, 2013; Deng *et al.*, 2017), and the A2 domain (Martin *et al.*, 2007; Aponte-Santamaria *et al.*, 2015). Once VWF is immobilized onto subendothelial collagen and subject to flow, hemodynamic drag forces stretch the macromolecule to adopt an elongated conformation, which facilitates the engagement of A1 to GPIbα (Barg *et al.*, 2007; Schneider *et al.*, 2007). Thus, VWF is mechanosensitive and responds to force by unfolding and exposing its A1 domain (VWF activation). Upon binding of GPIbα to immobilized VWF in the presence of shear, intraplatelet Ca^{2+} is rapidly triggered to activate integrin $\alpha_{IIb}\beta_3$. Thus, GPIb-mediated mechanosensing is the first step of the platelet adhesion and signaling cascade for which GPIb has long been indicated to be the initiating mechanoreceptor (Mazzucato *et al.*, 2002; Nesbitt *et al.*, 2002), and VWF-A1 is the mechanopresenter in this hemostatic process.

VWF–GPIbα binding kinetics

Platelet adhesion to the vascular surface usually occurs in a mechanically stressful environment of blood circulation. Upon vascular injury, VWF in plasma is immobilized onto the subendothelium. It becomes a ligand for mechanopresentation, because it presents the binding motif to GPIbα, the mechanoreceptor, on embarking platelets under shear flow. The rapid VWF–GPIbα association enables the capture of fast-flowing platelets, and rapid bond dissociation allows platelets to translocate (Doggett *et al.*, 2002, 2003; Kumar *et al.*, 2003; Yago *et al.*, 2008; Coburn *et al.*, 2011). In blood flow, physical transport—convection and diffusion—drives blood cells to collide with the vascular surface, bringing interacting receptors and ligands into close proximity (Yago *et al.*, 2007). The three distinct steps of the transport mechanism have been demonstrated to regulate the VWF–GPIbα association: tethering of platelet to the vascular surface, Brownian motion of the platelet, and rotational diffusion of the interacting molecules (Ju *et al.*, 2015c).

Moreover, flow shear results in force on VWF–GPIbα bonds to modulate their dissociation kinetics, eliciting catch bond to prolong bond lifetime at forces <22 pN (Yago et al., 2008; Ju et al., 2013). At forces >22 pN, VWF–GPIbα interaction displays the ordinary slip bond behavior. Such force-dependent kinetics governs the counterintuitive flow enhancement of platelet adhesion on VWF (Savage et al., 1996; Zhu et al., 2008a), which is crucial to the recruitment of platelets to sites of injury in the arterioles. On the other hand, VWF-A1 mutations exhibiting the phenotype of type 2B VWD form slip-only bonds with GPIbα and eliminate flow-enhanced platelet adhesion (Yago et al., 2008; Ju et al., 2013). Furthermore, force-dependent activation of VWF is also affected by its interaction with collagen upon immobilization at sites of vascular injury. DFS and two-dimensional affinity measurements suggest relative contributions of distinct VWF domains, such that the initial VWF capture is mediated by collagen interaction with the A3 domain while the subsequent VWF activation is mediated by interaction with the A1 domain (Ju et al., 2015a). Given that VWF represents an excellent example of a mechanopresenter (Figure 1B), the relative contributions of its distinct domains (and their synergy) to mechanopresentation remain as an interesting topic for future studies.

Taken together, the flow/force-enhanced platelet adhesion mediated by VWF–GPIbα involves at least three mechanisms that directly affect the binding kinetics: 1) relief of the VWF autoinhibitory mechanism involving interdomain associations within the A1A2A3 tridomain and between A1 and D'D3 to expose the GPIbα binding site; 2) enhancement of VWF–GPIbα association by transport; 3) enhancement of A1 interaction with GPIbα by the catch bond. As discussed in the section that demonstrates *Platelet mechanosensing via single GPIb*, the force-dependent kinetic properties of VWF–GPIbα interaction have a significant impact on platelet mechanosensing via GPIbα, not only in their effect on the duration of the force application, but also in both mechanotransmission and mechanotransduction.

Force-induced GPIbα domain unfolding mechanics

Two types of mechanical events have been observed when the GPIbα molecule is pulled by force, namely, unfolding of the LRRD and MSD (Ju et al., 2015b; Zhang et al., 2015). The former was identified using combined molecular dynamics (MD) simulations and BFP experiments (Ju et al., 2015b, 2016). The latter was identified by optical tweezers experiments and mutagenesis studies (Zhang et al., 2015). Pulling GPIbα on the platelet surface, both types of unfolding events are possible, but the two can be distinguished by their different unfolding lengths and by their differential susceptibilities to varying force waveforms. Unfolding of LRRD and MSD generates lengths of around 36 and 20 nm, respectively. Ramped force can unfold both LRRD and MSD (Figure 4, C and D), whereas clamped force can only unfold MSD (Figure 4, E and F). Unfolding of the juxtamembrane MSD following pulling on the GPIbα headpiece indicates the propagation of force along the GPIbα MP (macroglycopeptide) stalk, which defines its role of mechanotransmission.

Coupling between unbinding kinetics and the unfolding mechanics

Interestingly, the occurrence frequency of MSD unfolding was found to depend on the level of clamped force in the same manner as does the VWF–GPIbα bond lifetime. This is true for both cases of the WT VWF that forms a catch-slip bond with GPIbα and the type 2B VWD mutant VWF that forms a slip-only bond (Ju et al., 2016), despite the fact that MSD unfolding rate is accelerated by force, that is, behaving as a slip bond regardless of whether GPIbα is pulled by WT or the mutant VWF. These data suggest a coupling between the unbinding kinetics and the unfolding mechanics. Indeed, BFP experiments showed that LRRD unfolding prolonged VWF–GPIbα bond lifetime (Ju et al., 2015b, 2016). A simple interpretation for this is a force-induced fit mechanism wherein LRRD unfolding exposes cryptic binding site(s) for VWF-A1, as suggested by MD simulations (Ju et al., 2015b).

In contrast to the membrane-distal LRRD, the juxtamembrane MSD is separated from the VWF binding site by the >30-nm MP region. The highly glycosylated MP region is thought to be poorly structured and not known to propagate conformational changes allosterically (Ju et al., 2016). However, it can transmit tensile force from the LRRD to MSD. Thus, the simplest explanation for the coupling between VWF unbinding kinetics and MSD unfolding kinetics is that unfolding of MSD requires sustained force and cannot occur after force removal by VWF unbinding. Indeed, a mathematical model based on this idea predicts the MSD unfolding frequencies very well (Ju et al., 2016).

Importantly, the coupling between ligand binding kinetics and the domain unfolding mechanics generates cooperativity between LRRD and MSD unfolding, such that LRRD unfolding greatly increases the probability of MSD unfolding. This cooperativity occurs only when GPIbα is pulled by WT VWF but not by type 2B VWD mutant VWF, and reaches maximum at 25 pN, where the WT VWF–GPIbα bond lifetime is the longest (Ju et al., 2016).

MSD unfolding and GPIb mechanotransduction

Recent work has demonstrated that unfolding of MSD results in platelet intracellular signaling (Deng et al., 2016; Ju et al., 2016). The R. Li and X. Zhang groups have combined protein engineering and structural analysis with force ramp DFS via optical tweezers to identify the MSD (Zhang et al., 2015) and its Trigger sequence (Deng et al., 2016). Binding of VWF under physiological shear induced MSD unfolding and intracellular signaling in the platelet. Furthermore, mutations that unfolded the MSD and the Trigger sequence therein induced calcium fluxes, filopodia formation, and P-selectin expression in the absence of ligand binding (Deng et al., 2016).

At the level of single molecular interactions, the role of MSD unfolding in GPIb-mediated mechanosensing has been demonstrated using a fluorescence BFP (fBFP), which enables real-time single-molecule, single-cell correlative analyses of ligand binding kinetics, receptor domain unfolding, and intraplatelet calcium imaging (Ju et al., 2016). Single-platelet calcium imaging has revealed two types of intracellular Ca²⁺ fluxes induced in platelets interrogated by a VWF-coated probe that forms infrequent, sequential, and intermittent single bonds with GPIbα (Figure 4, G and H): 1) α type, which features an initial latent phase followed by a high spike with a quick decay (Figure 4G); and 2) β type, which features fluctuating signals around the baseline or gradually increasing signals to an intermediate level followed by a gradual decay to baseline (Figure 4H) (Ju et al., 2016). In-depth time-lapse correlative analyses have revealed that MSD unfolding is required to trigger α-type Ca²⁺. Furthermore, the GPIb example meets the criterion for receptor-mediated cell mechanosensing, that differential information embedded in the force waveform received by the receptor is transduced into distinct biochemical signals. Indeed, it was found that it is the durable force at an optimal level, not the transient force, that triggers maximum calcium. This provides a mechanistic explanation for the bleeding phenotype of VWD patients. The WT VWF–GPIbα catch bond enables sustained binding under high forces to unfold MSD and trigger robust Ca²⁺ for platelet activation. The conversion of the VWF–GPIbα catch bond to a slip bond in type 2B VWD

patients (Yago *et al.*, 2008; Ju *et al.*, 2013) cannot support sustained binding under high forces, and thus fails to trigger adequate mechanosensing (Ju *et al.*, 2016). Moreover, an interfering peptide that disrupts the interaction of the cytoplasmic adaptor protein 14-3-3ζ to GPIbα (Dai *et al.*, 2005) abolishes the α-type Ca^{2+}, implicating the role of 14-3-3ζ as a signal-transducing protein for GPIb-mediated mechanosensing (Ju *et al.*, 2016).

These findings concerning GPIb-mediated mechanosensing can be summarized as follows:

1. Mechanopresentation (Figure 1B, step 1): The ligand VWF undergoes local and global conformational changes to enhance the GPIbα binding capacity to its A1 domain.

2. Mechanoreception (Figure 1B, step 2): The LRRD binds the VWF-A1 and receives the force signal. Force induces LRRD unfolding, which in turn prolongs the duration of force application.

3. Mechanotransmission (Figure 1B, step 3): Force propagates from the LRRD through the MP stalk (Figure 4A) to induce unfolding of the MSD in the juxtamembrane region. The interplay between the VWF–GPIbα bond lifetime and time required to unfold the MSD drives them to follow the same force dependency. The coupling between ligand binding and receptor unfolding results in the cooperative unfolding of LRRD and MSD without the need for allosteric changes in the MP.

4. Mechanotransduction (Figure 1B, step 4): To convert mechanical cues to biochemical signals requires exposure of the Trigger sequence within the MSD and association of 14-3-3ζ to GPIb without inhibition by the interfering peptide.

Structurally, GPIbα may represent a class of mechanoreceptors, containing a distal ligand binding domain and a long, repeated sequence and/or a heavily glycosylated region to connect to the cell membrane. Notch may be another example in this class of receptors. Like GPIbα, Notch1 forms a catch-slip bond with ligand Jagged1 to prolong interactions in the range of forces required for Notch activation (Luca *et al.*, 2017). Pulling generated by endocytosis induces unfolding of the juxtamembrane negative regulatory region, resulting in shedding of its extracellular domain (Stephenson and Avis, 2012) and initiating Notch biochemical signaling inside the cell (Luca *et al.*, 2015). Although GPIbα bears little resemblance to Notch in sequence, structure, or function, their mechanosensing models are remarkably similar in three key aspects. First, both receptors form catch-slip bonds with their respective ligands to prolong the duration of mechanoreception. Second, both receptors contain a polypeptide sequence for long-distance mechanotransmission. Third, force induces unfolding of a juxtamembrane MSD, converting the mechanical cue into a conformational change. Therefore, it seems reasonable to propose that GPIbα may represent a fundamental and evolutionarily conserved signaling mechanism used by cells to sense their mechanical environment.

T-CELL MECHANOSENSING VIA TCR

Engagement of TCR by pMHC on antigen-presenting cells (APCs) to trigger intracellular signaling is central to T-cell development and function. The TCR complex includes the αβTCR heterodimer noncovalently associated with CD3εγ and CD3εδ heterodimers and a CD3ζζ homodimer (Figure 1C). The αβTCR binds pMHC ligand via the membrane distal end, but its very short CT contains no signaling motif. Conversely, the CTs of the CD3 chains contain a total of 10 immunoreceptor tyrosine-based activation motifs (ITAMs). TCR signaling has been extensively analyzed experimentally and modeled based on a number of hypothetical mechanisms. Yet it is still not clear how TCR–pMHC interaction gives the sensitivity and specificity for T-cells to recognize rare pathogenic antigens and discriminate them from abundant self-peptides. Nor is it clear how the TCR is triggered, that is, how information embedded in the pMHC is sensed by the TCR at the ligand binding site, transmitted across the membrane, and transduced into signaling events in terms of phosphorylation of CD3 ITAMs (van der Merwe and Dushek, 2011; Zhu *et al.*, 2013a; Malissen and Bongrand, 2015). Unlike GPIb, whose functions in hemostasis include providing adhesion force, hence giving force an ample opportunity to carry mechanical signals, TCR-mediated mechanosensing is a relatively new idea. In the next section, we compare features of TCR-mediated mechanosensing with those described earlier for GPIb-mediated mechanosensing. We suggest that viewing the TCR signaling process through the lens of the four-step model for receptor-mediated cell mechanosensing helps delineate key properties of T-cell antigen recognition.

TCR-mediated mechanosensing

Increasing evidence has demonstrated that the TCR can mediate T-cell mechanosensing. T-cells can sense the rigidity of anti-CD3 antibody–coated substrate at least in part by TCR signaling (Judokusumo *et al.*, 2012; O'Connor *et al.*, 2012). AFM measurements show that T-cells develop adhesion forces with pMHC-presenting APCs in a manner that correlates with the peptide's potency to induce T-cell calcium and interleukin-2 secretion (Lim *et al.*, 2011). Measurements by traction force microscopy (Hui *et al.*, 2015) and micro/nanoposts (Figure 3G) (Bashour *et al.*, 2014a,b) revealed that cytoskeletal forces are generated and exerted by T-cells during activation through TCR (Basu and Huse, 2016). As more direct evidence, several groups have observed intracellular calcium induction by exerting force through the TCR or CD3 chains, both tangential (Kim *et al.*, 2009; Li *et al.*, 2010; Feng *et al.*, 2017) and normal (Li *et al.*, 2010; Liu *et al.*, 2014a; Pryshchep *et al.*, 2014; Feng *et al.*, 2017) to the cell surface. More recently, oscillatory forces applied to TCR–pMHC bonds was shown to rescue F-actin–dependent T-cell signaling, decoupling the actin network from TCR proximal signaling (Hu and Butte, 2016). All these studies highlighted the importance of force in TCR signal triggering.

Although exerting external force to the TCR could induce T-cell signaling, it was not clear how the forces applied were related to endogenous forces experienced by the TCR. The rigidity-responsive mechanosensing and force-generation processes were clearly mediated by the TCR, but they occurred on the order of minutes when T-cells usually form an organized immunological synapse of adhesion and signaling molecules that convolute the independent mechanosensing of, and force exerted on, the TCR. These issues were addressed by two recent studies using the DNA hairpin force probe (Figure 3J), demonstrating that T-cells could exert 12-19 pN tension on pMHC via TCR and/or CD8 bonding (Liu *et al.*, 2016; Ma *et al.*, 2016). This finding also supports the proposal that, even at early time points, the TCR–pMHC bond is likely subject to mechanical forces (Malissen and Bongrand, 2015).

Force-modulated TCR–pMHC kinetics

Although most kinetic analyses of TCR–pMHC interactions were conducted when (or assumed that) no force was exerted on these bonds, several recent studies demonstrated that externally applied forces alter TCR–pMHC dissociation kinetics (Robert *et al.*, 2012; Liu *et al.*, 2014a; Das *et al.*, 2015; Hong *et al.*, 2015). Similar to VWF–GPIbα interactions, agonist pMHCs form catch-slip bonds with TCR, and like the type 2B VWD mutant VWF, weak agonist/antagonist pMHCs form slip-only bonds with TCR. This peptide-dependent formation of
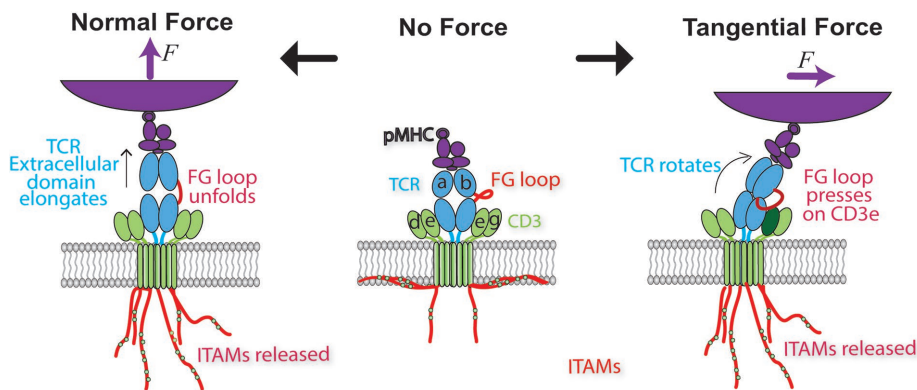
FIGURE 5: Models of how force may trigger TCR signaling. Middle, schematic of the ligated, unloaded, and untriggered TCR. Soluble pMHC binds to the TCR V domains, while the CTs of the TCR-associated CD3 chains remain buried in the lower leaflet of the cell membrane, preventing ITAM phosphorylation. Left, a force normal to the cell membrane pulls on the TCR, extending the length of the complex by ~10 nm. While the structural region responsible for such conformational change has not been identified, here the FG loop connecting the Cβ and Vβ domains is assumed to unfold to result in an extended conformer and in catch-bond formation. Force propagated across the TCR-CD3 connection is assumed to release the CD3 CTs for phosphorylation of the ITAMs. Right, when a force tangential to the cell surface is applied to the ligand binding site of the TCR that also experiences a lateral reaction force from its membrane anchor, a torque is generated to rotate the complex, which is assumed to allow the FG loop to press down on the CD3ε ectodomain to expose the cytoplasmic ITAMs in a piston-like manner.

catch and slip bonds enables force to amplify antigen discrimination (Liu *et al.*, 2014a; Das *et al.*, 2015; Hong *et al.*, 2015). Differential prolongation of bond lifetimes for rare agonists over abundant self-peptides can enhance context discrimination, a well-known property of T-cell antigen recognition, to distinguish between appropriate immune response and autoimmunity, respectively. The idea follows the TCR kinetic proofreading model, which postulates that completion of a series of reaction steps must occur while the TCR is bound to pMHC (McKeithan, 1995; Rabinowitz *et al.*, 1996) or upon rebinding (Dushek *et al.*, 2009; Jansson, 2011) to achieve T-cell activation.

Force-induced mechanical events and TCR mechanotransmission

Like GPIbα that can be unfolded by tension applied via engaged VWF, pulling a TCR–pMHC bond can induce structural changes in the molecular complex, yielding an ~10-nm extension (Figure 5, left). Interestingly, similar to GPIbα LRRD unfolding that prolongs GPIbα–VWF lifetime, this structural change impacts the TCR–pMHC dissociation such that catch bonds are only formed after the extension, suggesting a correlation between the binding kinetics and the structure of the TCR–pMHC complex. This idea has also been supported by other work (Das *et al.*, 2016; Schamel *et al.*, 2017). It is believed that the well-structured FG loop that is present in the Cβ chains of both TCR and pre-TCR contributes to such coupling, because its elimination diminished or abolished the catch bonds for TCR and pre-TCR, respectively, while stabilizing the FG loop greatly enhances those catch bonds (Das *et al.*, 2016). Moreover, the correlation between manipulating hydrophobic hot spots and influencing force-driven kinetic readouts such as double catch bonds highlighted the importance of studying TCR triggering under force (Das *et al.*, 2016).

The extension in the TCR–pMHC complex correlates with the biological activity of the peptide, suggesting a role of such a structural change in TCR-mediated mechanosensing (Das *et al.*, 2015, 2016). Because the location where this structural change occurs is far from cytoplasm signaling motifs, its role in mechanosensing

is likely that of mechanotransmission. The mechanoreception and mechanotransmission is naturally coupled, because the longer the application time, the more likely the force would induce structural changes in the TCR–pMHC complex. This coupling also allows the antigen discrimination property of TCR that produces distinctive force-dependent bond lifetimes for different antigens (Liu *et al.*, 2014a; Das *et al.*, 2015; Hong *et al.*, 2015) to be passed on from the mechanoreception step to the mechanotransmission step.

Models of TCR mechanotransduction and the effect of force direction

Both force and the change in the TCR–pMHC structure that induces extension propagate along the binding axis, until reaching downstream of the TCR C domains, where a conformational change is thought to be responsible for dislodging the CD3ε and ζ CTs from the inner leaflet of the plasma membrane, to make ITAMs accessible for phosphorylation, resulting in mechanotransduction (Figure 5) (DeFord-Watts *et al.*, 2011; Bettini *et al.*, 2014; Dobbins *et al.*, 2016; Li *et al.*, 2017b). This view is supported by a recent finding that three different conformational states of CD3ε cytoplasmic tails exist with various degrees of association with the plasma membrane (Guo *et al.*, 2017). The authors suggested that changes in these conformations could be triggered by mechanical force generated by TCR–pMHC binding. However, how the extension in the TCR–pMHC complex is related to conformational changes in the TCR-CD3 complex has not been defined. This is partially due to the fact that the structural data of the membrane-embedded TCR-CD3 complex are still not available.

The concept of making ITAMs more available for phosphorylation provides a basis for two models of TCR-mediated mechanotransduction. First, a structurally detailed mechanosensor model (Wang and Reinherz, 2012) proposes that lateral force applied via the pMHC generates a torque to rotate the αβTCR. With the TCRβ TMD acting as a fulcrum, the FG loop pushes on the CD3εγ to cause a piston-like movement downward through the membrane, exposing the cytoplasmic ITAMs for phosphorylation (Figure 5, right). The second model, which also highlights the importance of the TMD (Lee *et al.*, 2015), postulates a steady-state divarication of the CD3ζζ juxtamembrane regions, with the N-terminal CD3ζζ TMD acting as the pivot point. Upon pMHC engagement, a conformational change occurs, forcing the CD3ζζ CTs toward each other from the divarication, providing a potential means of transferring mechanical energy into biochemical energy by exposing ITAMs for phosphorylation by kinases (Lee *et al.*, 2015). Unlike the GPIb-mediated mechanosensing model, in which only the magnitude of force is considered, the above TCR-triggering models consider the direction of force as a key parameter (Figure 5). Also, unlike the GPIb-mediated mechanosensing model, in which the mechanoreceptor can only support linear tension, the TCR-triggering models require that the mechanoreceptor also supports rotational torque.

The mechanical changes in the TCR-CD3 complex may depend on the direction of force applied on the TCR–pMHC bond for several reasons. From a structural perspective, the TCR Cα and Cβ

domains vary in size and form different angles with their respective V domains, creating a cavity below the β chain (Kim *et al.*, 2012). Taken together with the CD3 heterodimers that organize around this asymmetry (Birnbaum *et al.*, 2014), force applied to the αβTCR may be productive only in specific directions. Furthermore, the FG loop, which connects the Vβ and Cβ domains, adds to the structural asymmetry and appears to play a functional role, as described earlier. From the ligand perspective, different peptide or antibody docking topologies of TCR have differential capacities to trigger T-cell signaling (Kim *et al.*, 2009; Adams *et al.*, 2011). When the force direction does not coincide with the line from the point of force application (which is determined by the ligand docking topology) to the membrane anchor point of the TCR-CD3 complex (which is determined by its asymmetric structure), a moment will be generated. This moment depends on both the magnitude and direction of force and on the lever arm that could be influenced by the length of the TCR–pMHC complex (which could be extended, as discussed earlier) (Kim *et al.*, 2012). It has therefore been suggested that tangential force, derived from lateral motions, may provide the specific directionality to trigger the TCR (Kim *et al.*, 2009).

However, it should be noted that, in reality, applying directional force through a micrometer-sized force probe to the cell does not guarantee the same directionality when the force is applied on the nanometer-sized molecule. Applying force tangential to the interface between a pMHC-coated bead and the T-cell (Kim *et al.*, 2009; Feng *et al.*, 2017) may cause the bead to pivot at the bead–cell anchor point to pull on the TCR–pMHC bond instead of shearing it. Conversely, pulling a bead away from the T-cell (Liu *et al.*, 2014a) could also induce shear if alignment is not perfect. Recently, both normal and tangential forces applied to T-cells through as few as a single antigenic MHC interaction were shown to induce calcium release, although tangential forces tended to improve activation efficiency (Feng *et al.*, 2017). Additionally, the surface roughness of the T-cell, due to dynamic microvilli, adds another layer of complexity to affect the force direction (Hivroz and Saitakis, 2016). Anisotropy may play an important role in TCR triggering, but further studies are required to address the influence of force direction.

Maintenance and amplification of TCR triggering

A sufficiently long TCR–pMHC bond lifetime would sustain the propagation of force waveform and allow conformational changes to reach the tail end of the TCR-CD3 complex. Yet the time to expose CD3 ITAMs is transitively finite. Is the window of opportunity for TCR signaling limited to the single TCR–pMHC bond lifetime? On the contrary, Liu and colleagues determined that intracellular calcium flux can be triggered if a T-cell accumulates durable TCR–pMHC bonds with a cumulative lifetime surpassing a threshold of 10 s within a 60-s window in the first 3 min of contact. Cells not meeting this threshold failed to trigger calcium, even if single bonds with relatively long lifetimes were experienced (Liu *et al.*, 2014a). This work unraveled a connection between mechanoreception and mechanotransduction in the process of TCR-mediated mechanosensing. Agonist pMHCs that form catch bonds with the TCR meet this requirement and hence can trigger T-cell activation, whereas antagonist pMHCs that form slip bonds with the TCR are defective in activating T-cells, due, at least in part, to the inability to support a durable force at sufficient levels. This may allow the T-cell to distinguish different ligands and react to the correct one only (Liu *et al.*, 2014a). Further, upon including CD8 to form the trimolecular complex, sustained calcium signaling can even be achieved by force-ramping the complex to bond rupture (Pryshchep *et al.*, 2014). These studies de-emphasize the need of single-bond durability in

robust mechanosensing and implicate a memory mechanism involving sequential accumulation of a number of bonds, allowing reconciliation between seemingly conflicting requirements of disengagement and signal maintenance within a serial triggering and kinetic proofreading framework.

The subsequent progression of the membrane proximal TCR signaling process is regulated by the spatiotemporal balance between kinases and phosphatases such as CD45. Highlighting this balance concept, the kinetic segregation model of TCR signaling postulates that phosphatases with large ectodomains are excluded by the narrow cell junction brought together by TCR–pMHC binding, tilting the balance to phosphorylation of ITAMs to trigger signaling (Davis and van der Merwe, 2006). While details of this model require further elaboration, the concept of force on the TCR–pMHC bond due to the large CD45 ectodomains is at the core of this model and must be considered in future revisions. Exactly what biochemical activity directly follows the above mechanotransduction remains elusive. Nonetheless, "come and stay" (Stepanek *et al.*, 2014), "warm-up" (Malissen and Bongrand, 2015), and "catch-and-release" (Katz *et al.*, 2017) models have been proposed to address the timing of the biochemical initiation. Specifically, they each describe how signaling molecules can nucleate amplification of T-cell signaling from the CD3 ITAM mechanotransduction.

From available data, a combination of the cumulative bond lifetime model (Liu *et al.*, 2014a) and models for TCR mechanosensing (van der Merwe and Dushek, 2011; Wang and Reinherz, 2012; Bettini *et al.*, 2014; Das *et al.*, 2015; Liu *et al.*, 2016) is summarized below:

1. Mechanopresentation (Figure 1C, step 1): pMHC as the mechanopresenter binds to the TCR, and a force is applied to this bond externally by relative cell–cell motion or spontaneous cell membrane motility, or internally by basal actomyosin transport of TCR clusters. Although the source of force can be internal to the T-cell, from the perspective of the TCR, force is modulated and presented by the pMHC for the TCR to sense.

2. Mechanoreception (Figure 1C, step 2): The TCR forms a specific bond with the pMHC to support the force, thereby acting as the mechanoreceptor. Force amplifies the information embedded in the pMHC, eliciting catch bonds to strengthen engagement with agonists but slip bonds to weaken engagement with weak agonists/antagonists. In this way, the mechanically modulated information is received in a form readily transmittable across the TCR-CD3 complex.

3. Mechanotransmission (Figure 1C, step 3): The catch bond may be further amplified by the FG loop, which may undergo structural changes to stabilize the extended state of the stressed TCR–pMHC complex (Das *et al.*, 2015). The observation that peptide potency correlates with mechanical extension of the TCR–pMHC complex suggests that information embedded in the pMHC has been translated into a mechanical variable from the binding site to the site of structural transition under force (Das *et al.*, 2016). This mechanical change further propagates to the CD3 chains, resulting in the release of CD3 CTs from the membrane.

4. Mechanotransduction (Figure 1C, step 4): Exposure of the CD3 ITAMs permits phosphorylation, which initiates further signal propagation. This is regulated by the balance of kinases and phosphatases and occurs in a cumulative manner.

Recently, emerging cancer immunotherapies such as the TCR-based chimeric antigen receptor approach have demonstrated high response rates but lack broad applicability (Lim and June, 2017). Genetically modifying TCRs of autologous, tumor-infiltrating T-cells

(Lim and June, 2017) to target a vast array of antigens on solid tumors also appears to be a promising strategy to combat cancer (Maus and June, 2016). By further vetting the four-step process of TCR mechanosensing, one could more rationally engineer TCRs and chimeric antigen receptors to detect and respond exclusively to tumor antigens and neoantigens. Receptors could be designed to amplify different responses between cancerous and healthy cells with minute differences in the antigen structure and abundance, through distinctive force-regulated binding and mechanosensing (Hinrichs and Restifo, 2013). To take such a reverse-engineering approach requires further understanding of TCR-mediated mechanosensing. For example, specific conformational changes under force within the TCR V domains to elicit catch bonds have yet to be identified. Additionally, because conformational changes were found through the FG loop only when the TCR–pMHC complex was under force, it will be instructive to monitor the CD3 CTs under force to study the force transmission beyond the cell membrane. Elucidating these processes will inform how the T-cell can detect and respond to appropriate antigens with such exquisite sensitivity and specificity, key knowledge needed to improve immunotherapies.

INTEGRIN-MEDIATED CELL ADHESION AND MECHANOSENSING

Integrins mediate many cellular processes, including adhesion, spreading, migration, proliferation, and differentiation (Roca-Cusachs

et al., 2009; Elosegui-Artola et al., 2014; Qiu et al., 2014). As physical linkages for the mechanochemical coupling between the ECM and the intracellular structures (Li et al., 2016b), integrins have long been known for their role in mediating force transmission from the ECM across the cell membrane (Volk et al., 1990; Welch et al., 1990). The discovery that integrins are a mechanoreceptor was made more than two decades ago in an elegant experiment using MTC, showing that twisting ligand-bearing beads bound to β_1 integrins caused endothelial cells to stiffen (Wang et al., 1993). The integrin family consists of 18 α and 8 β subunits that combine to form 24 $\alpha\beta$ heterodimeric membrane receptors. Each integrin subunit contains an N-terminal ectodomain consisting of a large head with the ligand binding site and a long leg, a single-pass TMD, and a short C-terminal CT (Figure 6A). The heads and the upper legs of the $\alpha\beta$ heterodimer form the headpiece that connects with the α and β lower legs at the knees (Figure 6A).

In a resting state, integrin adopts a bent conformation with a closed headpiece (Figure 6, B and Ei). Upon activation, integrins undergo a series of conformational changes: ectodomain extension, $\alpha\beta$ separation below the headpiece (including the ectodomain lower leg, TMD, and CT), and hybrid domain swing-out and headpiece opening (Takagi et al., 2002, 2003; Xiao et al., 2004; Nishida et al., 2006; Xie et al., 2010) (Figure 6, B and E, middle, right). The coordinated transitions between these conformations are essential for the cell to regulate integrins' adhesive and signaling functions
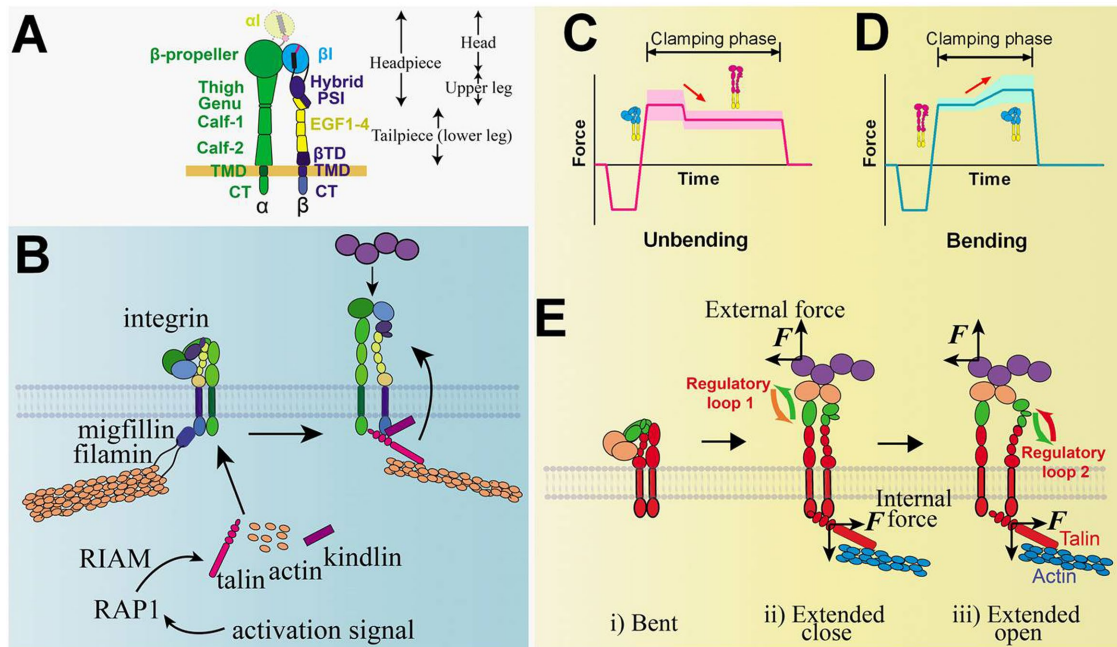

FIGURE 6: Integrin structure and signaling models and illustrative BFP signals that detect integrin bending and unbending. (A) Integrin structure. Domains of the integrin and the ranges of integrin head, upper leg, headpiece, and tailpiece (or lower leg) are annotated. αI domain that exists only in αI-bearing integrins is distinguished by a lighter color and dashed lines. (B) A model of integrin inside-out signaling mediated by RAP1 and RIAM. (C, D) Illustrative BFP force vs. time traces depicting respective integrin unbending (C) and bending (D) conformational change events, denoted by red arrows. The conformations of the integrin are inserted accordingly, with red and blue indicating the extended and bent conformers of the ectodomain, respectively. Widths of the shaded regions reflect the thermal fluctuation amplitude of each trace in the clamping phase. (E) Postulated model of integrin-mediated mechanosensing without prior talin engagement. External force pulls on the i) bent integrin headpiece and allosterically induces ii) ectodomain extension, iii) hybrid domain swing-out, and $\alpha\beta$-subunit separation below the headpiece due to an endogenous lateral force pulling on the βCT. The $\alpha\beta$ separation at the CT presumably recruits intracellular molecules and initiates downstream signaling. The ligand, integrin domains, and talin are colored based on their respective roles in the four-step mechanosensing model (cf. Figure 1). Proposed regulatory loops 1 (between mechanoreception and mechanotransmission) and 2 (between mechanotransmission and mechanotransduction) are denoted.

dynamically (Takagi *et al.*, 2002; Nishida *et al.*, 2006; Campbell and Humphries, 2011; Springer and Dustin, 2012; Cheng *et al.*, 2013; Zhu *et al.*, 2013b; Nordenfelt *et al.*, 2016; Su *et al.*, 2016). Such mechanical changes are accompanied by biochemical changes, making integrins a key component of a cell's mechanosensing machinery. Especially, the separated integrin βCT can associate with talin, kindlin, and several other adaptors to transduce mechanical/biochemical signals (Arnaout *et al.*, 2005; Luo and Springer, 2006). In inside-out signaling, cytoplasmic signals are generated from other cell receptors and relayed by an integrin from the βCT to the headpiece for high-affinity ligand binding. Moreover, it is generally assumed that integrin conformational changes are allosteric, hence also operating reversely to achieve outside-in signaling, in which extracellular ligand binding acts as a signal to propagate from the integrin headpiece to the βCT to induce intracellular signaling. Although outside-in signaling of integrins has long been recognized, the mechanical aspects of this process often are not sufficiently emphasized. We suggest that integrin outside-in signaling in many cases is a mechanosensing process and hence can be analyzed by our four-step model.

Mechanopresentation by the ECM

Besides blood flow, (sub)endothelium, and adjacent adherent cells, the ECM represents a major mechanical environment sensed by integrins for many cell types. The mechanical properties of the ECM, for example, stiffness, can directly affect cell functions (Engler *et al.*, 2006; Fu *et al.*, 2010). Interestingly, in addition to passively receiving mechanical cues from the ECM, cells can also actively modify the ECM as a response of mechanosensing. For example, certain tissues exhibit a "stress-relaxation" property, such that stretching decreases its stiffness (Chaudhuri *et al.*, 2016); conversely, some biological gels and fibrin networks display a "strain-stiffening" property, such that force-induced deformation increases their stiffness (Storm *et al.*, 2005; Jansen *et al.*, 2013). Such properties add a dynamic confounding factor into the ECM mechanopresentation in the integrin-mediated cell mechanosensing (Jansen *et al.*, 2017). In-depth study of cell mechanosensing in this context requires techniques that can adjust the system stiffness in real-time. An example of this is the feedback loop added to an AFM to tune the cantilever stiffness "felt" by the cell in real-time (Lam *et al.*, 2011).

Force-modulated integrin binding kinetics and mechanoreception

The integrin binding kinetics is modulated by force in many biological processes, for example, in leukocyte inflammatory response and in platelet thrombus formation, wherein hemodynamic forces acting on rolling or adherent cells are balanced by adhesive forces on integrin–ligand bonds. Like GPIb and TCR, several integrins have been shown to form catch-slip bonds with their ligands, allowing force to prolong bond lifetimes (Chen and Zhu, 2013). These include $\alpha_5\beta_1$–fibronectin (Kong *et al.*, 2009), $\alpha_L\beta_2$–ICAM-1 (intercellular adhesion molecule 1) (Chen *et al.*, 2010), $\alpha_4\beta_1$–VCAM-1 (vascular adhesion molecule 1) (Choi *et al.*, 2014), $\alpha_M\beta_2$–ICAM-1 (Rosetti *et al.*, 2015), and $\alpha_V\beta_3$–fibronectin (Chen *et al.*, 2016; Elosegui-Artola *et al.*, 2016) interactions. Structural analyses (Kallen *et al.*, 1999; Luo *et al.*, 2007a) and molecular dynamics simulations (Jin *et al.*, 2004; Xiang *et al.*, 2011) on αI-bearing integrins suggest that pulling on the metal ion–dependent adhesion site (MIDAS) of the αI domain causes its anchored α7 helix to shift downward like a piston relative to the upward-moving αI domain, thereby allosterically inducing the αI domain to bind ligand more stably (Chen *et al.*, 2010). Here, the anchor of the αI domain is not at the point of its C-terminal inser-

tion to the β-propeller domain but is provided by the binding of an intrinsic ligand at the end of the α7 helix to the βI domain MIDAS, which also forms an interdomain catch bond (Chen *et al.*, 2010). Using this "dual-catch" mechanism, the suppression of $\alpha_M\beta_2$–ICAM-1 catch bonds by an $\alpha_M\beta_2$ variant R77H (Rosetti *et al.*, 2015), which is associated with systemic lupus erythematosus (SLE) (Nath *et al.*, 2008; Han *et al.*, 2009), can be well explained. The difficulty here is that R77H occurs in the β-propeller domain away from the αI domain MIDAS, which cannot directly alter the αI ligand binding epitope. However, mechanical analysis suggests that R77H loosens the connection of αI to the β-propeller domain, thereby shifting a greater portion of the ligand-transmitted force to the αI–βI interdomain bond. Consequently, before the total force on the external bond (between the ligand and the αI domain MIDAS) reaches a sufficient level to pull the αI domain upward, the component force on the internal bond (between the αI domain α7 helix and the βI domain MIDAS) already exceeds the optimal level. The resulting short-lived slippery internal bond cannot effectively pull down the α7 helix to induce conformational changes in the αI domain MIDAS, thereby suppressing its ability to form a catch bond with the external ligand. This example underscores the pathophysiological relevance of integrin catch bonds. Conversely, cellular signaling can change a receptor's ligand binding behavior under force. For example, the $\alpha_4\beta_1$–VCAM-1 catch bond is suppressed by inside-out signaling upon binding of sema3E to plexinD1 (Choi *et al.*, 2014). This in turn regulates the signaling outcomes of mechanosensing.

Force is inevitably involved in many cases of integrin signaling, owing to the immobilization of the ligand and cytoskeletal linkage of the integrin. Many studies have demonstrated that integrin signal transduction exhibits force sensitivity, recognizing integrins' role as a mechanoreceptor (Lefort and Ley, 2012; Case and Waterman, 2015). Mutations that reduce integrin binding, for example, those in the α1/α1′-helix of the $\alpha_{IIb}\beta_3$ ligand binding βI domain and adjacent to the MIDAS (ADMIDAS) region, cause defects in outside-in signaling in transfected cells (Raborn *et al.*, 2011; Zhang *et al.*, 2013a). Conversely, T188I, a heterozygous ligand binding–increasing mutation in the β_1 integrin headpiece, promotes cell spreading (Evans *et al.*, 2003). Furthermore, like TCR and GPIb, the catch-bond property of an integrin mediates its mechanoreception (Janostiak *et al.*, 2014; Qiu *et al.*, 2014; Sun *et al.*, 2016). For example, the endothelial surface molecule Thy-1 (CD90) forms a slip bond with integrin $\alpha_5\beta_1$ or syndecan-4 when only one receptor is presented. Thy-1 forms two additive slip bonds at low forces with the two receptors when both are present. However, higher forces induce cooperative binding of both receptors to Thy-1, forming a trimolecular catch bond (Fiore *et al.*, 2014). This "dynamic catch" correlates with inhibition of intracellular tension– and substrate rigidity–dependent FAK activity, indicating its relevance to mechanosensing and, with alterations in tumor cell metastasis, suggesting its pathophysiological relevance (Fiore *et al.*, 2014).

Force-induced integrin conformational changes and integrin mechanotransmission

Unlike GPIb and TCR, whose extended or unfolded conformations are induced by force, integrins can exist in three distinct global conformations in the absence of external force: bent with a closed headpiece (Figure 6Ei), extended with a closed headpiece (Figure 6Eii), and extended with an open headpiece (Figure 6Eiii) (Springer and Dustin, 2012). The transitions between these stable conformations may occur spontaneously, giving rise to the coexistence of multiple conformers in equilibrium (Xiao *et al.*, 2004; Zhu *et al.*, 2008b; Ye *et al.*, 2010). From a mechanical standpoint, the multidomain

quaternary structure of integrins resembles a protein machine, with structural parts for force transmission and moving parts for conformational motion connected by ratchets, ropes, and hinges. It is therefore intuitive that the integrin conformation would modulate the transmission of force from the ligand binding site across the main ectodomain down to the cytoplasmic anchor or vice versa. An example is the SLE-associated $\alpha_M\beta_2$ variant discussed earlier, where the single-residue replacement R77H is suggested to tilt the partition of forces between the N- and C-termini of the αI domain that respectively transmit to the β-propeller and βI domains (Rosetti et al., 2015).

Conversely, force can directly induce integrin conformational changes. MD simulations have observed that force can activate the headpiece in silico (Puklin-Faucher and Vogel, 2009), swing out the hybrid domain (Zhu et al., 2008b), separate the αβ tailpieces (Zhu et al., 2008b), and extend the ectodomain (Chen et al., 2011). Real-time observations of force-regulated dynamic bending (Figure 6C) and unbending (Figure 6D) of single integrin $\alpha_L\beta_2$ (Chen et al., 2012) and $\alpha_V\beta_3$ (Chen et al., 2016) using the BFP show that force-induced integrin unbending tilts the conformational equilibrium to favor the extended over the bent conformation. Specifically, tensile force increases the rate (how soon it occurs) and speed (how fast it proceeds), and therefore the frequency, of unbending, and at the same time, decreases the corresponding parameters of bending in a magnitude-dependent manner (Chen et al., 2012, 2016). Thus, integrins are mechanosensitive via a hinge-motion mechanism (Figure 2C) to allow force to facilitate their extension (unbending). The extension in turn facilitates swing-out of the hybrid domain by freeing it from close contact with the tailpiece (Zhu et al., 2008b). Thus, along with force, conformational changes can also propagate in the integrin in a relayed manner. The force-modulated propagation of conformational changes transmits mechanical signals along the molecule, which is an important part of the integrin mechanotransmission step.

The cytoplasmic linkage between integrins and actin is thought to be critical to stabilizing integrins in the extended-open conformation (Zhu et al., 2008b). Recent structural analyses and MD simulations suggest a "cytoskeletal force model," in which lateral pulling on the βCT via talin/kindlin bound to actin retrograde flow could pull the α and β CTs and tailpieces apart to stabilize the extended-open conformation for high-affinity ligand binding. Using integrin βCT-TSMod fusion (Figure 3L) constructs in cell-spreading experiments (Nordenfelt et al., 2016), it has been found that an activated integrin requires the balance between internal forces exerted by retrograde actin flow and external forces applied through engaged ligands by flow-induced shear or substrate stretch. By using the DNA-based force probes, the external force on integrin has been estimated to range from 4–13 pN at the initial spreading (Zhang et al., 2014) and ~40 pN at a stable adhesion (Wang and Ha, 2013). With the TSMod method, the internal force transmitted across the integrin has been estimated as 2.5 pN in focal adhesion (Grashoff et al., 2010).

Mechanical coupling of integrin mechanoreception and mechanotransmission

Integrin conformation and affinity are closely coupled: a bent integrin has a low affinity for ligand. Upon the activation, the hybrid domain swings out to open the headpiece for high-affinity ligand binding. For αI-absence integrins, this requires the headpiece to transition through six intermediate conformations, starting from a closed to a fully open βI (Zhu et al., 2013b). The opening of the headpiece is allosterically associated with the ectodomain extension (Liu et al., 2015b). Conversely, locking the integrin in the bent or extended-closed conformation prevents the emergence of the

high-affinity state (Li et al., 2017a). Promoting ectodomain extension, TMD and CT separation and hybrid-domain swing-out by point mutation or functional activating agents greatly enhances integrin reactivity (Vanhoorelbeke et al., 2009; Rosetti et al., 2015; Su et al., 2016). Although supported by many studies, not all aspects of this model have been validated, leaving room for alternative models. For example, a recent study revealed a bent but high-affinity conformation in the β_2 integrins, implying that ectodomain extension is not necessarily required for the integrin activation (Fan et al., 2016).

Similar to the force-induced GPIb binding reinforcement by LRRD unfolding (Figure 4B) (Ju et al., 2015b), force-induced extension of $\alpha_L\beta_2$ immediately prolongs its binding lifetime (Chen et al., 2012). In addition, cyclic forces applied to integrin $\alpha_5\beta_1$–fibronectin and $\alpha_L\beta_2$–ICAM-1 bonds substantially reinforce their lifetimes (Kong et al., 2013), suggesting a highly localized force-induced dynamic affinity–conformation interplay within the molecule. Considering the coupling between integrin conformation and affinity, integrin mechanoreception (Figure 1D, step 2) and mechanotransmission (Figure 1D, step 3) may be able to provide feedback to each other in a back-and-forth "regulatory loop" (Figure 6Eii, regulatory loop 1), providing force a role in the integrin affinity maturation process.

Signal transmission across the membrane and downstream mechanotransduction

Downstream of integrins and the linked cytoskeletal proteins, mechanotransduction involves the recruitment and phosphorylation of kinases. For example, FAK is phosphorylated by integrin signaling (Harburger and Calderwood, 2009) and regulates Rho-family GTPase activation, which mediates cell migration (Mitra et al., 2005). As another example, following integrin signaling, the Src-family protein tyrosine kinases contribute to the formation of integrin-mediated firm adhesion (Ginsberg et al., 2005; Giannone and Sheetz, 2006; Jansen et al., 2017). Previously, it was believed that induction of these signals requires integrins to cluster, and mechanotransduction is via rearrangement of a cytoskeletal structure like actin (de)polymerization and accumulation of cytoskeletal proteins (Miyamoto et al., 1995; Yamada and Miyamoto, 1995). Yet more recent work has suggested that single integrins may be capable of transducing mechanical signals as well (Harburger and Calderwood, 2009; Lefort et al., 2009; Hu and Luo, 2015). Below we provide three models of single integrin-mediated mechanotransduction that rely on different components of the molecular network connected to integrin. These models are not mutually exclusive; rather, they may respectively modulate different stages/scenarios of cell functioning.

1. The αβ-subunit separation below the headpiece is the most likely mechanical event that allows a single integrin to transmit signal across the membrane (Kim et al., 2003). As mentioned earlier, during mechanotransmission, the extension of integrin is stabilized by a tensile force pulling on the headpiece. Using molecular dynamic simulations on an integrin ectodomain structure, Zhu and colleagues found that a combination of a tensile force normal to the cell membrane and a lateral force on the lower β leg applied on an extended integrin swings out the hybrid domain and separates the lower legs (Zhu et al., 2008b). Integrin βCT is engaged to a number of adaptor proteins, including talin, kindlin, filamin, and migfilin, among others (Figure 6B), which provide constraints to its lateral diffusion (Zhang et al., 2004; Roca-Cusachs et al., 2012). Therefore, the above evidence from Zhu et al. (2008b) suggests that pulling on the headpiece of an extended integrin that is not well aligned with its cytoplasmic

anchor may result in a lateral component force on the β tail causing it to detach from the α tail. The αβ separation in the CT may in turn unmask binding/catalytic sites within the cytoplasmic domains (e.g., enable talin association), resulting in initiation of biochemical signaling and the fulfillment of mechanotransduction (Jani and Schock, 2009) (Figure 6E).

2. It is widely accepted that talin binding to integrin βCT represents a final common step in integrin activation (Shattil *et al.*, 2010). For this reason, talin can play a major role in the mechanotransduction step beneath the membrane. Talin associates with integrin βCT through its head domain and with actin through its rod domain, making the sequences in between susceptible to conformational change upon force pulling (Critchley, 2009). In integrin outside-in signaling, force transmitted through the βCT unfolds the attached talin (Yao *et al.*, 2016), which in turn recruits vinculin and facilitates downstream signaling (Yao *et al.*, 2014). A recent study has provided support for a talin-dependent mechanotransduction mechanism in relation to cell sensing of substrate stiffness. On a soft surface, integrin–ligand engagement is weak and fails to unfold talin. On a stiff surface, by contrast, integrin binding is reinforced by a catch bond, allowing talin to unfold before ligand dissociation (Elosegui-Artola *et al.*, 2016; Jansen *et al.*, 2017). Because the αβ separation below the headpiece would affect the stability of the extended-open conformation of the integrin (Zhu *et al.*, 2008b), a second "regulatory loop" between mechanotransduction (Figure 1D, step 3) and mechanotransmission (Figure 1D, step 4) may exist (Figure 6Eiii, regulatory loop 2). The two regulatory loops described here may even work cooperatively to allow back-and-forth communications among mechanoreception, mechanotransmission, and mechanotransduction (Figure 6E).

3. As force propagates through intracellular structures, several other proteins besides talin that are indirectly linked to integrins may also change conformation and/or function. Examples include vinculin unfolding that exposes the buried motifs (Huveneers and Danen, 2009), p130Cas extension (Sawada *et al.*, 2006), Src activation (Wang *et al.*, 2005), myosin translocation on actin (Figure 2E) (Cross, 2006), and deformation of the nuclear membrane (Wang *et al.*, 2009). These events respectively facilitate focal adhesion maturation (vinculin, p130Cas, and Src), cell deformation (myosin translocation), and directed gene expression and nuclear transport (nuclear membrane deformation), and thus represent additional choices of intracellular mechanotransduction.

Among the three mechanosensing-mediating receptors discussed in this review, integrins represent the most sophisticated system, due to its feature of bidirectional signal transduction, multiple conformational states, and a combination of conformational changes associated with its activation. While multiple mechanisms of integrin-mediated mechanosensing coexist, we can nevertheless summarize the integrin mechanosensing process into the following four steps:

1. Mechanopresentation (Figure 1D, step 1): Integrin binds to the ligands in its headpiece and external force is applied to this bond by relative cell–matrix motion or spontaneous cell membrane movement.

2. Mechanoreception (Figure 1D, step 2): The ligand binding site of the integrin headpiece receives the ligand together with the force signal. Catch bonds are formed to reinforce the binding state under a tensile force.

3. Mechanotransmission: Force propagates along the integrin, unbends integrin through a "hinge" mechanism (Figure 6Eii), and swings out the hybrid domain. In certain scenarios, force directly propagates onto talin (Figure 1D, step 3) or other cytoplasmic molecules, and the integrin unbending and hybrid domain swing-out may not be necessary.

4. Mechanotransduction: Propagation of the conformational changes leads to the αβ separation at the lower leg, TMD, and finally CT to expose cytoplasmic sequences to recruit signaling molecules (Figure 6Eiii). Force propagated through the βCT unfolds MSDs in talin rod and exposes multiple cryptic vinculin binding sites. The recruited vinculin then recruits more F-actin and strengthens the talin–actin linkage (Figure 1D, step 4). In addition, mechanotransduction may also be accomplished via conformational changes of several other cytoplasmic proteins with varied functions.

OTHER CELL MECHANOSENSING MODELS

Besides the aforementioned examples, other molecular systems have been shown to be used by cells to sense their mechanical environment. These systems do not necessarily follow the proposed four-step model. However, certain steps are shared, despite the variations among different biological systems and distinctive functions to be served. Especially, the step of mechanotransduction has to be included in any mechanosensing mechanism, because it is the step of converting mechanical signals to biochemical signals. Here, we will briefly discuss other cell mechanosensing models that include some, but not all, of the four steps of receptor-mediated cell mechanosensing.

In the sound reception of an auditory system, the mechanical signal adopts the form of a soundwave, which is a pure physical signal instead of a mechanical–biological combination. Thus, the mechanopresentation step is not required and is replaced by sound generation. The mechanoreception step, on the other hand, is required, but exists in another form—instead of force-bearing binding to a ligand, the soundwave exerts mechanical load on the hair bundles to cause vibration and deflection. Following that is the mechanotransmission step, wherein the force borne in the deflecting hair bundles propagates across connecting molecules to reach the anchorage of the cell surface ion channels. Force then controls the opening and closing of the mechanically gated ion channels to convert to the first chemical signal, resulting in mechanotransduction with the "clamp movement" mechanism (Figure 2B) (Gillespie and Walker, 2001; Gillespie and Muller, 2009). Adopting a similar mechanism, mechanosensing has also been observed in bristle-based haptic systems of animals, for example, flies (Gillespie and Walker, 2001). Primary cilia that extend from most mammalian cell types have been proposed to perform cell mechanosensing of pure physical signals. Although the underlying mechanotransduction mechanism of this process is still under debate (Delling *et al.*, 2016), its mechaotransmission should also be achieved by the force-induced deflection of the primary cilium (Hoey *et al.*, 2012).

In comparison, the force-sensitive ion channels of somatosensory systems adopt another form of mechanosensing. It also lacks mechanopresentation, with a mechanical force being physically exerted onto the skin cell membrane to trigger the mechanoreception. But in contrast to the auditory system, the mechanotransmission here is the propagation of force on the cell that causes it to deform and change shape. The deformation leads to cell membrane stretching, and opens up ion channels that adopt the "deformation" mechanosensitive mechanism (Figure 2A) to achieve mechanotransduction. This mechanosensing mechanism was suggested

long ago (Orr *et al.*, 2006) and has recently been demonstrated on mouse epidermal Merkel cells (Maksimovic *et al.*, 2014). The same mechanism is shared by muscle mechanosensing, in which stretching of the membrane of the vascular smooth muscle cells opens cation channels. In a similar manner, red blood cells release ATP in response to mechanical deformation, with the only difference being that the mechanotransducer here is the cytoskeletal spectrin–actin network, which is rearranged under force, instead of ion channels (Wan *et al.*, 2008).

Yet in some other systems, the cell can use surface structures to directly receive mechanical signals without binding to a ligand, hence having a mechanoreceptor but no mechanopresenter. For example, glycocalyx and G proteins on the endothelial cell surface have been found to directly transduce shear stress for mechanosensing without binding to any ligand (Chachisvilis *et al.*, 2006; Makino *et al.*, 2006; Orr *et al.*, 2006). Corresponding mechanisms have been proposed (Fu and Tarbell, 2013).

Although examples in this review only discuss monovalent molecular interactions, multivalent interactions do exist in many receptor–ligand systems. For example, P- and E-selectins (but not L-selectin) are dimeric and can form dimeric bonds with the dimeric P-selectin GP ligand 1 (Ramachandran *et al.*, 2001; Zhang *et al.*, 2013b); fibrinogen and VWF are both capable of binding multiple copies of their corresponding receptors on the same or even different platelets (Rooney *et al.*, 1998; Zhou *et al.*, 2012). The association and dissociation of these complexed bonds follow multistep kinetic mechanisms that involve intermediate steps with additional rate parameters. Also, the force burden is, likely unevenly, dispersed onto multiple bonds, making the force dependency of the bond lifetime more complicated (Friddle *et al.*, 2012). Nonetheless, it is likely that a multimeric ligand has a higher chance to engage cell receptors for more frequent mechanopresentation. The overall bond lifetime is also likely prolonged, because dissociated members of the receptor–ligand cluster are still kept within close proximity by other members, remaining associated so that they can quickly re-engage, resulting in more effective mechanoreception. Although receptor cross-linking by multimeric ligand is known to facilitate cell signaling in many situations, multivalency may or may not guarantee stronger mechanosensing. While clustering creates a higher local concentration of signaling molecules to strengthen signaling, the formation of more bonds must be accompanied by the decrease of force dispersed over each of the bonds, which may weaken the signaling of each receptor. Should mechanosensing adopt a threshold mechanism that requires certain amplitude of force on single receptors, excessive force dispersion could even eliminate signaling. In addition, despite not having a dimeric/multimeric structure, some proteins contain dual or multiple sites that can bind independently or cooperatively. For example, fibronectin contains a synergy site in the III_9 domain that can strengthen the binding of the III_{10} domain RGD sequence to integrin $\alpha_5\beta_1$ (Friedland *et al.*, 2009; Kong *et al.*, 2009). Thy-1 can simultaneously bind integrin $\alpha_5\beta_1$ and syndecan-4 in a force-induced cooperative manner (Fiore *et al.*, 2014). Accordingly, the mechanoreception is reinforced in both cases. Conversely, cellular signaling can directly affect the multimericity of a molecular system. For example, when pMHC binds TCR to induce signaling, the co-receptor CD8 is upregulated to bind the pMHC pre-engaged with the TCR, which converts the TCR–pMHC bimolecular bond to a TCR–pMHC–CD8 trimolecular bond (Jiang *et al.*, 2011; Casas *et al.*, 2014; Liu *et al.*, 2014b).

CONCLUSIONS

Research in the past few decades focused on developing biochemical and molecular biological approaches, which allowed scientists to identify and characterize molecular players of mechanosensing pathways. Recently, new biophysical nanotools have moved the mechanobiology field into a new era, when it has become possible to address a fundamental question: how are mechanical cues presented, received, transmitted, and transduced by cell surface and intracellular proteins? Furthermore, the time has come to elucidate the design and working principles of the mechanosensing apparatus. As summarized in this review, the use of these nanotools allows the investigation of receptor-mediated cell mechanosensing in many biologically significant contexts, including how platelets balance their functions between hemostasis and thrombosis, how T-cells recognize pathogens, how stem cells differentiate into distinct cell types, how cancer cells metastasize, and how neutrophils migrate to fight infections. More importantly, the combined kinetic analysis of molecular interactions and mechanical analysis of the molecular conformational changes reveal the working mechanisms of each mechanosensing receptor and its MSDs—GPIbα unfolds the LRRD and MSD, TCR–pMHC increases its length, and integrins bend and unbend between the upper and lower legs. In the future, full characterization of these and other modular structures of mechanosensing machineries will provide synthetic biology design principles for a generic mechanosensing machine. The similarities and differences between the GPIb and TCR systems imply that, in addition to the general principles, each receptor may have its own details to accommodate its specific function. Future studies will relate the mechanosensing mechanisms of distinct receptors to their physiological context and allow customization based on the general principles and re-engineering of the details.

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