Fall 12-15-2020

Single-Fluorophore Sensors for Mechanical Force in Living Cells

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January 20th, 2021

Abstract

Mechanotransduction is the process by which a mechanical stimulus is converted to a cellular signal. This process is heavily influential of cell morphology, differentiation, and behavior. However, altered levels of mechanical stimuli are also found in many pathological contexts. For example, cancerous cells have stiffer surrounding tissue than healthy cells, and research suggests that this alters cell behavior and promotes metastasis. Despite these findings, the cellular processes behind these signaling alterations remain widely unknown. Understanding these cascades is critical, as involved proteins can give us a deeper understanding of the role of mechanotransduction, and certain proteins can potentially be targeted by drug therapeutics.

This thesis reviews existing methods used to study mechanotransduction and force within the cell, and specifically investigates the benefits of single-fluorophore tension probes. Moreover, the idea of a novel tension probe, based on protein-protein interactions and bond-breaking, is introduced and developmental steps are outlined.

Acknowledgements

The study is supported by funds from UConn Summer Undergraduate Research Fun (SURF) and the Health Research Program (HRP) (award to SK), and NIH (YIW, GM117061). Special thanks to the Coric Family for the SURF 2020 Named Award. Additional thanks to my incredible mentors in the Wu Lab: Dr. Yi Wu, Dr. Prem Shrestha, Yuezhe Li, and Milda Stanislauskas.

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I. Introduction

The classical ideology of 'structure dictates function,' has been widely described in a biological context. The mechanical features of a cell, such as whether its extracellular matrix is soft or stiff, have been found to determine cell behavior, and ultimately tissue function (1). This process is called ***mechanotransduction***, and occurs when a mechanical stimulus is converted to a cellular signal. Transmembrane proteins like integrin facilitate this process in response to changing extracellular environments (2). For example, in response to soft or stiff extracellular matrices, integrin receptors can initiate the formation of focal adhesions (Figure 1). Focal adhesions are integrin clusters that serve as 'signaling hubs' (3). These hubs recruit components like talin and vinculin to their sites, and couple to intracellular F-actin to play a large role in downstream cellular signaling effects (4). Depending on the extracellular ligand present, these signaling events can be highly influential of cytoskeletal structure, cellular morphology, and stromal cell behavior (5, 6). Moreover, stiff mechanical substrates can promote F-actin and integrin dependent activation of YAP1 and TAZ transcription factors, which can drive gene expression and promote differentiation (6). Additionally, the altered microenvironment of the cell can then go on to influence the behavior of its surrounding cells (5).

Altered levels of stiffness within the extracellular matrix are present in pathology as well. Present-day literature outlines several processes in which malignant tumors alter cellular mechanics and contribute to increased stiffness of local somatic tissue (4). Prior research has found that the primary cause can be attributed to an imbalance between the rates of deposition and degradation of the extracellular matrix

(ECM) (4). Classical properties of pathologically stiffer tissues include a decrease or absence of tumor necrosis factor, TNF-α, and increased levels of transforming growth factor beta (TGF-β) (4, 7). The increased rate of crosslinking causes the ECM to accumulate much faster than it can be degraded, contributing to the larger and harder masses that are characteristic of most cancers (1). There's also strong evidence showing that stiffer substrates help malignant cells facilitate pro-proliferatory as well as pro-migratory behaviors (4). This is because malignant cells have specialized integrin receptors coupled to their F-actin cytoskeleton interiors (4). These receptors allow cancer cells to recognize harder substrates, and ultimately alter the cell's normal process of mechanotransduction. Additionally, like under normal physiological conditions, the altered interface between the cancerous cells and their extracellular environment leads to the formation of focal adhesions (4). Their subsequent interactions with the ECM lead to the downstream activation of molecules like MAP Kinase, which promotes cellular growth, while simultaneously downregulating tumor suppressors such as PTEN (4).

Despite what is already known about mechanotransduction pathways, many of the intricacies involving altered signaling pathways are still unknown. Understanding common properties amongst mechanotransduction cascades can help scientists to improve upon diagnostic as well as therapeutic techniques, as well as to gain an overall deeper understanding of cell behaviors. Characterization of how cells behave in environments of altered stiffness would be most useful with the use of a tension biosensor. The following is a literature of review of existing mechanotransduction systems, technologies, and their respective limitations. Additionally, I review the

benefits of single-fluorophore fluorescent probes, and introduce a prospective tension sensor based on bond-breaking interactions.

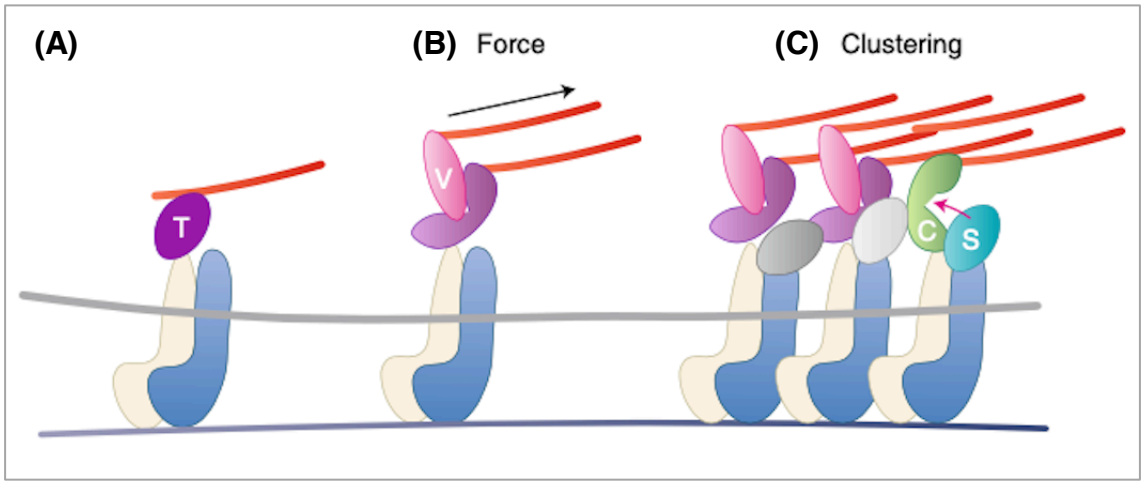

Figure 1 *Reprinted by permission from Copyright Clearance Center: Springer Nature, Nature Cell Biology, Mohammadi et al, 2018*
Schematic of force application and subsequent focal adhesion formation. **(A)** Transmembrane integrins (blue and white) spanning cell membrane (grey line) are coupled to F-actin (red) via talin protein (T; purple). **(B)** Force is applied to the system, more pronounced by stiffer substates, and talin unfolds. The conformational change allows vinculin (V; pink) binding. **(C)** Vinculin recruits more actin filaments, and promotes clustering.

II. Existing Tension Sensors, and Limitations

IIa. Grashoff et al., VinTS

Currently, a majority of tension sensors involve a double-fluorophore system and FRET pairing. One primary example of a mechanical sensor is the genetically encoded sensor, VintTS published by Grashoff et al. (3). VinTS was designed as a fluorescent resonance energy transfer (FRET) pair with an elastic linker derived from a spider silk protein (3). The construct is meant to be inserted within vinculin, allowing imaging to take place *in vivo* (3). When tension is applied on the system, the elastic domain extends, and the FRET pair is pulled apart resulting in a weaker FRET signal (3). The signal is measured before and after tension is applied, and the difference is

calculated to determine relative mechanical force (3). Although VinTS is a useful model for detecting the presence of increased mechanical force, it has many limitations. To begin with, the range of forces measurable by VinTS is confined by the strength of its elastic linker. The spider silk derivative is only sensitive to weak forces up to a few pN, such that stronger forces will lead to a saturation of signal. Additionally, the availability of the linker is restricted to what is available in nature, and may not be easily tuned for optimal elasticity. Moreover, VinTS has limited dynamic range and sensitivity. Due to the large sizes of both the FRET donor and acceptor molecules, the initial distance and resting signal of the pair is inherently weak. The low sensitivity of the system is highly unfavorable, since the threshold of force to trigger downstream cascades is still unknown. Finally, using FRET imaging limits the ability to fully elucidate how various signaling pathways interact simultaneously during a downstream event (8).

IIb. Chen et al. & Zhang et al., Oligonucleotide Approaches

In 2014, Chen et al. published a genetically encoded, single-oligonucleotide tension probe (8). The system is made up of a fluorophore, and its corresponding quencher, located on opposite ends of the oligo (Figure 2A). This arrangement forms a hairpin structure in which one end binds a substrate, and the opposite end binds an integrin (8). When both ends are bound to appropriate ligands, and force is applied, the force ‘unzips’ the hairpin structure. The applied tension unwinds the hairpin, subsequently separating the quencher from the fluorophore, and results in an observable fluorescence emission (8). The single oligo design makes this sensor quite robust, and allows the sensor to observe the adhesion maturation process over long periods of time

without shearing (9). However, the use of a single linker provides a limited distance between the fluorophore and quencher when unzipped, and can result in a lowered dynamic range when force is applied to the system.

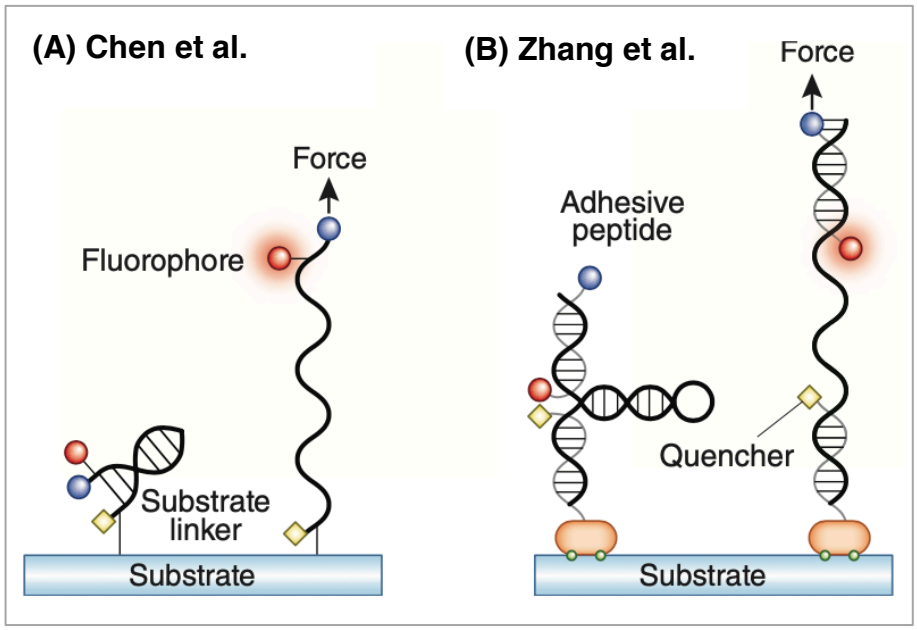

Figure 2
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(A) Single-oligonucleotide tension probe developed by Chen et al. Applied force unzips the hairpin structure, and separates the fluorophore from its quencher.

(B) Tri-oligonucleotide tension probe developed by Zhang et al. ligand and anchoring oligos may be modified without affecting intermediate hairpin.

Comparatively, Zhang et al. published another tension module in 2014, which is composed of three oligos rather than one (Figure 2B)(1). The first of the three oligo consists of a quencher linked to the extracellular substrate, and the second contains an adhesive peptide and fluorophore (1). Interestingly, the third oligo acts as an intermediate, and hybridizes to the first two as another hairpin structure (1). When force is applied to the system, the hybrid oligo unzips such that the first and second oligos separate, and the fluorophore emits a detectable/stronger signal (1). The benefit of this design is such that the ligand and anchoring oligos may be modified without adjusting the hairpin domain. This allows the user to generate a library of sensors using hairpin oligos which unzip upon application of varying forces (1, 9). However, the system's dependency on hybridization makes it less sturdy, and more likely to shear when

compared to the single-oligo design by Chen et al. (9). Therefore, this module is better suited for models involving lower magnitudes of force (1).

The major disadvantage of the oligonucleotide approach is that it may only be used in the extracellular environment. These constructs are not genetically encoded, and therefore must be formed via chemical synthesis. Thus, it would be very difficult to study intracellular events with these cells, since these systems would have to be inserted into cell of interest. That being said, these probes are useful when studying tension between cells, or between cells and their extracellular matrices.

IIc. Rief et al., Optical Tweezers and Extension to FRET probes

Although less common, there are also single-fluorophore tension probes used in mechanotransduction research. In 2012, Rief et al., published an optical tweezer construct which utilized a single-molecule competition assay. The group was able to demonstrate a force-sensing mechanism in human filamin A through the use of a single-molecule optical tweezer mechanism (10). Cytoskeletal protein filamin interacts with membrane proteins integrin and GPIbα. Binding sites for these proteins are located from domains 16 to 24 of filamin and become available at the domain 24 via dimerization and activation (10). In this system, interacting peptides are directly fused to the N-terminus of domain 21 on filamin, with a 6-residue amino acid linker providing flexibility (10). Disulfide bonds then tether the construct to dsDNA molecules in order to facilitate force application (10). Under load, the optical tweezer system undulates between bound and unbound states. When unbound, a tagged ligand in solution will compete to bind the available receptor in a way that is directly observable by

researchers. (10). Rief et al. found that an increased magnitude of force subsequently induced a conformational chain in filamin, and elevated downstream interactions with the cytoskeletal protein (10).

As a collaborative project, Grashoff and Rief et al., expanded on the initial VinTS model by developing a new method to improve the VinTS model using single-molecule force spectroscopy (11). The elastic linker between the FRET pair of VinTS is a spider silk protein with a fixed spring constant. The silk protein linker is relatively weak, and therefore cannot measure the higher forces typically applied across integrin receptors (12). Using optical tweezers, and single-molecule force spectroscopy, the two labs were able to generate additional molecular springs with unique properties and characteristics (11). Using this method, they were able to generate two new talin biosensors with additionally improved upon dynamic ranges (11, 12).

III. Benefit of Single Fluorophore Molecular Tension Probes

The primary obstacle FRET imaging methods face is their inability to allow for sensor multiplexing. Current approaches are widely limited to monitoring just two subjects, which cannot appropriately capture a downstream signaling event in real time (13). This largely restricts researcher's understanding of mechanotransduction pathways, as nearly all signaling processes within the body including tension mechanics, and involve a complex network of dynamics and crosstalk. Therefore, it is essential to form sensors which allow for sensor multiplexing (13).

Single-fluorophore based biosensors have been found to successfully monitor multiple activities simultaneously *in vivo* (13). Specific construct formats such as

single-fluorophores based on circularly permuted fluorescent proteins (cpFPs) have also been found to be increasingly efficient (13). Moreover, they generally offer an increased magnitude of dynamic range, and make more of the fluorescent spectrum available for sensor multiplexing (13).

Despite the benefits of single-fluorophore systems, two-parameter multiplexing still remains common across both double and single-fluorophore imaging techniques (13). Color variant fluorophores have been developed in recent years which hold potential to expand the number of experimental subjects at a given time, but these variants have currently only been used for imaging with calcium ions (7). Current reservations and limitations surrounding single-fluorophore probes include a difficulty in controlling the density of sensors genetically encoded and expressed, as well as the difficulty of creating probes with diverse sensitives (3, 5). However, there is the potential to create a novel tension sensor with a tunable range of measurable forces. I believe that a novel sensor with an alternative mechanism of signaling can product more favorable results.

IV. Prospective Sensor Based on Chemical Bond-Breaking

IVa. Background

Pre-existing research has explored how various cytoskeleton proteins structurally adapt in response to mechanical force. Bell et al. investigated this topic in 1978, discussing how the varying strengths of chemical bonds dictate protein behavior in response to stress (7). More recent research has delved deeper, identifying proteins such as talin and filamin which undergo changes involving the breaking and reforming of chemical bonds, in order to accommodate an applied force (10, 14). This thought process has given rise to the proposed creation of a novel type of mechanical sensor based on bond-breaking amongst protein-protein interactions as a means of measuring mechanical force. By taking advantage of existing chromophores and fluorescence modifiers, there lies potential to develop a novel tension sensor with an expanded range of measurable forces, and an improved dynamic range compared to the existing FRET sensors (Figure 3).

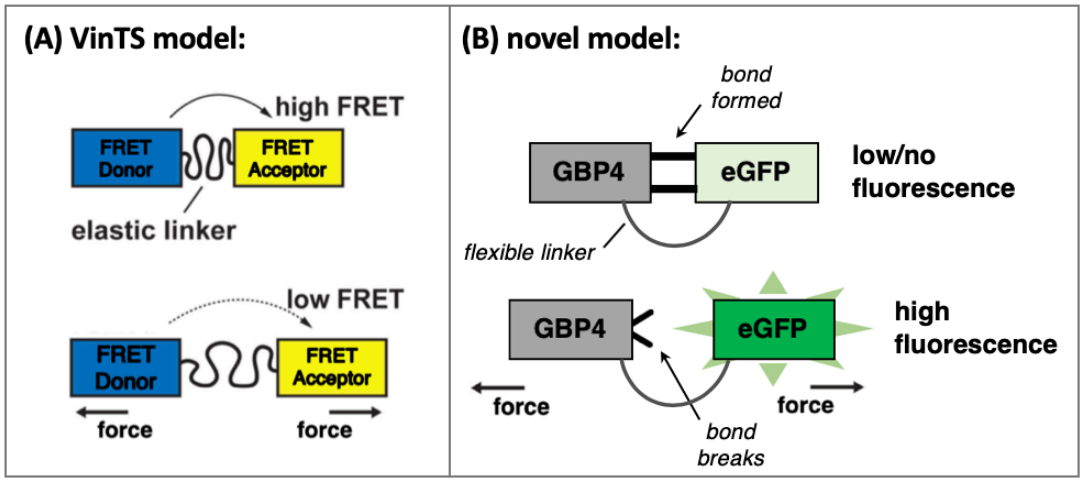

Figure 3 (A) *Adapted from Grashoff et al.* The VinTS model uses FRET imaging to measure the onset of mechanical force. **(B)** The proposed tension sensor is based on bond-breaking between protein-protein interactions. Signaling based on bond breaking, rather than FRET, can lead to a tension sensor with higher sensitivity and an expanded dynamic range.

The initial inspiration for this novel tension sensor, **J**ust an **E**GFP-**D**erived **I**ndicator (JEDI), were nanobodies designed to bind green fluorescent proteins (GFPs) (15). Upon binding, these GFP-binding proteins (GBPs) alter the chromophore in such a way that changes its fluorescence intensity (Figure 4) (15). One particular protein, GBP4, decreased fluorescence in the bound-state, and after binding the enhanced form of GFP, eGFP, caused an 8-fold decrease in fluorescence (15).

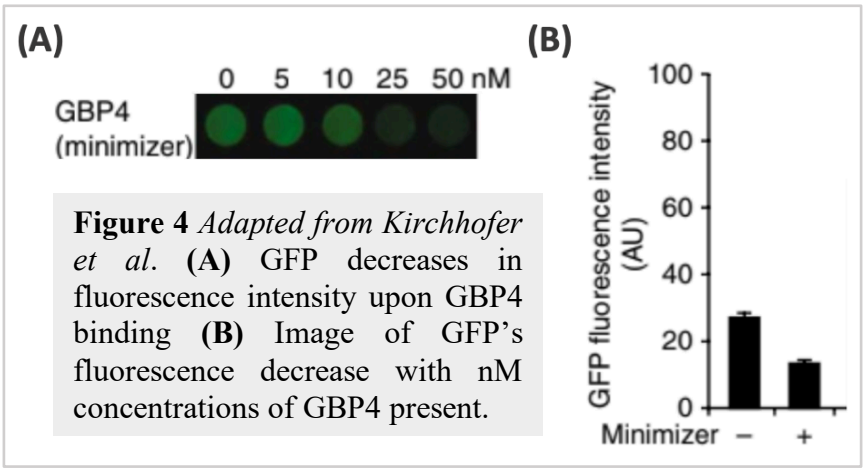

Figure 4 *Adapted from Kirchhofer et al*. **(A)** GFP decreases in fluorescence intensity upon GBP4 binding **(B)** Image of GFP's fluorescence decrease with nM concentrations of GBP4 present.

The fluorescence shift can be attributed to a key amino acid, $Arg168^{GFP}$, identified at the eGFP-GBP4 interface (Figure 4)(15). $Arg168^{GFP}$ exists in two confirmations: extended or bent. Without external influence, $Arg168^{GFP}$ adopts a bent conformation, resulting in an ionized chromophore and an absorbance peak at 488nm (15). Contrastingly GBP4, promotes the residue's extended conformation, and adjacent eGFP molecules adopt a neutral state, shifting the absorbance peak from 488nm to 405nm (15). The onset of GBP4 binding is observed as a noticeable decrease in fluorescence when imaging at 488nm, and serves as a promising research tool in the field of mechanotransduction. The novel complex can potentially be inserted within protein domains that regularly experience tensile force, such as vinculin. When a certain level of tensile stress is applied to a protein of interest, GBP4 will dissociate from eGFP

resulting in an observable fluorescence increase. However, certain adjustments can be made to improve the binding mechanism.

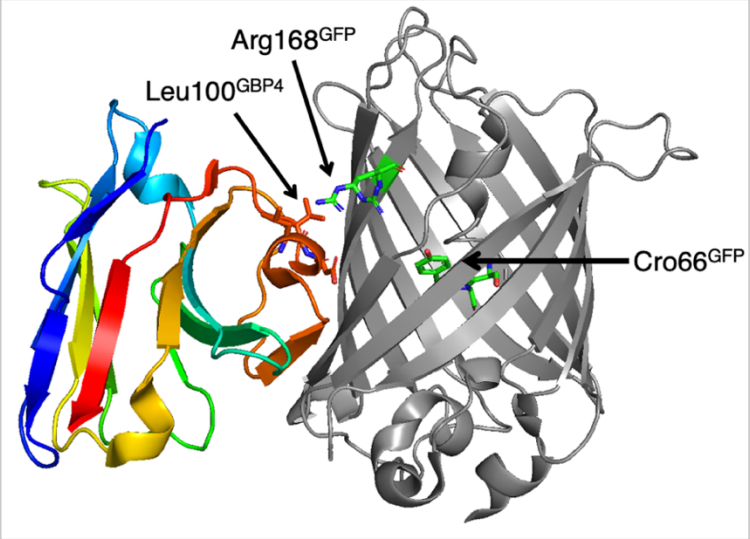

Figure 5
Adapted from Kirchhofer et al.

GBP4 residues, like at the Leu100 site promote the extension of Arg168^{GFP} with 50% efficiency.

Extended, Arg168^{GFP} induces the neutral state of Cro66^{GFP}, causing decreased fluorescence at 488nm.

IVb. Directed Evolution and Random Mutagenesis

The novel sensor can be adjusted by tuning identified residues located at the GBP4-eGFP interface. If successfully executed, the novel sensor has the potential for an exceptional dynamic range, and the ability to measure a wide range of mechanical forces. This can be completed via directed evolution and random mutagenesis. GBP4 currently promotes the extended Arg168 ^{GFP} conformation with 50% efficiency. The crystallized arrangement at the binding interface does not show many stabilizing residues around Arg168 ^{GFP} (Figure 5). By introducing stabilizing mutations, the frequency of the extended conformation can increase, resulting in a stronger fluorescence decrease upon binding.

Two key locations that have been identified as critical to the fluorescence of GFP. 100KLN (Kabat numbering for nanobodies) are three amino acids near Arg168 ^{GFP} that may stabilize its extended form with random substitutions (15). The second site is 100J, a cysteine residue on GBP4 forming a disulfide bond near Arg168 ^{GFP} (15). Specially designed ssOligo primers can introduce mutations at these sites by Gibson Assembly (GA). GA is a highly favorable method of mutagenesis, and the use of ssDNA oligos eliminates the need for mutagenesis by PCR, unnecessary restriction sites, and any potential scarring (16). Moreover, ssDNA oligos carry the exact mutations wanted in NNK format, minimizing codon redundancy, and making subsequent bacterial screening more time efficient (17). After cloning, colonies can be screened for favorable behaviors using ratiometric measurements between 405 and 488nm (Figure 6).

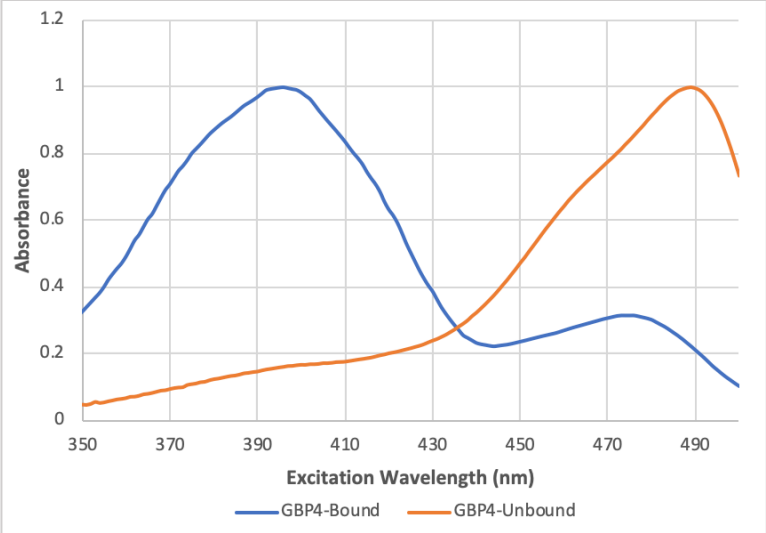

Figure 6 *Adapted from Kirchhofer et al.* Absorbance spectra of GBP4 in its bound (blue) vs unbound (orange) states. Construct colonies can be chosen for further characterization based on a high ex405/ex488 ratio. Corrupted constructs may be ruled out based on their lack of 405nm excitation, indicating unwanted behavior.

Measuring ratiometrically lets researchers identify optimized protein constructs independent from their relative concentrations, such that the focus can be on the dynamic range of each colony. Moreover, the existing linker between eGFP and GBP4 is short, so unless severe disruption occurs at the mutated interface, it can be assumed

that we the bound-state of the novel complex is being observed. This process of cloning and screening may be repeated multiple times to create a library of recombinant DNA with fixed mutations that produce favorable behaviors.

IVc. Characterization of Variable Binding Affinities

In addition, the current eGFP-GBP4 complex has a very strong binding affinity, and requires a significant amount of force before bond-breaking occurs. However, having multiple iterations of the complex with varying affinities would permit experiments to occur across a wide range of conditions, and with variable magnitudes of force. Since the binding of GBP4 attenuates the excitation strength of eGFP at 488nm, binding affinity can be measured by titrating GBP4 candidates into a solution of eGFP with a fixed concentration. This can be conducted by purifying candidate proteins, and measuring spectra in conjunction with a fluorescence spectrometer.

IVd. Modeling *in silico*

Once a library of novel sensors with varying magnitudes of measurable forces has been created, the construct can be subjected to experiments in cells. However, prior to conducting these experiments, experimental designs may be optimized using computational modeling. The differentiation of mesenchymal stem cells is strongly influenced by substrate stiffness and mechanical memory, and therefore serves as a useful system for tension sensor simulations (6). Moreover, there is a published mathematical model by Peng at al. for mechanical sensing in mesenchymal cells, which one can recreate and extend to the novel sensor (6). The model incorporates YAP and

TAZ transcription factors' downstream signaling effects in response to changing levels of environmental stiffness (6). Depending on the level of mechanical force present, different transcription factors will be activated or inhibited, and will ultimately dictate stem cell differentiation (6). By adjusting the mesenchymal stem cell model's parameters, one can observe biological outcomes and sensor readouts over various conditions *in silico* and help optimize our experimental designs for the sensor going forward. Being able to create these models is incredibly useful for wet lab work, as their calculated outputs can help make experimental designs more efficient, and direct certain hypotheses.

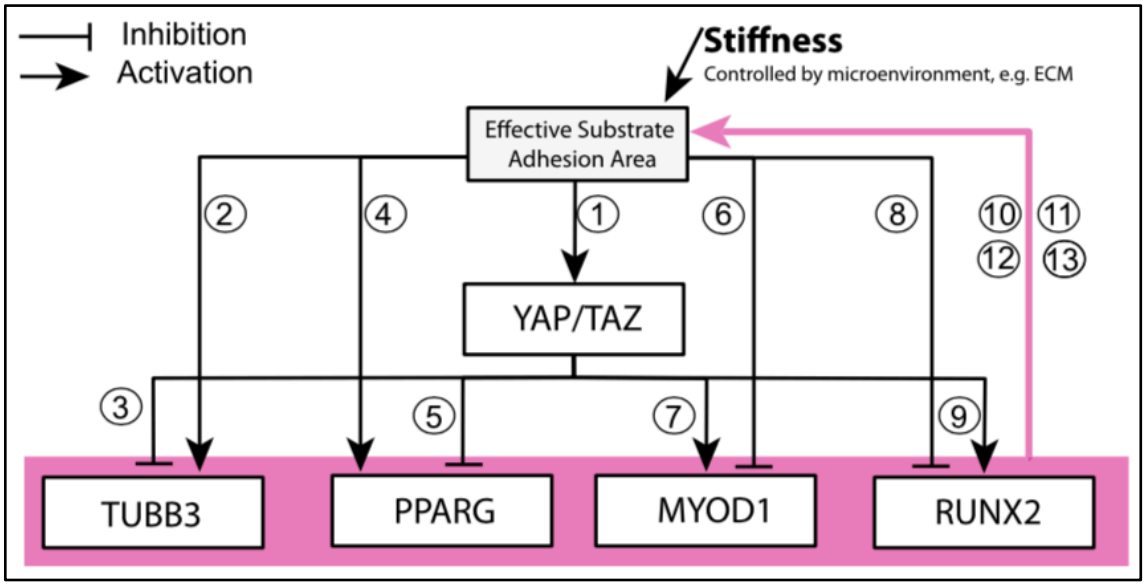

Figure 7: Seven core mechanisms of mesenchymal stem cell fate
Adapted from Peng et al. The differentiation of mesenchymal stem cells (MSC) is strongly influenced by substrate stiffness and mechanical memory. Therefore, it serves as a useful system for tension simulations. Here, the seven core mechanisms are defined *in silico*

V. Expected Results and Significance

This sensor, if successfully developed, can be transfected in cells which regularly experience force. If tagged to certain proteins of interest, researchers may begin characterizing the behavior of a subject of interest under high-stress conditions. Once finalized, this novel sensor has the potential to serve as an important tool with academic as well as clinical significance. The sensor can ultimately be used to understand behaviors of cells in the context of varying thresholds of force, or identify proteins with high involvement in stiff substrate mechanotransduction. Pinpointing these proteins can lead to a deeper understanding of the cell's basic mechanotransduction cascades, as well as aid in the development of new targeted therapies against tension-related pathologies. In fact, the novel system itself can be redesigned to deliver drugs in response to interactions with areas of increased tension. For example, if a bound nanobody-GFP complex were exposed to an environment experiencing a specific level of tensile force, the construct would be pulled apart and expose the nanobody end. The nanobody can subsequently act as an actuator, and bind other components *in vivo* that resemble GFP in order to sequester them or initiate downstream signaling events. This can lead to the development of a novel actuator which can perturb cells in response to mechanical force.

Finally, this methodology may be extended to other ligands and used to create a range of novel sensors that use bond breaking as a mode of measurement with respect to fluorescent imaging. The collected data from this development could serve as an important foundational step towards a new range of available tension.

VI. Concluding Remarks

Mechanotransduction is the process by which a mechanical stimulus is converted to a cellular signal, and has many important roles in cell functioning as well as in pathologies. However, the exact processes behind mechanotransduction-dependent signaling cascades have not been fully characterized, and to do so requires the use of a biological tension sensor. There are currently a variety of available fluorescent-protein tension probes, most of which utilize double-fluorophore FRET pairing. That being said, there has been an increasing demand for single-fluorophore tension probes, such that sensor multiplexing may take place. Moreover, many existing tension sensors used have a low dynamic range, and have a limited breadth of measurable forces.

After a deep dive into existing biosensor technologies, a prospective tension sensor has been introduced. Derived from nanobodies, a novel sensor's binary modality of fluorescent emission can provide an increased dynamic range, and its single-fluorophore design may allow for sensor multiplexing. When completed, the construct has the potential to lead to new discoveries in mechanotransduction signaling pathways, as well as serve as an important foundational step towards a new range of available biosensors.

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