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A DNA-Encoded FRET Biosensor for Visualizing the Tension across Paxillin in Living Cells upon Shear Stress

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Abstract: Paxillin is a potential participant in the direct intracellular force transmission which is considered as the foundation of cells sensing and responding to extracellular environment. However, the detection of tension across paxillin has not been achieved due to lacking microsized tools. Herein, a paxillin tension sensor (PaxTs) based on Fluorescence Resonance Energy Transfer (FRET) technique was constructed. PaxTs can be expressed and assembled to FA sites spontaneously to visualize the tension across paxillin with FRET efficiency of ~62.4% in living cells. The tension across paxillin was found to decrease upon shear stress, in which the membrane fluidity and contractility of actin acted as cushions. It is observed that paxillin participates in the pathway of cell membrane-cytoskeleton-FAs for force transmission upon mechanical force in real time visualization, which provides a promising new method to investigate the direct intracellular force transmission in biology and technology.

Introduction

The migration of cells is a complicated and coordinated process related to numerous signaling pathways. It plays a significant role in various physiological and pathological process including cancer invasion and metastasis[1]. Migrating cells experience various forces and the relevant signal proteins are activated asymmetrically in space to establish cell polarity as a reaction to the surrounding mechanical environment in the early stage of migration [2]. The establishment of polarity is very rapid and recognized to be in connection with the direct participation of stress transmission through cytoskeleton[3]. As the initial sites of mechanotransduction occurring on cells, focal adhesions are micron-sized multi-component protein complexes that assemble at the front edge and disassemble at the rear edge dynamically in the process of cell migration[4] . Cells are anchored to extracellular matrix (ECM) with integrins in FAs, a family of transmembrane proteins that can sense the mechanical properties of surroundings [5]. In addition, FAs are also linked to cytoskeleton, which establishes a connection to ECM[6]. This structure, with numbers of structure proteins contained in FAs, provides the foundation for the transduction of extracellular mechanical signals into intracellular responses[7].

Paxillin, a main component of FAs, is anchored to FA sites on the plasma membrane by its four LIM domains on the C-terminal region and connected to cytoskeleton by its five LD motifs on the N-terminal region[8]. During cell movement and migration, paxillin is recruited at nascent FAs at the front of cells to assemble the adhesion complex and also necessary to disassemble FAs at the rear end of cells[9]. Instead of exhibiting enzyme activity, paxillin acts as a scaffolding protein to provide docking sites for multiple FA-associated proteins to facilitate the formation of the complex. Many of the binding partners to paxillin have been proved to have close relationships with the rapid establishment of cell polarity or force transmission[10]. Thus, paxillin contacting those proteins extensively enables itself to participate in force transmission and the subsequent cell polarity establishment, implying a potential direct pathway for force transfer through the structure consisting of ECM, FAs and cytoskeletons. However, how force is transferred across paxillin is not clear because of lacking effective tools to visualize the paxillin tension on a micro scale.

Previously, a tension sensor module (TSMod) in which an elastic domain was inserted between a pair of fluorophores was developed to measure the force through the nano-spring by fluorescence resonance energy transfer (FRET) technique. The application of tension stretched the nano-spring which was compact without tension and decreased FRET. Using tension sensors constructed with TSMod, paxillin and talin in FAs were proved to under mechanical tension[11]. In this study, a paxillin tension sensor (PaxTS) was designed with a modified tension sensor module and verified on its ability to visualize the force transmission across paxillin in living cells. With PaxTS biosensor, the stress condition of paxillin during shear stress application was analyzed and the results demonstrated the stress transmission pathway of cell membrane-cytoskeleton-FAs in cells. The stress transmission across paxillin within cellular FAs was directly related to the fluidity of cell membrane and the integrity of cytoskeleton, especially the microfilament system.

Results and Discussion

The two major sets of motifs in paxillin, the LIM domains in the carboxyl terminal and the LD domains close to the amino terminus, are essential parts for its localization to FAs and cytoskeleton. The fluorescence protein pairs ECFP/Ypet was connected with an improved nano-spring[12], which formed a unique structure converting tension changes to FRET efficiency. The structure was inserted between LD12 domain and LIM1234 domain to construct a FRET biosensor named PaxTS, which was used to detect the tension across paxillin upon shear stress (Fig 1A). A LD12-less contrast biosensor (PaxDL, Fig1B) was

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constructed to confirm whether PaxTS was assembled correctly to FA sites. PaxTS expressed steadily in U-2 OS, CHO and SH-SY5Y cells with prominent plaques respectively with some presented in the cytosol and nucleus, while no obvious plaques could be observed with the expression of PaxDL (Fig 1C, 1D). The results demonstrated that PaxTS was recruited to FAs spontaneously instead of endogenous paxillin with superior specificity and adaptability.

To evaluate the relationship between the tension across paxillin and FRET ratio, hypotonic treatment with H_2O was applied to cells, which stretched the cytoskeleton by water swelling and increased the tension across paxillin. Cells expressing PaxTS showed low FRET ratio after hypotonic treatment (Fig 2A-2D). Furthermore, when adding more volume of H_2O (1/2/3 times) into culture medium respectively, PaxTS displayed lower FRET ratio (Fig 2E, Supplementary Movie 1-3). It suggested that the FRET ratio was corresponding to the force sensed by paxillin and PaxTS achieved its desired function. On this basis, hypertonic treatment with sucrose solution of 0.025 g/ml was applied after the hypotonic treatment with 1-time volume of H_2O for 10 min, and the FRET ratio increased after decline (Fig 2F-2H, Supplementary Movie 4). In addition, removing shear stress after application for 10 min, the FRET ratio recovered nearly to the ground state (Fig S1). Both the results proved the reversibility of PaxTS. Generally, these results indicated that PaxTS can be used as a tool to detect the force transmission in living cells.

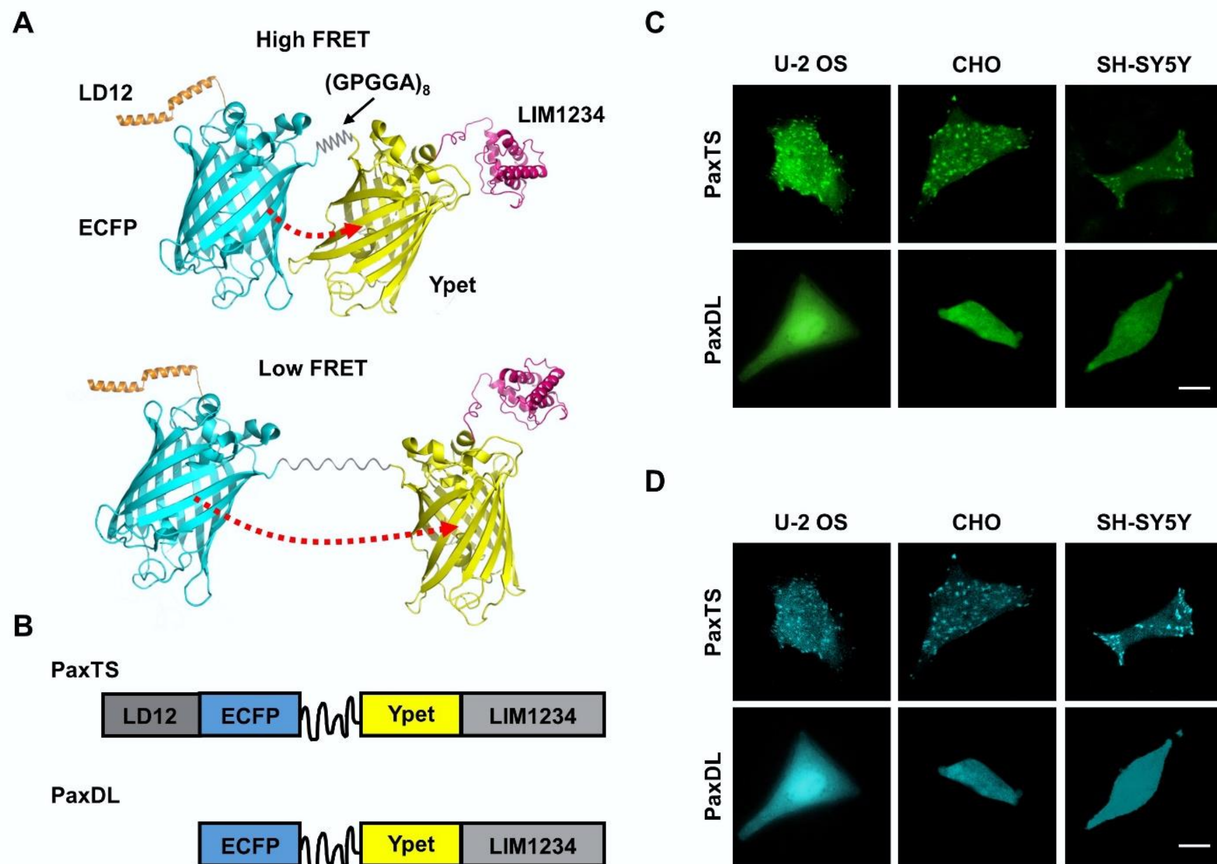

Figure 1. The PaxTS and its expression in multiple living cells. A) The mechanism of PaxTS. B) The structure of PaxTS and the derived biosensor PaxDL. C) The images of PaxTS expressing in U-2 OS cells, CHO cells and SH-SY5Y cells (Ypet channel). Scale bar: 20 μm. D) The images of PaxTS expressing in U-2 OS cells, CHO cells and SH-SY5Y cells (ECFP channel). Scale bar: 20 μm.

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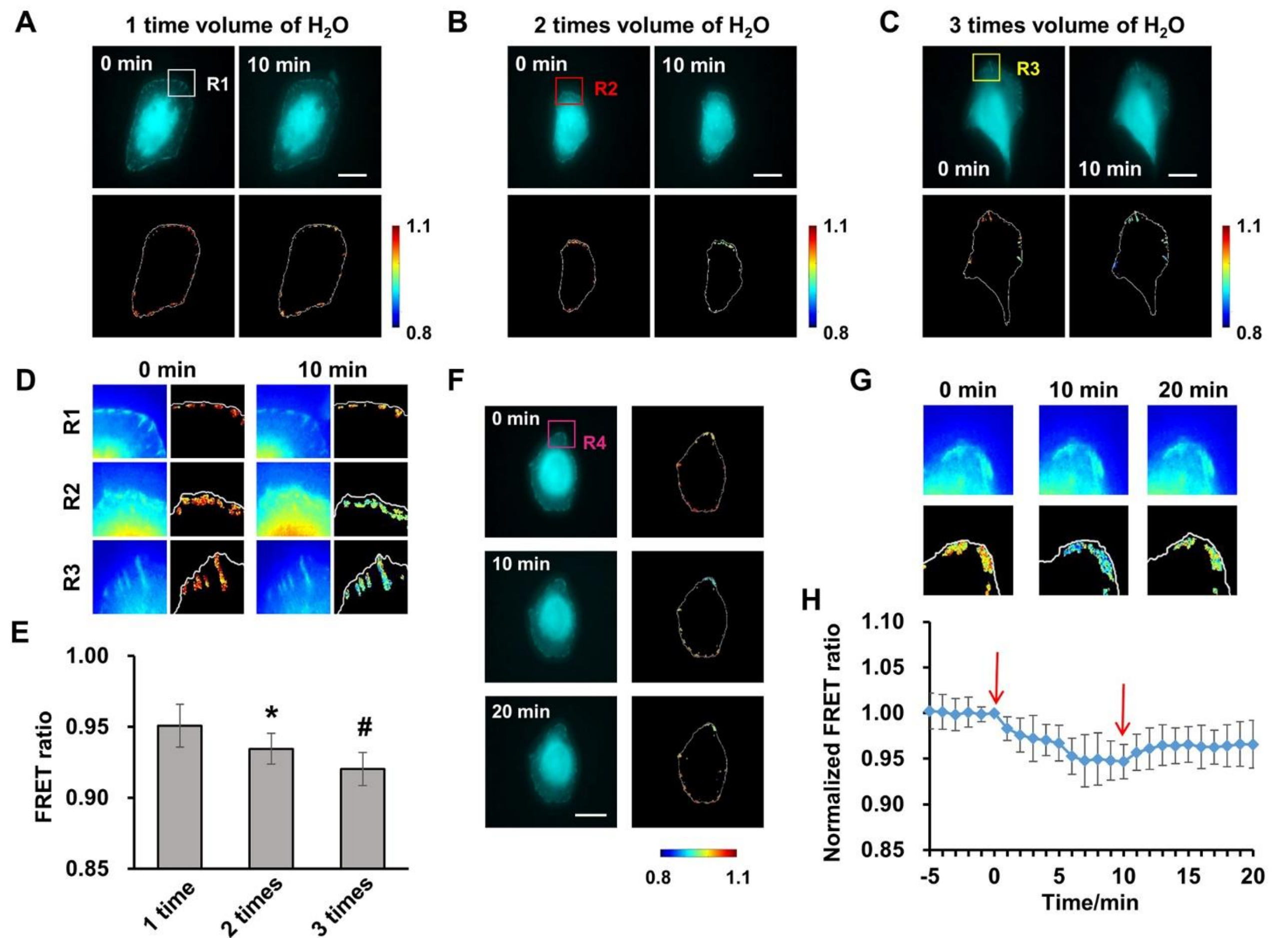

Figure 2. The verification experiments of PaxTS. A-C) FRET ratio decreased differently after hypotonic treatment with different volumes of H_2O. The volume of H_2O was 1/2/3-times volume respectively from Fig A to Fig C. The top line showed the live-cell images of PaxTS (ECFP channel), and the bottom line exhibited the segmentation results of FAs, corresponding to the top line. Scale bar: 20 µm. D) The partial enlargement of Region 1 (R1), Region 2 (R2) and Region 3 (R3) from A-C. E) The FRET ratio at 10 min after three kinds of hypotonic treatment (n=5, 6, 5, respectively). * represented there was an obvious difference between 1-time group and 2-times group. # represented there was an obvious difference between 2-times group and 3-times group. F) FRET decreased after hypotonic treatment but increased with hypertonic treatment. The left column showed the live-cell images of PaxTS after hypotonic treatment and hypertonic treatment (ECFP channel). 1-time volume of H_2O was applied at 0 min and sucrose solution of 0.025 g/ml was applied at 10 min. The right column exhibited the segmentation results FAs, corresponding to the left column. Scale bar: 20 µm. G) The partial enlargement of Region 4 (R4) from F. H) The time series of FRET ratio after hypotonic and hypertonic treatment (n=8) with normalized. Red arrows represented the application time of hypotonic and hypertonic treatment.

The structure of paxillin implied that the maintaining of its internal tension under static state probably depended on the membrane and cytoskeleton. Without shear stress application, the FRET efficiency indicated by PaxTS was ~62.4%. Destroying microfilaments with Cytochalasin D (CytoD) pre-incubation at 2 µmol/l for 1 h and depolymerizing microtubules by 1 µmol/l of nocodazole (NOCO) for 1 h increased the FRET ratio of PaxTS significantly ($p<0.05$), promoted the FRET efficiency to ~66.9% and ~65.8% respectively (Fig S2A). However, inhibiting the microfilaments contractility by the inhibitor of myosin light chain kinase (MLCK), ML-7, at 5 µmol/l for 1 h leaded the FRET ratio to be higher than control group but lower than treatment of CytoD or NOCO ($p<0.05$, Fig 3A, 3B), as well as increase the FRET efficiency to ~63.9% (Fig S2A). These results exhibited that paxillin endured tension in static cells and the tension was maintained by both microfilaments and microtubules.

Paxillin has a close relationship with other FA-associated proteins and interacts with cytoskeleton, which probably is the key point in the pathway of mechanical signals transduced into chemistry signals. To evaluate the force transferred across paxillin, cells transfected with PaxTS were exposed to 20 dyn/cm^2 of shear stress for 10 min. FA sites at the periphery were segmented as described in Method. The FRET ratio on those sites increased sharply by ~11% within 1 min and finally increased by ~14% (Fig 3C, 3D). It indicated that the tension across paxillin was decreased due to shear stress application.

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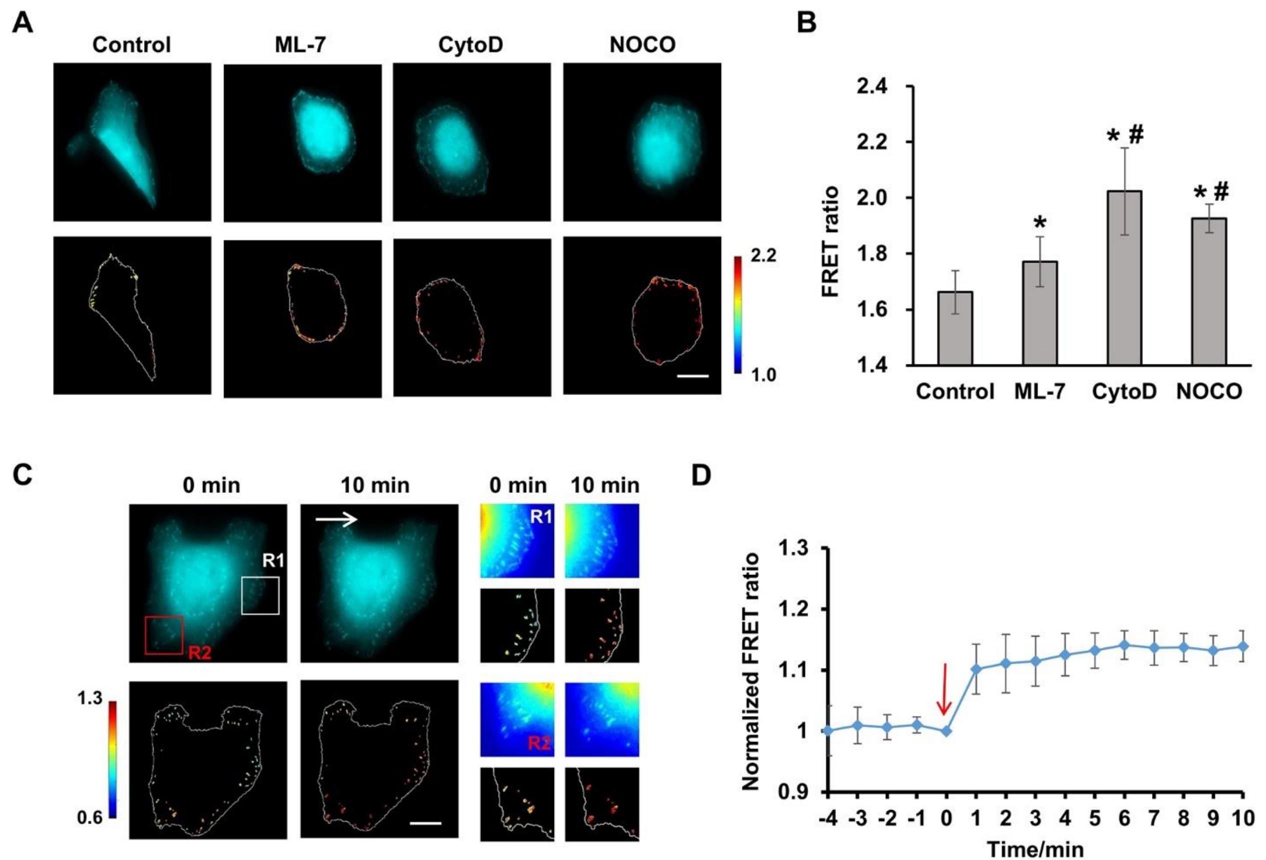

Figure 3. The expression of PaxTS in living cells under different conditions. A) Cells with PaxTS treated with different drugs. The top line showed the live-cell images of PaxTS in static state with treatment of 5 μmol/l of ML-7, 2 μmol/l of Cytochalasin D (Cyto D) and 1 μmol/l of nocodazole (NOCO) respectively. The bottom line exhibited the segmentation results of FAs, corresponding to the top line. Scale bar: 20 μm. B) The histogram of FRET ratio in control group (n=7), ML-7 group (n=8), CytoD group (n=5), and NOCO group (n=7). * represented there was an obvious difference compared to control group. # represented there was an obvious difference compared to ML-7 group. C) FRET ratio increased upon shear stress. The top line showed the live-cell images of PaxTS upon shear stress including the partial enlargement of Region 1 (R1) and Region 2 (R2). The bottom line exhibited the segmentation results of FAs, corresponding to the top line. The white arrows showed the direction of shear stress application. Red arrow represented shear stress applied at zero time. Scale bar: 20 μm. D) The time series of FRET ratio upon shear stress.

The plasma membrane is probably related to mechanotransduction closely, since paxillin-linked FAs sites on the membrane are the initial locations of force sensing[4b]. Considering membrane fluidity is the most notable physical feature of cell membrane, the pre-incubation of benzol alcohol (BA) at 45 mmol/l for 15 min was used to enhance membrane fluidity[13], and then the shear stress of 20 dyn/cm^2 was applied. Compared to control group, the result revealed that when membrane fluidity was enhanced, the increase of the FRET ratio induced by shear stress was significantly dampened after 10 minutes of flow application ($p<0.05$, Fig 4A,4B,4D-4F Supplementary Movie 5, Supplementary Movie 6). However, treatment of cholesterol (CHO) at 0.1 mmol/l for 3 h to reduce membrane fluidity has no obvious effect on FRET ratio (Fig 4C-4F, Supplementary Movie 7). Thus, higher membrane fluidity restrained the shear stress-induced tension decline across paxillin, while inhibiting fluidity has no obvious effect.

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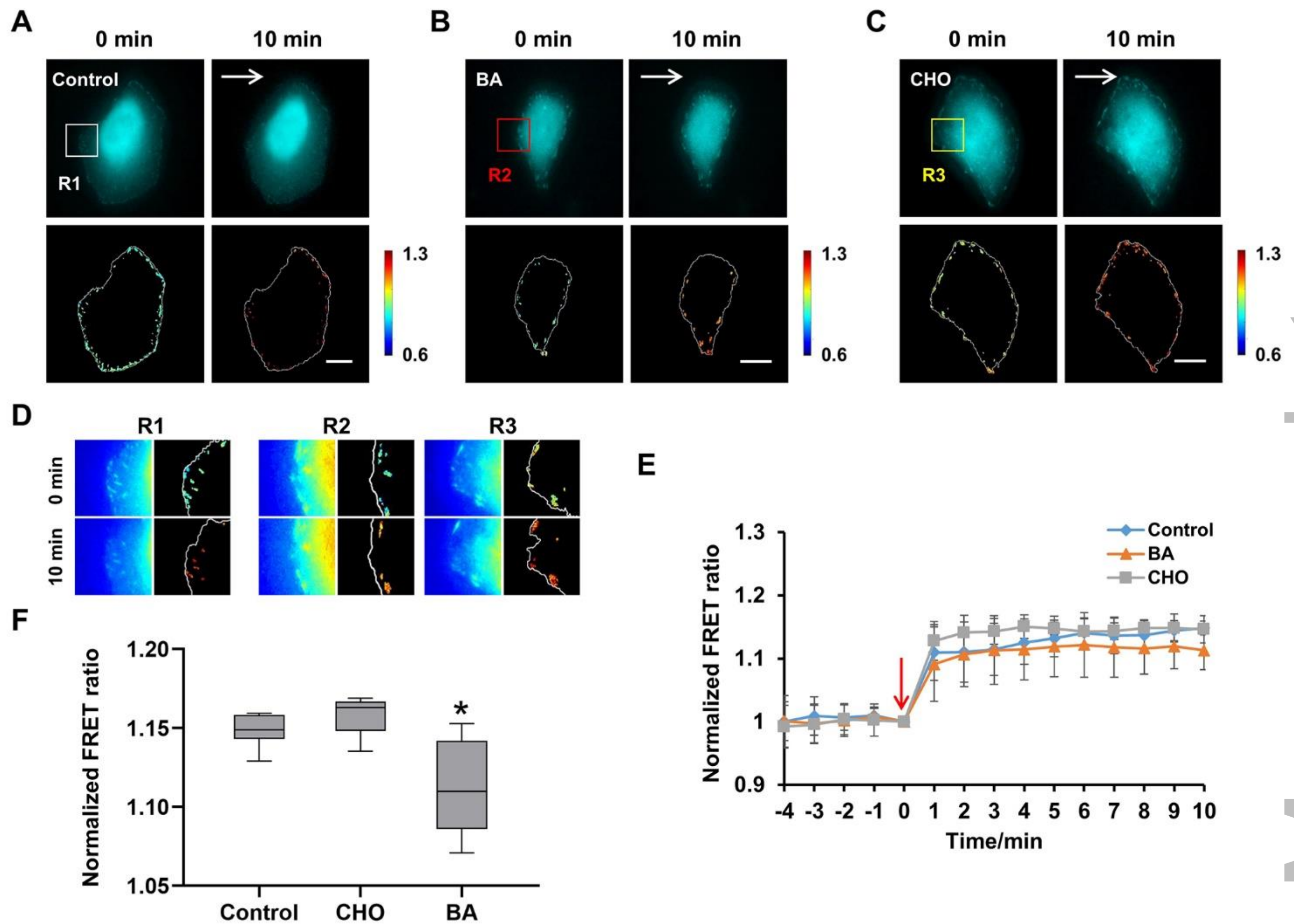

Figure 4. The tension across paxillin upon shear stress was affected by membrane fluidity. A) Live-cell images from ECFP channel of PaxTS under 20 dyn/cm^2 of shear stress without drug application and the segmentation results. Scale bar: 20 μm. B-C) Live-cell images from ECFP channel of PaxTS under 20 dyn/cm^2 of shear stress with 45 mmol/L benzol alcohol (BA) or 0.1 mmol/L of Cholesterol (CHO), and the segmentation results of FAs. The arrows showed the direction of shear stress application. Scale bar: 20μm. D) The partial enlargement of Region 1 (R1), Region 2 (R2) and Region 3 (R3) from A-C. E) The time series of FRET ratio of control group (n=6), BA group (n=5) and CHO group (n=6), after normalized. Red arrow represented shear stress applied at zero time. F) The box plot of FRET ratio in control group and BA/CHO group. * represented there was an obvious difference compared to control group.

In addition to cell membrane, paxillin is also associated with cytoskeleton, which is involved in mechanical signaling[14]. Thus, the cytoskeleton may also participate in force transfer involving paxillin. When the contractility of microfilaments was eliminated without destroying intact structure by the pre-treatment of the inhibitor of myosin light chain kinase (MLCK), ML-7, at 5 μmol/l for 1 h before applying shear stress (Fig 5A, 5B)[13], an increase of FRET ratio was observed within 1 min after the onset of shear stress, and the FRET ratio remained steadily at a higher level during flow application compared to control group (Fig 5E-5G, Supplementary Movie 8). Conversely, although destroying the microfilament by Cytochalasin D (CytoD) pre-incubation at 2 μmol/l for 1 h caused FRET ratio to increase significantly, the increment was lower than the control group and the group of ML-7 treatment ($p<0.05$, Fig 5C,5E-5G, Supplementary Movie 9). Similarly, after the application of 1 μmol/l of nocodazole (NOCO) for 1 h, which depolymerized microtubules, the change of FRET ratio was significantly lower than the control group (Fig 5D–5G, Supplementary Movie 10). These results demonstrated that the integrality of both microfilaments and microtubules was essential for force transfer mediated by paxillin.

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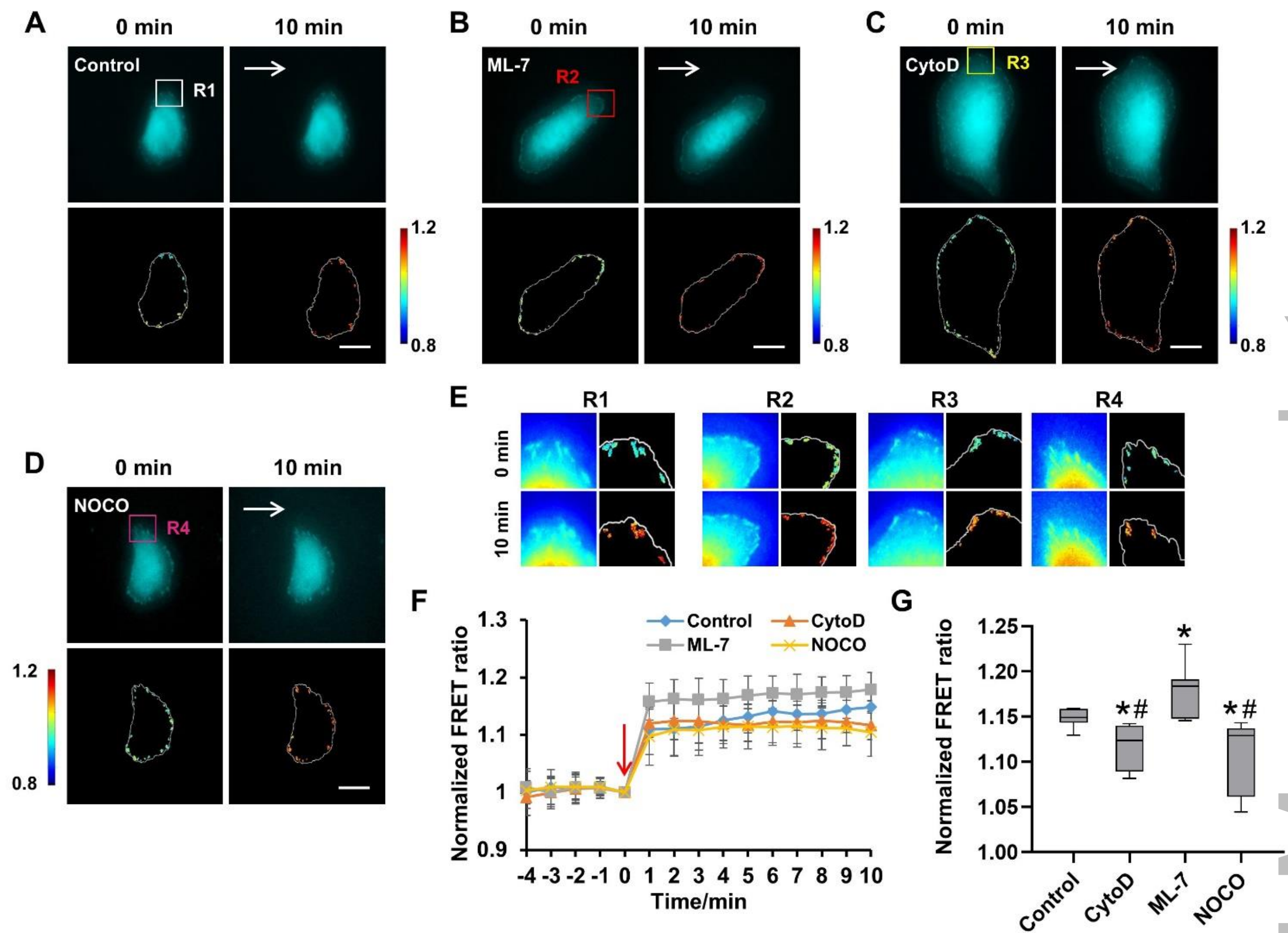

Figure 5. The tension across paxillin upon shear stress was affected by cytoskeleton. A-D) Live-cell images from ECFP channel of PaxTS under 20 dyn/cm^2 of shear stress after treatment with different drugs and the segmentation results of FAs. Fig A exhibited the control group in which cells were applied with shear stress without any drug treatment. From Fig B to Fig D, the drug treatments were 5 μmol/l of ML-7, 2 μmol/l of Cytochalasin D (CytoD) and 1 μmol/l of nocodazole (NOCO) respectively. The arrow showed the direction of shear stress application. Scale bar: 20 μm. E) The partial enlargement of Region 1 (R1), Region 2 (R2), Region 3 (R3) and Region 4 (R4) from A-D. F) The time series of FRET ratio of control group (n=6), CytoD group (n=6), ML-7 group (n=7) and NOCO group (n=5), after normalized. G) The box plot of FRET ratio in control group and CytoD/ML-7/NOCO group. * represented there was an obvious difference compared to control group. # represented there was an obvious difference compared to ML-7 group.

Based on previous studies, there should exist a force transmission pathway in cells to activate cell polarity[3c, 13]. As mentioned above, paxillin contacts several proteins related to force transmission and subsequent cell polarity, which provides a possibility that paxillin also participates in the process of force activating cell polarity directly. However, previous works focused more on the dynamics of paxillin and its interaction with other FA proteins, while the mechanical tension across paxillin and the potential force transmission pathway are still unclear probably due to lack of effective tools. The paxillin-based FRET biosensor PaxTS was designed based on the tension sensor module reported previously[11a], inserting a tension measuring module which consisted of a pair of fluorescence proteins and a nano-spring between the two domains, to visualize and measure the tension across paxillin in real time. Compared to the previous tension sensor for vinculin[11a], the FRET pairs in PaxTS was changed to ECFP/YPet and the original DNA sequence of nano-spring $(GPGGA)_8$ was improved into a series of nonredundant sequence following the degeneracy of codon (shown in Methods) to overcome the difficulties in constructing objective plasmids. All LIM domains from LIM1 to LIM4, integrally called LIM1234, were reserved in PaxTS. However, among the LD domains, LD1 and LD4 are combined with actin by actopaxin to link paxillin to cytoskeleton, while LD3 and LD5 are not related to the connection[8a]. In addition, LD4 was also removed owing to the fact that it was affected by the interaction between LIM and other proteins[15]. Only LD1 and LD2 (short as LD12) were adopted in the biosensor structure to ensure that all efficiency of energy transfer was attributed to the length changes of nano-spring in PaxTS, not the intermolecular conformation changes. Although it is possible that PaxTS cannot perform exactly the same function as endogenous paxillin protein due to its incomplete paxillin domains, the existing structure of PaxTS ensured its target to observe whether force can be transferred to the location of paxillin. The expression of PaxTS in living cells exhibited clear plaques, indicating that PaxTS replaced endogenous paxillin automatically to assemble to FAs where the biosensor should be located to as the design. It is notable that the endogenous paxillin was reserved, thus there would be a competition which could decrease the ability of PaxDL to locate to FAs and further the competitiveness with

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endogenous paxillin declined. The failing in competition would lead to the fact that PaxDL showed no focal adhesions.

The module of tension measuring used in PaxTS has already been proved accurate and effective in previous study[12], thus the emphasis of evaluating PaxTS was put on whether the module could work normally. Based on the structure, the nano-spring was stretched upon force application and the FRET ratio decreased when the tension across paxillin was enhanced[11b]. The water swelling of cells caused by hypotonic treatments stretched the plasma membrane and cytoskeleton and further increased the tension across paxillin. Correspondingly, PaxTS also showed a gradually lower FRET ratio after different levels of hypotonic treatments, which implied a correspondence between the tension across paxillin and FRET ratio. After hypotonic treatment, the hypertonic treatment increased the FRET ratio after the decline caused by hypotonic treatment, due to the shrinking of cells which resulted in the relaxation of cell membrane and cytoskeleton. However, the hypertonic treatment failed to increase the FRET ratio to the ground state. It was probably owing to that the changes of intracellular ionic strength and cytoskeletal transitions triggered by hypotonicity were unable to recover to the original state with hypertonicity in the observation time. In addition, hypotonicity and hypertonicity may affect different components of cytoskeleton in different methods[16], which may also disturb the recovery of FRET ratio to ground state. With an in-depth analysis of the relationship between the FRET-proportional value and the actual force magnitude by theoretical calculation[11a, 17], the tension across paxillin measured by PaxTS was ~2 pN in cells (Fig S2), which is in the same order of magnitude as vinculin, a traditional mechanosensor[11a]. These results demonstrated that PaxTS expressed steadily in various kinds of cell, located to FA sites correctly and responded to force linearly and reversibly. In summary, PaxTS is an effective visual tool for observing tension across paxillin and exploring the force transmission between FAs and cytoskeleton.

The tension across paxillin decreased upon shear stress proving that paxillin participated in the force transmission between plasma membrane and FAs. This result is somewhat surprising considering that the tension across paxillin did not increase with shear stress application. The interesting phenomenon could be due to the cytoskeleton dynamics upon shear stress. In cytoskeleton, there is a contractile network of both branched and unbranched actin filaments and myosin underlying and being attached to the membrane, named cellular cortex[18]. Due to the close association of the actin network and the membrane, these two components should be considered as a composite material together, which means that the cytoskeleton could be affected by membrane for purely mechanical reasons[19]. When cells are pulled along the direction of flow, the force exerts on membrane and cellular cortex together, and further stretches the actin filaments which are linked to paxillin. In response to stretch, the cytoskeleton becomes softer and more fluid-like accompanying with a decrease of its prestress, while the actin filaments are the main contributors in cytoskeleton softening which is induced by stretch[20]. With the release of press stress in actin filaments, the tension across paxillin also decreases.

Plasma membrane is the barrier of the whole cell to separate cells from the external environment, and also known as the first structure to sense extracellular signals. Considering the structure of paxillin, the continuous deformation of the lipid membrane at the upper surface, where the shear stress directly acts on, stretches the cytoskeleton unavoidably, which promotes the force to affect the tension across paxillin. Interestingly, the tension across paxillin decreased less at a higher level of membrane fluidity, as revealed by the results that PaxTS was stretched more slightly when membrane fluidity was enhanced. In this process, the membrane seems to act as a 'buffer' rather than a structure merely sensing force or stretching the cytoskeleton mechanically. The function of buffer here is not offsetting or eliminating but dispersing caused by the redistribution of membrane tension upon shear stress, supported by the research that high fluidity helps to homogenize membrane tension during flow application[12]. The redistribution makes membrane tension more uniform and further causes multidirectional stretch to cytoskeleton, and hence the stretching on paxillin caused by force is attenuated even though the cytoskeleton might be stretched more acutely when fluidity is enhanced. In contrast, inhibiting membrane fluidity had no significant effect on the tension across paxillin, which is consistent with the previous report that the stiff membrane is adverse to membrane tension redistribution[12]. Besides, other mechanical changes of membrane upon shear stress application, such as the internal movements of phospholipid molecules, possibly also affect the force transmission in a direct manner. The movements of phospholipid molecules intensify with higher fluidity, which contributes to the redistribution of membrane tension.

Besides plasma membrane, paxillin is also associated with actins to establish a link from plasma membrane to cytoskeleton with the cooperation of actin-binding proteins. Since the cytoskeletal network is considered to sense and transmit force intracellularly [21], it is acceptable that cytoskeleton participates in the force transmission pathway involving paxillin. In static state, the stretch of cytoskeleton to paxillin disappeared with the destroy of cytoskeleton, which led the tension across paxillin to decrease. The measurements showed that the paxillin in FAs also endured tension which was maintained by cytoskeleton even under static state. Interestingly, inhibiting the contractility of microfilaments led to a different result compared to depolymerizing the structure, which exhibited that the cytoskeleton structure and the prestress existed in actin networks were both important to maintain the tension across paxillin in statics state, but functioned differently.

Correspondingly, our results displayed that the tension across paxillin changed after being exposed to shear stress for 10 min when microfilaments disassembled, but significantly less than control group. It indicated that microfilaments participate in the pathway of force transmission across paxillin upon shear stress in addition to maintaining the tension under static state. However, inhibiting the contractility of microfilaments performed a larger change of the tension across paxillin than the control group, while depolymerizing microfilaments led to a lesser extent. It is probably due to the inhibition of the contractility eliminates the elastic responding of microfilament system to force, and the force thus directly acts on paxillin without any cushion. As another component of cytoskeleton, microtubules were found that its destroying also decreased the tension change across paxillin caused by shear stress, which was probably due to the association of paxillin with cytoskeleton. Paxillin is linked to microfilaments by actopaxin and other actin-binding proteins directly, while microtubules are connected with paxillin through microfilament in an indirect manner[8b, 22].

Nevertheless, there existed an interesting phenomenon that the tension across paxillin still decreased slowly after the transient enhancement following the onset of flow application. However, when the microfilaments were destroyed or the contractility was inhibited, the tension across paxillin stabilized immediately and remained during the whole application. It implied that microfilaments were able to participate in the regulation of tension across paxillin indeed upon shear stress, and functioned as a 'cushion' rather than a simple transmitter. It is well known that cells show a conserved trend to counteract external tensile forces as well as to reduce the peak shear stresses when being exposed to flow and by the rearrangement of actin filaments along the direction of force[23]. Similarly, the 'cushion' would be related to the dynamics of stress fibers upon shear stress. The microfilaments network shows no clear directionality under static state. When force is sensed by plasma membrane and transmitted along actin, it is dispersed to different intracellular sites actually, not only to FAs and paxillin, to form a cushion. Meanwhile, in 2D-cultured cell, the filamentous actin depolymerizes as the earliest response of cytoskeleton to shear stress[24]. The rapid decrease in actin filaments amount results in a decline of the ability of microfilaments to disperse the cytoskeleton strain caused by membrane deformation and a sustained tension change of paxillin

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in a short period of time after shear stress application, which is generalized as a cushion function. Agree with the observation in 2D cultured cells, strain softening of cytoskeleton showing decreased elasticity as well as increased hysteresivity in 3D culture, which lead the cytoskeleton to react slowly to stretch upon shear stress. It is also consistent with the definition of cushion function in the tension changes across paxillin induced by shear stress.

Conclusion

In this paper, a FRET biosensor named PaxTS was constructed to visualize the tension across paxillin, providing a useful tool for exploring how the tension across paxillin is regulated upon shear stress. The results proved that paxillin endured tension in cells and there existed a novel manner to regulate the tension across paxillin upon mechanical force. The regulation was proposed to show a relationship with the plasma membrane deformation upon shear stress and the chain reaction of cytoskeleton strain. The plasma membrane and actin network acted as cushions together to mitigate the effect of shear stress on paxillin. These results suggested that the force applied on the upper surface of the cell did affect the tension across the paxillin protein located on the sites adhered to substrate with the assistance of cytoskeleton. It also supported that there existed a potential pathway in cells to transfer force to local subcellular locations directly and probably further activate the cell polarity establishment, while aberrant cell polarity and migrating ability are recognized as main precipitating factors in various diseases[25], such as cancer invasion and metastasis. Exploring the intracellular pathway of force transmission involved with paxillin provides a new path to understand the diseases in depth. However, the distinct pathway of shear stress regulating the tension across paxillin is still incomplete. How the force is transmitted from the plasma membrane to cytoskeleton or from cytoskeleton to paxillin, and whether the actin binding proteins related to paxillin such as actopaxin participated in the regulatory pathway are interesting questions for future investigations.

Experimental Section

Design and establishment of PaxTS biosensor

The biosensor named PaxTS consisted of four sections, LD12 domain, LIM1234 domain, molecule spring and ECFP/Ypet fluorescent protein pairs for FRET. In the biosensor construction, LD12 domain was a section of LD domain which connected paxillin with cytoskeleton, while LIM1234 domain located paxillin to FA sites. The two sections were linked by an improved nano-spring (DNA sequence shown below), which could convert stress across paxillin to the distance between the fluorescent proteins pair.

5'GGTCCAGGAGGCGCAGGACCTGGCGGGGCTGGACCGG
GTGGCGCGGGACCCGGCGGAGCCGGCCCAGGTGGGGCG
GGCCCTGGTGGTGCTGGTCCGGGAGGGGCAGGGCCCGGA
GGTGCC-3'

To demonstrate the biosensor was located correctly to FA sites, a contrast biosensor without LD12 domain was designed and named as PaxDL (Fig 1B). The whole water swelling was uploaded as supplementary. The two biosensors mentioned above were constructed into pcDNA3.1(+) plasmids for expression in U-2 OS, CHO and SH-SY5Y cells, respectively.

Cell culture and transient transfection

Before transfection, U-2 OS cells were cultured with the Roswell Park Memorial Institute (RPMI-1640, Bioind) containing 10% fetal bovine serum (FBS, Bioind) and 100 unit/ml penicillin (Hyclone). CHO cells were cultured with the Ham's F 12 nutrient medium (F12, HYCLON) containing 10% FBS, 100 unit/ml penicillin and 100 mg/ml sodium pyruvate (GIBCO). SH-SY5Y cells were cultured with Dulbecco's modified Eagle medium (DMEM, HYCLON) containing 10% FBS, 100 unit/ml penicillin and 100 mg/ml sodium pyruvate (GIBCO). Different DNA plasmids were transfected into cells by the transfection reagent, Lipofectamin 3000 (Thermo Fisher Scientific). After transfection for 24 h, cells were passed onto fibronectin-coated cover slips and cultured with 0.5% FBS for 12 h before laminar flow application.

Flow systems

A classis parallel-plate flow chamber was applied to provide laminar flows, and modified to fit for dynamic observations under a FRET microscope[10a]. The glass slides seeded with separated U-2 OS cells were covered by a silicone gasket and a cover glass, and exposed to shear stress which can be calculated as equation (1):

$$\tau = \frac{6\mu Q}{bh^2} \quad (1)$$

Here τ = fluid shear stress (Pa), μ = fluid viscosity of solution (cp), Q = flow rate (cm^3/s), b = width (mm), h = height (mm). In this work, μ = 0.82 cp (dulbecco's modified eagle medium (DMEM) containing 0.5% fetal bovine serum (FBS)), b = 13 mm, h = 0.508 mm. Lamina shear stress was set to 20 dyn/cm^2 by adjusting fluid flow in the chamber[26]. The flow experiments were done 37 °C with 5% CO_2 to maintain the pH at 7.4.

Microscope image acquisition

The microscope image acquisition set-up contained an inverted microscope (Olympus, IX73) and a color camera (Olympus, DP74). The two-channel fluorescent images were taken alternatively by switching the excitation light paths through dichroic mirror. A 420DF20 filter and a 455DRLP dichroic mirror were set as excitation for ECFP, and two emission filters, 480DF30 and 535DF25, for ECFP and Ypet) All fluorescence images were collected on an isolated single cell by Software (Cellsens Dimension) with an interval of 1 min for each shoot on a single cell.

Image analysis

The software package used to analyze the stress changing of paxillin based on Matlab (Mathworks; Natick, MA, RRID:SCR_001622) contained three different sections, pretreatment, FAs identity and ratio calculation. First, all fluorescence images from different channels of a same sample were read and marked. The background of each image was set as the mean value of the fluorescence intensity from four corners and subtracted to avoid disturbance of background. The cell body was detected according to the ECFP image by a segmentation algorithm combined with Otsu's method with an adaptive detection threshold. The global threshold renewed automatically to adapt the intensity changing in ECFP images. After that, the initial segmentation result was dealt by a K-means clustering combined with a low-pass filter, to further improve the accuracy of detection at vague edges. The second section to identify FA sites was based on K-means clustering mainly. A Gaussian filter was applied to the image without background which was named ImageA, and then the original image without background was subtracted from ImageA to achieve a FA-enhanced image. After that, the enhanced image was handled with K-means clustering again to segment FA sites. Based on the current segmentation result, the distances of each FA to its nearest edge (DAE) and the distance to the cell centroid (DAC) were calculated, and the ratio of these two parameters (DAE/DAC) was set as a standard to describe positions of FAs. Cells were divided into layers according

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to the standard, allowing that only the outermost FAs sites (distance ratio>0.85) were remained for further analysis. With the final segmentation results of ECFP and Ypet channel, the tension across paxillin was calculated as an averaged FRET ratio of Ypet/ECFP at FA sites (Fig S3).

Statistical analysis

All FRET ratio (YPet / ECFP) data were normalized by their basal level at zero point in the same cell. The zero point was considered as the last time point before shear stress application. Statistical analysis used in the study was Two-tailed t-test function contained in the Excel software (Microsoft) to evaluate the statistical difference between groups. The data population numbers (n) here are the number of cell samples in each group. Difference was determined as significant when the p-value was smaller than 0.05. All means involved in the manuscript were modified by standard deviation.

Data and Code Availability Statements

The MATLAB source code generated during this study are available as Supplementary Software 1.

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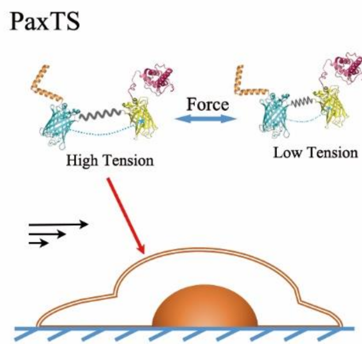

Visualizing the tension across paxillin in real time. This work provides a DNA-encoded biosensor based on Fluorescence Resonance Energy Transfer(FRET) technique to detect the tension across paxillin in living cells. The results indicate that there exists a force transmission pathway described as ‘membrane-cytoskeleton-FAs’ and involved with paxillin upon mechanical force.

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