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Receptor-mediated endocytosis generates nanomechanical force reflective of ligand identity and cellular property

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Abstract

Whether environmental (thermal, chemical, and nutrient) signals generate quantifiable, nanoscale, mechanophysical changes in the cellular plasma membrane has not been well elucidated. Assessment of such mechanophysical properties of plasma membrane may shed lights on fundamental cellular process. Atomic force microscopic (AFM) measurement of the mechanical properties of live cells was hampered by the difficulty in accounting for the effects of the cantilever motion and the associated hydrodynamic force on the mechanical measurement. These challenges have been addressed in our recently developed control-based AFM nanomechanical measurement protocol, which enables a fast, noninvasive, broadband measurement of the real-time changes in plasma membrane elasticity in live cells. Here we show using this newly developed AFM platform that the plasma membrane of live mammalian cells exhibits a constant and quantifiable nanomechanical property, the membrane elasticity. This mechanical property sensitively changes in response to environmental factors, such as the thermal, chemical, and growth factor stimuli. We demonstrate that different chemical inhibitors of endocytosis elicit distinct changes in plasma membrane elastic modulus reflecting their specific molecular actions on the lipid configuration or the endocytic machinery. Interestingly, two different growth factors, EGF and Wnt3a, elicited distinct elastic force profiles revealed by AFM at the plasma membrane during receptor-mediated endocytosis. By applying this platform to genetically modified cells, we uncovered a previously unknown contribution of Cdc42, a key component of the cellular trafficking network, to EGF-stimulated endocytosis at plasma membrane. Together, this nanomechanical AFM study establishes an important foundation that is expandable and adaptable for investigation of cellular membrane evolution in response to various key extracellular signals.

Keywords

atomic force microscope; endocytosis; mechanical force; plasma membrane

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

1 | INTRODUCTION

The mammalian cell plasma membrane is a lipid bilayer separating extra- and intra-cellular components. It functionally transduces environmental signals via a diverse array of membrane-bound proteins. Chemical or thermal signals induce dynamic organization of lipids and proteins in the plasma membrane (Bernardino de la Serna, Schütz, Eggeling, & Cebecauer, 2016). Whether these extracellular stimuli generate quantifiable, nanoscale, mechanophysical features in the plasma membrane has not been well studied. Specifically, it is not clear whether biochemical alterations in the plasma membrane will lead to substantial changes in the mechanical properties of the membrane. Assessment of such biophysical properties of the plasma membrane may provide important insights about the membrane evolutions at subcellular level, potentially shedding lights on key cellular events related to responding to environmental stimuli.

One of the fundamental cellular processes that involve active plasma membrane reorganization is endocytosis. During endocytosis, macromolecules are internalized by cells through small double-membrane vesicles emerged from plasma membrane. The formation of these endocytic vesicles employs strategies that most notably utilize clathrin- or caveolae-dependent pathways. In clathrin-dependent pathway, the coat protein clathrins are recruited to the cargo-concentrated membrane microdomains bound by clathrin adaptor proteins (APs) (Conner & Schmid, 2003; Gupta, Kloster, Rodionov, & Bakke, 2006; Kirchhausen, 1999, 2012; Owen, Collins, & Evans, 2004; Touz, Kulakova, & Nash, 2004; Traub, 2009). Clathrin-independent endocytic pathway is mediated by caveolin family proteins at cholesterol and glycosphingolipid enriched membrane microdomains, also known as lipid rafts (Lajoie & Nabi, 2010; Razani, Woodman, & Lisanti, 2002; Simons & Ehehalt, 2002; Simons & Toomre, 2000). Insertion of caveolin into these microdomains drives the formation of flask-shaped membrane invaginations (Anderson, 1998; Parton & Simons, 2007). In both pathways, the recruitment of a key small GTPase, dynamin, by a variety of accessory proteins, to the pinching site of the membrane enables scission and release of vesicles into the cell. This process exclusively depends on the hydrolysis of GTP by dynamin (Marsh & McMahon, 1999; Praefcke & McMahon, 2004). Once internalized, the cargos travel to membrane-bound early endosomes, where they are further sorted to recycling endosome, late endosome, or lysosome for signaling or degradation (Sorkin & von Zastrow, 2009; Wolfe & Trejo, 2007). The trafficking of endocytic vesicles critically relies on a cytoplasmic ATPase motor, dynein, which dictates the retrograde vesicular movement along the microtubules. Small chemical molecules that inhibit dynamin or dynein activities are effective blockers of endocytosis (Firestone et al., 2012; Hill et al., 2005; Kirchhausen, Macia, & Pelish, 2008; Macia et al., 2006; Roossien, Miller, & Gallo, 2015). Likewise, small molecules that disrupt clathrin function or lipid raft organization have been shown to impact endocytosis (Allen, Yu, Donati, & Rasenick, 2005; Ares & Ortiz, 2012; Auriac, Willemetz, & Canonne-Hergaux, 2010; Ros-Baro et al., 2001; Sarnataro et al., 2009; von Kleist et al., 2011).

Ligand-induced receptor-mediated endocytosis is a particularly important cellular event initiated by extracellular ligands, such as growth factors, at plasma (Sorkin & von Zastrow,

2009). During this process, ligand engagement of the plasma membrane-localized receptors triggers an internalization of the ligand-receptor complex through clathrin-dependent or -independent pathways (Marsh & McMahon, 1999; Rappoport, 2008). Key stem cell growth factors, such as the epidermal growth factor (EGF) and the Wnt proteins, have been demonstrated to induce receptor-mediated endocytosis to transduce, maintain, or terminate corresponding cellular signaling.

Atomic force microscope (AFM) technologies have been developed and utilized to interrogate and/or manipulate live cellular and subcellular structures in a biologically-friendly aqueous environment (Dufrene et al., 2017; Kasas, Stupar, & Dietler, 2017). Both morphological and/or mechanical properties of a live cell or subcellular compartment can be examined with nanoscale spatial and force resolutions. AFM measurement of mechanical properties (e.g., elasticity) of live cells (Butt, Cappella, & Kappl, 2005), however, has been hampered by the difficulty in accounting for the effects of the cantilever motion and the associated hydrodynamic force on the mechanical measurement (Ren, Yu, Gao, & Zou, 2013b). These challenges have been addressed in our recently developed control-based AFM nanomechanical measurement protocol (Cinar & Sahin, 2014; Ren et al., 2013b). Our methods enabled a fast, noninvasive, broadband measurement of the real-time changes of plasma membrane elasticity in live mammalian cells (Ren, Yu, Gao, & Zou, 2013a, 2013b; Yu et al., 2015, 2016).

Here, we utilized this nanomechanical protocol to monitor in real-time the variations of the elasticity in live mammalian cells. We hypothesize that the dynamic organization of plasma membrane during ligand-stimulated endocytosis, for example, processes such as the formation of membrane curvature, membrane invagination, and fission, generates significant alternation in the mechanical behaviors of plasma membrane. We demonstrate that the changes of the plasma membrane elasticity in live cells sensitively correlate with the membrane status in response to thermal, chemical, and growth factor stimuli known to influence endocytosis. We further show that different ligands induce distinguishable nanomechanical alterations, whereas cells with a single genetic manipulation produce distinct force signatures at plasma membrane in response to identical ligands.

2 | RESULTS

2.1 | AFM instrumentation for noninvasive measurement of membrane elasticity in live mammalian cells

Elasticity of cell membrane is one of the most fundamental and important mechanical characteristics of live mammalian cells. Using AFM, the elasticity of cell membrane can be quantified by applying an excitation force of known profile (amplitude as a function of time) from the cantilever probe to the cell (Figures 1a and 1b), measuring the indentation generated, and then computing the Young's modulus (i.e., elastic modulus) from a chosen probe-sample contact mechanics model (Butt et al., 2005). In this work, Hertz contact model (Butt et al., 2005) as given below was chosen, as the adhesion force effect was negligible in our measurements (see "AFM" in the Method section):

$$F_z(t) = \frac{4}{3} \frac{E \sqrt{R} \Delta z^3}{1 - \gamma^2}$$

where E is the Young's modulus, F_z and Δz are the force applied and the indentation generated, respectively. R and γ are the tip radius and the Poisson ratio of the cell, respectively.

2.2 | Temperature alteration and endocytic inhibitors alter membrane elasticity at atomic level

Endocytosis is a temperature-dependent process (Punnonen, Ryhanen, & Marjomaki, 1998; Tomoda, Kishimoto, & Lee, 1989; Weigel & Oka, 1981). We first assessed the Young's modulus of the plasma membrane of HeLa cells incubated at various temperatures. We detected a temperature-dependent difference in membrane elastic modulus, with an 11 (+/-0.85) kPa Young's modulus at 37 °C compared to a 5 (+/-0.3) kPa at 4 °C (Figure 1c). Intermediate membrane elastic modulus was detected at 25 °C. To monitor in real-time the evolution of the mechanical behavior (Young's modulus) of cell membrane in response to altered temperatures, we performed continuous measurement on Young's modulus in live cells that underwent a dynamic temperature alterations. As cells were gradually warmed up from 4° C to 25 °C, we observed a steady increase in membrane tension, which stabilized around 10 (+/-0.76) kPa when the temperature reached 25 °C (Figure 1d). Conversely, when temperature was slowly decreased from 37 °C to 25 °C, cells exhibited a gradual reduction of Young's modulus, which ultimately stabilized around 10 kPa. Interestingly, we were able to detect a wider fluctuation in Young's modulus at higher temperatures (e.g., 25 °C and 37 °C) that were likely attributable to the enhanced membrane activities, as they were absent from cells at 4 °C when only static membrane activity was observed (compare 1–200 s vs. 800–1000 s, green line, Figure 1d). Since endocytotic rate normally reduces to negligible level below 10 °C (Tomoda et al., 1989), our results of plasma membrane mechanophysical properties suggested that the cell surface elasticity reflected as Young's modulus positively correlated with cellular endocytosis activity.

To examine the above correlation in a more specific manner, we tested plasma membrane elasticity in live cells in response to a potent endocytosis inhibitor dynasore. Dynasore is a membrane permeable reversible noncompetitive small molecular inhibitor for dynamin, which is required for membrane fission and vesicle release in both clathrin-dependent and caveolae-dependent endocytosis (Henley, Cao, & McNiven, 1999; Henley, Krueger, Oswald, & McNiven, 1998). We used two cell biology methods, dextran uptake and EEA1 staining, to assess endocytosis in parallel to AFM analysis on cells under treatment. Compared to vehicle-treated cells, cells pre-incubated in dynasore-containing medium for 2 hr demonstrated a reduced endocytic uptake of dextran (Figure 1f) and a decreased number of early endosomes marked by EEA1 (Figure 1g). AFM examination of live cells that were pre-incubated with dynasore revealed a consistent reduction of Young's modulus (Figures 1d and 1e). The lowest detected elastic modulus dropped by over twofolds to around 5 kPa (Figure 1d), a value similar to cells that were measured at 4 °C. As dynasore was shown to block clathrin-dependent vesicle formation and endocytic pathway within seconds (Macia et

al., 2006), we also examined the acute effect of dynasore on cellular membrane tension immediately following 80 μ M of dynasore addition to the medium. Interestingly, we observed a gradual decrease in elastic modulus after dynasore addition (purple line, Figure 3b). Within 900 s, the Young's modulus decreased to approximately 5 kPa, strongly suggesting that the reduction of membrane indentation highly correlates with a reduced dynamin-mediated endocytosis (Figure 1h).

We then tested the effect of a different endocytosis inhibitor, ciliobrevin, which specifically blocks dynein-mediated retrograde movement of endocytic vesicles along microtubules (Figure 2a) (Day et al., 2015; Le Droguen, Claret, Guichet, & Brodu, 2015). Cells pretreated with ciliobrevin A (CBVA) for 1 hr showed a reduction in dextran uptake and in the number of early endosomes (Figure 2b). AFM analysis of CBVA-treated live cells exhibited a drastically lower elastic modulus than vehicle-treated cells, suggesting that blockage of dynein-mediated retrograde transport of endocytic vesicles from the plasma membrane by CBVA reduced membrane elasticity, consistent with an inhibited endocytosis (Figure 2c).

2.3 | The cell surface nanomechanical property reflects plasma membrane composition and organization

We reasoned that as here the elasticity was measured by exerting an external force to the outer surface of cellular plasma membrane, the change of Young's modulus likely reflected the fluidity or rigidity of the membrane, which is often influenced by the composition and configuration of lipids. We then employed small molecules known to perturb the lipid bilayer composition or organization, and asked whether they would induce detectable elastic alterations by AFM. Chlorpromazine (CPZ) usually inserts its hydrophobic cationic portion in the lipid bilayer of cell membrane by binding to polyphosphoinositides (Figures 2a and 2e). By abrogating the PI(4,5)P₂-dependent membrane recruitment of AP2 adaptor, CPZ has been shown to block clathrin-mediated endocytosis (Sofer & Futerman, 1995). The literature also suggested a tendency for CPZ to suppress clathrin-independent endocytosis (Vercauteren et al., 2010). Cells treated with CPZ for 2 hr showed a reduced uptake of dextran into small vesicular puncta, which was accompanied by a clear reduction of EEA1 positive early endosomes (Figure 2d). However, in contrast to the effects of dynasore and ciliobrevin, CPZ treatment almost doubled the elastic modulus in live HeLa cells up to 22 kPa (red line, Figure 2e), indicating that the Young's modulus here reflected an increased membrane rigidity upon CPZ anchoring to the lipid bilayer. This notion was also supported by treating cells with Filipin, a small molecule that binds with high affinity to cholesterol in plasma membranes, forms globular deposits, and disrupts the planar organization of the membrane (Figure 2h). Filipin-treated cells exhibited even stiffer membrane with the Young's modulus quadrupled to nearly 50 kPa (yellow line, Figure 2e), an effect that was in agreement with the reported cellular plasma membrane viscosity and rigidity in Filipin-treated cells (Bonneau et al., 2010). Filipin treatment also inhibited dextran uptake into small vesicles as well as reduced the number of early endosome (Figure 2g), consistent with its reported blockage on non-coated endocytosis (Orlandi & Fishman, 1998; Schnitzer, Oh, Pinney, & Allard, 1994). Thus, the increased membrane tension in CPZ- or Filipin-treated cells was not reflective of endocytic rate in these cells; instead it indicated the membrane rigidity.

We further tested the effects of M β CD, a small molecule that extracts cholesterol from cellular membranes (Vercauteren et al., 2010) (Figure 2j). Strikingly, addition of M β CD led to an initial fluctuation of membrane elasticity in the first 200 s, followed by a rapid decrease of elastic modulus around 300 s (Figure 2j). The Young's modulus became stabilized around 2 kPa that likely represented the minimal membrane elasticity in these cells. M β CD-treated cells showed a reduced dextran uptake and decreased number of early endosomes (Figure 2i), consistent with its reported inhibition on endocytosis in various cell lines (Vercauteren et al., 2010). Taken together, although CPZ, Filipin, and M β CD have similarly inhibitory effects on endocytosis, these pharmacological molecules elicited mechanistically distinct nanoscale elasticity profiles at plasma membrane, which should reflect their different actions on membrane configuration and composition.

2.4 | AFM detects ligand-dependent nanomechanical profiles during receptor-mediated endocytosis

Above chemical molecules are potent membrane disruptors, the effects of which are not physiologically relevant for normal cellular activities. We next asked whether our AFM analysis could detect mechanical alterations in receptor-mediated endocytosis triggered by endogenously expressed ligands. EGF has been well studied for its effect on receptor-mediated clathrin-dependent endocytosis (Vieira, Lamaze, & Schmid, 1996). HeLa cells that were serum-starved for 30 min showed an enhanced uptake of dextran at 2 hr after EGF addition. This effect was blocked by dynasore (Figures 3a and 3b), consistent with the notion that EGF stimulates receptor endocytosis. We performed AFM measurement on serum-starved live cells receiving a single addition of EGF. Remarkably, AFM detected an almost immediate surge of the Young's modulus upon EGF addition to the culture medium (red line in Figure 3c). It took less than 60 s for the Young's modulus to reach the peak, which was followed by a wide range of oscillation between 14 and 17.5 kPa in the next 10 min. The modulus returned to baseline after approximately 15 min, reflecting an overall rapid but transient EGF effect on the plasma membrane elasticity. This robust EGF-induced surge of Young's modulus was blocked by pre-incubating cells with dynasore for 2 hr (green line in Figure 3c), strongly suggesting that the detected EGF-triggered membrane tension was endocytosis-related. Interestingly, cells treated with dynasore alone, starting at 0 s, showed a gradual decline of Young's modulus, whereas EGF treatment (in the presence of dynasore) appeared to antagonize the inhibitory effect of dynasore (compare green versus purple lines, Figure 3c). These results suggested that EGF rapidly stimulated receptor-mediated endocytosis, which was blocked by dynasore. In the presence of dynasore, there remained some EGF-receptor engagements at cell surface.

We then postulated whether the AFM-detected EGF effects could be seen for other growth factors that also stimulate receptor endocytosis. We thus tested the effect of Wnt3a, which represents another important extracellular factor required for stem cell renewal and adult tissue homeostasis. Wnt binds its receptor Frizzled and co-receptor LRP6 on plasma membrane. The formation of Wnt/Frizzled/LRP6 tertiary complex triggers an endocytotic internalization via clathrin- or caveolae-mediated pathways (Feng & Gao, 2015). Similar to EGF treatment, Wnt3a addition to live serum-starved HeLa cells increased Young's modulus to a peak value around 17 kPa (yellow line in Figure 3d). Interestingly, even though both

Wnt3a and EGF elicited similar maximal amplitudes (Figure 3e), in comparison to EGF, the effect of Wnt3a was almost five times slower lasted over two times longer (Figures 3d, 3f, and 3g). It took an approximately 300 s to reach maximal Young's modulus upon Wnt3a addition (Figures 3d and 3f), as opposed to less than 60 s for EGF (Figures 3c and 3f). However, Wnt3a-induced oscillation of membrane elasticity at an elevated modulus was sustained for more than 30 min. Just like EGF, the detected membrane effect of Wnt3a was reflective of receptor endocytosis as it was blunted by either dynasore treatment (orange line in Figure 3d), or by lowering the cellular temperature to 4 °C (purple line in Figure 3d). When cells gradually warmed up from 4 °C to 25 °C, this inhibitory effect was released, revealing the membrane effect elicited by Wnt3a (1200–1600 s, purple line in Figure 3d). These results collectively suggested that Young's modulus was capable not only to detect receptor endocytosis but also to reveal distinct membrane effects elicited by different ligands.

2.5 | Single genetic manipulation changes membrane nanomechanics during receptor endocytosis

We next postulated whether the growth factor effects seen in HeLa cells could be reproduced in other cell lines. We chose the Caco2 cells, a colonic epithelial cell line, and performed EGF-stimulated endocytosis experiments. EGF elicited a largely similar change in the membrane elasticity in Caco2 cells (Figure 4a), suggesting that EGF induced similar nanoscale changes in plasma membranes of both cell types. We then sought to investigate whether our AFM measurement was capable of identifying the nanomechanical differences in receptor endocytosis profile from cells with a genetic alteration in the membrane trafficking machinery. For this purpose, we selectively depleted the small GTPase Cdc42, which plays critical roles in the organization of actin cytoskeleton and in the regulation of vesicular trafficking. We have recently shown that Cdc42 contributes to adult stem cell renewal (Sakamori et al., 2012) and colonic tumor progression (Sakamori et al., 2014). However, it was not clear whether Cdc42 is involved in EGF-stimulated receptor endocytosis. We first derived a stable Cdc42-deficient Caco2 cell line by knocking down the endogenous gene via lentiviral expression of Cdc42 specific siRNA (Figure 4b). Remarkably, when serum starved live *Cdc42*-deficient cells were stimulated with EGF, there was a drastic delay in the response to EGF stimulation by approximately 15 min, compared to the control scramble knockdown cells, as reflected by the increase of Young's modulus (Figure 4a). Cdc42 deficiency diminished both the rate of increase in Young's modulus by 22-fold, and the amplitude of Young's modulus by over threefold (Figures 4a and 4c). The slow increase of Young's modulus at a rate of 0.06 kPa/s to a maximum of 57.5 kPa in *Cdc42*-deficient Caco2 cells (as opposed to 1.3 kPa/s in control cells to a maximum of 219.4 kPa) suggested that there was some remaining but weak cellular response to EGF in the absence of Cdc42, even though the overall response was significantly dampened in the knockdown cells. Taken together, AFM-mediated real-time measurement of Young's modulus was capable to quantitatively assess the ligand-specific receptor endocytosis in genetically manipulated cells.

3 | DISCUSSION

To our knowledge, this is the first study utilizing AFM to quantitatively assess nanomechanical property at plasma membrane during receptor endocytosis in live mammalian cells. Most of existing AFM studies of the endocytosis process have been focused on the morphological changes of live cells. For example, high-speed AFM has been used in combination with optical microscopy to elegantly capture the morphogenesis such as filopodia, membrane ruffles, pit formation, and endocytosis in COS-7, HeLa cells and hippocampal neurons (Shibata, Uchihashi, Ando, & Yasuda, 2015; Shibata, Watanabe, Uchihashi, Ando, & Yasuda, 2017). Time-resolved images of chemical-induced lamellar bodies of living alveolar type II cells was captured by low-force contact mode AFM, although with limited resolution on live specimens (Hecht et al., 2011). In addition, AFM imaging captured the uptake of liposomes on human coronary artery endothelial cells (HCAECs) (Zaske, Danila, Queen, Golunski, & Conyers, 2013). Scanning raised domains at the inner cell plasma membrane and co-identifying of cargo-receptors and clathrin by using a combination of AFM and fluorescent microscopy predicted the potential occurrence of signaling and endocytosis in fixed RBL-2H3 mast cells (Frankel et al., 2006). Our work contrasted with above studies by revealing the mechanical evolution of cell membrane and the underneath biological pathways during endocytosis. By utilizing the control-based nanomechanical protocol developed by us (see Methods below) (Ren et al., 2013a, 2013b), we were able to reliably quantify the nanomechanical properties (Young's modulus) of the cells over a large range of force spectrum, which allowed us to quantitatively monitor in real-time the nanomechanical evolution of the cell membrane during endocytosis, with or without genetic alternations and/or biochemical modifications.

At a global scale, the rate of cellular endocytosis is influenced by environmental temperature and nutrient abundance (Etxeberria, Baroja-Fernandez, Munoz, & Pozueta-Romero, 2005; Grahammer et al., 2017; Punnonen et al., 1998). During our early AFM instrumental development and study, we reported a decreased Young's modulus in serum-starved HeLa cells, which likely reflected a decreased endocytosis rate (Ren et al., 2013b). In this study, the dynamic changes in cellular plasma membrane, such as the alteration of basal rate of endocytosis at different temperatures, or the membrane compositions altered by various pharmacological molecules, were sensitively reflected in the elastic modules detected by AFM. It is important to note that despite of being inhibitory to endocytosis for various small chemical molecules, AFM was capable of revealing their distinct mechanistic actions on membrane properties. In general, our data suggest that higher membrane tension appears to be associated with enhanced endocytic rate induced by stimulative ligands at steady states. The enhanced tension could be a result from a reduction of plasma membrane from endocytosis or a cytoskeletal reorganization at the cortical membrane. For instance, the clathrin-dependent endocytosis requires reorganization of the cytoskeleton network at the membrane-cortex region. The reorganization of actin into comet-like tails is important for micropinocytosis (Merrifield et al., 1999) while remodeling of membrane-associated actin on endosomes may be generally important for membrane budding (Harder, Kellner, Parton, & Gruenberg, 1997; Lamaze, Fujimoto, Yin, & Schmid, 1997). Thus, in addition to a loss of surface membrane during endocytosis, cytoskeleton tension generated at plasma membrane

is likely an important contributor to the detected nanomechanical alteration and fluctuation. In addition, the potential alteration of membrane lipid composition following endocytosis may be another contributing factor to the elasticity change. This notion was supported by our results on CPZ or Filipin treated membranes, where anchoring of these amphipathic molecules to the phospholipid or cholesterol led to a stiffening of the membrane. At this moment, we do not have evidence to suggest that the observed elastic changes are predominantly determined by any single parameter of the above factors. We tentatively conclude that the plasma membrane elasticity change may result from multiple factors at steady states. Furthermore, change in cellular membrane tension can in turn affect cellular trafficking. It has been shown that high membrane tension tends to drive exocytotic membrane traffic to increase plasma membrane area and lower tension while low membrane tension favoring endocytosis; mitotic cells have high membrane tension and a nearly terminated endocytosis (Raucher & Sheetz, 1999). Overall, our AFM platform was sensitive enough to discern the changes in live cells, when their membrane homeostasis was perturbed by a physiologic or chemical stimuli that affect endocytosis.

One of the most interesting findings from this study was the capability of our AFM to identify differences in mechanical force profiles from two different growth factors, namely EGF and Wnt3a, in terms of their demonstrated influences, for example, rapidness, duration, and amplitude, on membrane elasticity. The observed rapid effect of EGF as well as the relatively slow but sustained effects of Wnt3a are in good agreement with cell biology studies that assessed the endocytic vesicle formation at plasma membrane (Carmon, Lin, Gong, Thomas, & Liu, 2012; Sigismund et al., 2008). The uncovering of a blunted EGF response in *Cdc42*-deficient cells by our AFM analysis is conceptually novel and important, which suggested that loss of *Cdc42* may impair the cellular response to EGF, a critical growth factor that promotes cell growth and survival in the adult stem cell niche. We have previously reported that *Cdc42*-deficient mouse intestinal stem cells were outcompeted by wild type stem cells in vivo (Sakamori et al., 2012), and *Cdc42*-deficient organoids failed to propagate in vitro (Sakamori et al., 2014). These new AFM data pointed to a potentially important mechanism that may contribute to the reduced self-renewal capacity of *Cdc42*-deficient stem cells. The involvement of *Cdc42* in EGF-induced endocytosis and signal transduction has not been formally examined and is important for further investigation at molecular levels. Overall, our study demonstrated the capacity of AFM in determining real-time alteration of mechanical properties at live cell plasma membrane. Future coupling of AFM nanomechanical analysis with morphological and molecular studies is anticipated to gain insights into membrane dynamics and cellular response to environmental signals.

4 | MATERIALS AND METHODS

4.1 | AFM

A commercial AFM system (Dimension ICON, by Bruker-Nano, Inc., Santa Barbara, CA) was used to measure all the cell mechanical properties. The live cells were measured in cell culture medium by using a MLCT-C cantilever, of which the spring constant at 0.01 N/m was calibrated via the thermal tune method (Bruker-Nano, Inc.). To avoid the substrate effect and keep consistence of the measurement conditions, all of the measurements were

performed at the top of the cell over the cell nucleus. The probe radius was calibrated by using a tip-radius calibration sample, and a silicon sample was used as the hard reference sample for all the indentation measurements (as described below). To minimize the cantilever drift due to the temperature fluctuation caused by the heating of the laser, the AFM system was thermally equilibrated for 40 min before the measurements.

To monitor the Young's modulus evolution during the endocytosis process (with or without the treatments and/or genetic modifications as described above), we measured the indentation on the cell membrane by using the CNM protocol (Ren et al., 2013a, 2013b). First, a stable probe-sample interaction was maintained throughout the measurement under a preload force of amplitude at 0.05–0.08 nN, then a desired force profile (of given amplitude and force load rate) was continuously exerted to the cell while both the cantilever deflection, $d_c(t)$, and the displacement of the fixed end of the cantilever (i.e., the end of the cantilever that is fixed to the AFM scanner), $z_c(t)$, were measured. In all the cell measurements, the desired force profile was set as a 1 Hz triangle wave of amplitude at 0.2–0.3 nN, with a duration of 600–1200 s (to monitor the time-elapsed evolution of Young's modulus). After the cell measurement, the recorded cantilever deflection $d_c(t)$, was regarded as the desired deflection profile to be tracked on the silicon reference sample under the same preload force condition. The modeling-free iterative control (MIIC) technique (Kim & Zou, 2013) was used to ensure that precision tracking of $d_c(t)$ was achieved on the silicon sample (with RMS tracking error within 2%), and the displacement of the fixed end of the cantilever measured on the reference silicon sample, $z_h(t)$, was recorded. Finally, the indentation in the cell membrane, $\Delta_z(t)$, was obtained as the difference of the displacement of the cantilever fixed end on the cell respect to that on the silicon sample (Ren et al., 2013a), that is,

$$\Delta_z(t) = z_c(t) - z_h(t)$$

Next, the force applied was computed via the relation $F_c(t) = k_c d_c(t)$ with k_c the spring constant of the cantilever calibrated a priori. The measured indentation and force signals were used to compute the Young's modulus via the Hertz model in Eq. (1) above (where the Poisson's ratio of $\gamma = 0.5$ was chosen (Butt et al., 2005; Schillers, Walte, Urbanova, & Oberleithner, 2010)).

The graphed AFM plots represent consistent results obtained across multiple different cells measured in each individual experiment, with the same profiles reproduced in one or more independent experiments performed on different days.

4.2 | Cell culture

Culture of HeLa and Caco2 cells has been described by us (Sakamori et al., 2012; Sun et al., 2017). Briefly, cells were grown in a Dulbecco's Modification of Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, MT10013CM) containing 10 % fetal bovine serum (Sigma, St. Louis, MO, F2442) and 1 % Penicillin/Streptomycin solution (Gibco, 15070063, Thermo Fisher Scientific), and were passaged with Trypsin-Ethylene-Diamine-Tetraacetic-Acid (Trypsin-EDTA, Gibco™ Fisher 25-200-072) digestion every 3 days. A total of 1×10^6 HeLa cells were then seeded into a 60 mm cell culture dish (Thermo Fisher Scientific Nunc,

Catalog No. 12-565-97) containing 5 ml of fresh complete culture medium and were grown overnight to a complete confluent monolayer. To knockdown CDC42 in Caco2 cells, human CDC42-specific lentiviral transduction particles (clone ID TRCN0000047628; Sigma–Aldrich) was used at 1–5 multiplicity of infection with 8 µg/ml Polybrene. Stable cells were selected by puromycin. Non-Targeting scrambled shRNA Control Transduction Particles (Sigma, SHC203V) were used as negative controls. For EGF and Wnt3a experiments, cells were serum-starved for 12 hr prior to the growth factor addition.

4.3 | Small molecular chemicals and growth factors

The sources and working concentrations of chemical and growth factors used in this study are: Dynasore (Sigma, D7693) at 80 µg/ml, and Ciliobrevin (Tocris 4529) at 100 µM, Filipin (Sigma, F4767) at 2 µg/ml, CPZ hydrochloride (Sigma, C8138) at 50 µg/ml, MβCD (Sigma, C4555) at 5 mM, EGF (Life Technologies, PMG8043) at 100 ng/ml, and recombinant mouse Wnt3a (R&D Systems, Minneapolis, MN) at 100 ng/ml.

4.4 | Dextran uptake assay, immunofluorescence, and confocal microscopy

For dextran uptake, cells with or without treatment were incubated with 100 µg/ml Dextran-568, or Dextran-488 (10,000 MW, Anionic, Fixable, ThermoFisher Scientific) for 30 min at 37 °C. After washing with PBS, cells were fixed with 4% paraformaldehyde (PFA), washed with PBS, and stained for actin with Phalloidin-488 (Thermo Fisher Scientific, A12379), for 20 min at room temperature. After washing with PBS for three times, cells were dried in dark, and mounted with ProLong® Gold Antifade Mountant (Thermo Fisher Scientific, P36930) before imaging with Zeiss LSM confocal microscope.

Immunofluorescent analysis has been previously described by us (Das et al., 2015; Yu, Nie, et al., 2014; Yu, Yehia, et al., 2014). Briefly, cells with or without treatment were washed with PBS twice and fixed with 4% PFA at room temperature for 15 min. Cells were then washed with PBS and blocked with 10% donkey serum in PBS with 0.1% Triton X-100 for 1 hr at room temperature. Cells were incubated with EEA1 antibody (Cell Signaling Technology, Beverly, MA, #3288), at 1:200 dilution in blocking buffer over-night at 4 °C. Cells were washed with PBS and incubated with Alexa-555 conjugated anti-rabbit secondary antibody (1:500) and Phalloidin-Alexa-488 (1:3000) at room temperature for 1 hr before washing and mounting.

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FIGURE 1.

AFM detects thermal and chemical effects on nanomechanical membrane elasticity. (a) Schematic diagram showing the setup of AFM and the vertical movement of the cell-contacting probe that applies force on plasma membrane of a live cell in medium. (b) An AFM scanning image of live HeLa cells. (c) AFM detected different Young's modulus of HeLa cells at 37 $^{\circ}\text{C}$ (blue), 4 $^{\circ}\text{C}$ (green), and 25 $^{\circ}\text{C}$ (red). (d) Real-time measurement by AFM on cells in medium with temperature changes from 4 $^{\circ}\text{C}$ to 25 $^{\circ}\text{C}$ (green) and from 25 $^{\circ}\text{C}$ to 4 $^{\circ}\text{C}$ (blue). Cells pre-treated with Dynasore showed an overall decreased Young's modulus (red line). (e) Dynasore pretreatment reduced membrane elasticity by half. (f and g) Dynasore treatment reduced dextran uptake and EEA1 positive early endosomes. (h)

Schematic diagram illustrating the blockage of endocytosis by Dynasore may alter the plasma membrane mechanical property

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FIGURE 2.

The AFM elasticity property reflects the composition and organization of plasma membrane.

(a) Schematic diagram illustrating clathrin-mediated endocytosis and the molecular targets of Ciliobrevin A (CVBA) and CPZ. (b) CVBA treatment reduced dextran uptake and EEA1 endosome. (c) AFM detected a clear decrease in Young's modulus in CVBA-treated cells. The diagram above illustrates the blocked Dynein-dependent retrograde movement of vesicles. (d) CPZ treatment inhibited dextran uptake and EEA1 endosome. The diagram illustrates that CPZ binding to PI4,5P2 lipids on membrane potentially rigidifies the membrane. (e) AFM detected increased Young's modulus in CPZ- and Filipin-treated cells. (f) A diagram illustrating caveolae mediated endocytosis. (g) Filipin treatment decreased dextran uptake and EEA1 endosomes. (h) A diagram illustrating that anchoring of Filipin to

cholesterol bends and stiffens the membrane. (i) M β CD treatment of cells reduced dextran uptake and EEA1 endosomes. (j) AFM detected a rapid decrease of elasticity following an initial fluctuation of modulus in response to addition of M β CD. A diagram above shows the extraction of cholesterol from cellular plasma membrane, potentially contributing to the decreased membrane stiffness

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FIGURE 3.

AFM detects ligand-dependent changes in elastic modulus during receptor-mediated endocytosis. (a and b) Dextran uptake was stimulated in serum starved HeLa cells by EGF. This effect was blocked by dynasore. (c) Upon addition of EGF to the medium, AFM detected an immediate surge of Young's modulus (compare red to blue line). This effect of EGF was completely blocked by dynasore treatment (green line). Fresh addition of dynasore alone gradually decreased Young's modulus to 5 kPa during a time course of 800 s (purple line). (d) Similar effects were observed after addition of Wnt3a, however the response was slower and the duration of response was longer (compare yellow to blue line). This Wnt3a-induced increase in Young's modulus was blocked by dynasore treatment (orange line) or by lowering the temperature (purple line). (e) The effect of EGF on the magnitude of Young's

modulus was similar to the effect of Wnt3a. (f and g) EGF elicits a much rapid increase of Young's modulus than Wnt3a

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FIGURE 4.

AFM detects changes in elastic modulus in cells with a genetic alteration. (a) AFM detected EGF-stimulated Young's modulus increase, at a rate of 1.3 kPa/s, in control Caco2 cells (red line). In CDC42-knockdown (KD) cells, there was a rate of 0.06 kPa/s increase in Young's modulus. No significant difference between serum starved control and CDC42-KD cells was observed under unstimulated condition. (b) Western blot analysis for CDC42 confirmed stable knockdown of this gene in CDC42-KD cells. (c) AFM detected a nearly 5-time higher in the EGF-stimulated amplitude of Young's modulus in control cells than CDC42-KD cells