Plectin linkages are mechanosensitive and required for the nuclear piston mechanism of three-dimensional cell migration

Pragati C. Marks\(^a\), Breanne R. Hewitt\(^a\), Michelle A. Baird\(^b\), Gerhard Wiche\(^c\), and Ryan J. Petrie\(^a\)*

\(^{a}\)Department of Biology, Drexel University, Philadelphia, PA 19104; \(^{b}\)Cell and Developmental Biology Center, National Heart Lung and Blood Institute, NIH, Bethesda, MD 20892; \(^{c}\)Department of Biochemistry and Cell Biology, Max F. Perutz Laboratories, University of Vienna, Vienna, Austria

**ABSTRACT** Cells migrating through physiologically relevant three-dimensional (3D) substrates such as cell-derived matrix (CDM) use actomyosin and vimentin intermediate filaments to pull the nucleus forward and pressurize the front of the cell as part of the nuclear piston mechanism of 3D migration. In this study, we tested the role of the cytoskeleton cross-linking protein plectin in facilitating the movement of the nucleus through 3D matrices. We find that the interaction of F-actin and vimentin filaments in cells on 2D glass and in 3D CDM requires actomyosin contractility. Plectin also facilitated these interactions and interacts with vimentin in response to NMII contractility and substrate stiffness, suggesting that the association of plectin and vimentin is mechanosensitive. We find that this mechanosensitive plectin complex slows down 2D migration but is critical for pulling the nucleus forward and generating compartmentalized intracellular pressure in 3D CDM, as well as low-pressure lamellipodial migration in 3D collagen. Finally, plectin expression helped to polarize NMII to in front of the nucleus and to localize the vimentin network around the nucleus. Together, our data suggest that plectin cross-links vimentin and actomyosin filaments, organizes the vimentin network, and polarizes NMII to facilitate the nuclear piston mechanism of 3D cell migration.

**INTRODUCTION**

The generation of intracellular forces is a critical step governing the movement of cells across two-dimensional (2D) surfaces and through three-dimensional (3D) matrices (Yamada and Sixt, 2019; Bodor et al., 2020). Critically, how those forces are generated and applied to facilitate cell motility can be modified in response to the physical cellular environment. On 2D surfaces and in 3D collagen matrices, fibroblasts migrate using low-pressure protrusions called lamellipodia. In these cells, NMII contractility responds to matrix rigidity to strengthen integrin-based adhesions, which can slow cell motility (Doyle et al., 2012). In contrast, cells migrating through 3D matrices rely on NMII-mediated contractility either in front of or behind the nucleus to help pull or push it forward, respectively (Petrie et al., 2014; Wu et al., 2014; Lomakin et al., 2020; Lee et al., 2021). Further, cytoskeletal cross-talk plays an important role in 3D nuclear movement (Martini and Valdeolmillos, 2010; Petrie et al., 2014). Understanding how NMII contractility and cytoskeletal cross-talk are coordinated with power nuclear movement through 3D matrices will help illuminate how cells migrate through tight spaces in matrices throughout the tissues of the body.

In primary human foreskin fibroblasts (HFFs) migrating through cross-linked 3D CDM, tropomyosin 1.6 (Tpm 1.6) recruits NMII to filamentous actin (F-actin) to pull the nucleus forward in combination with vimentin intermediate filaments and the nucleoskeleton-cytoskeleton cross-linking protein nesprin 3 (Sao et al., 2019). While this nuclear piston machinery is at least partially assembled on rigid 2D surfaces, it is activated in response to cross-linked 3D matrices (Petrie et al., 2012, 2014). As the nucleus is pulled forward like a piston in these cells, it generates compartmentalized pressure where the nucleus separates an anterior high-pressure cytoplasmic...
FIGURE 1: NMII activity promotes actin-vimentin interactions. (A) Structured illumination microscopy of vimentin (green) and F-actin (magenta) networks in HFFs. While both filamentous networks are abundant in the cytoplasm, we detected no significant colocalization of vimentin and F-actin relative to our positive colocalization control where the F-actin network was simultaneously labeled with two different colored phalloidins \((n \geq 20, N = 3)\). * \(p = 0.0001\).
compartment from a lower pressure compartment at the rear of the cell. This high-pressure helps to generate pressure-based lobopodial protrusions which rely on both NMII activity and cell-matrix adhesion (Petrie et al., 2014; Petrie and Yamada, 2016). It is currently unclear how the anteriorly polarized actomyosin filaments are linked to the cytoplasmic vimentin network to transmit the pulling forces generated by the piston machinery to the nucleus. Further, it is not clear how the assembly of this pulling machinery is regulated.

Plectin is a large cytolinker protein that is responsible for cross-linking vimentin intermediate filaments to a large variety of intracellular structures. For example, the C-terminal domain of plectin preferentially binds vimentin, whereas the binding specificity of the N-terminal domain can vary based on which particular plectin splice isoform is expressed (Wiche et al., 2015). The N-terminal domain of different plectin isoforms can cross-link vimentin with actin (Sutoh Yoneyama et al., 2014; Jiu et al., 2015), and these connections contribute to 2D lamellipodia formation and migration (Jiu et al., 2015). Based on these previous studies, we tested the hypothesis that plectin cross-links between vimentin and F-actin are needed for NMII activity to pull the nucleus forward like a piston to generate compartmentalized pressure and migrate efficiently through 3D matrices. We find that plectin responds to 2D matrix rigidity to help cross-link vimentin and F-actin in primary human fibroblasts. Further, these cross-links are needed to polarize vimentin and NMII during 3D migration, as well as to generate compartmentalized pressure by helping to pull the nucleus forward.

RESULTS

Actin and vimentin interactions depend on actomyosin contractility on 2D surfaces and in 3D matrices

To determine where actin and vimentin interact within HFFs, we first quantified the relative colocalization between the actin and vimentin networks in fibroblasts on 2D using structured illumination microscopy (Figure 1, A and B). While there was robust colocalization between F-actin stained with a mixture of two different fluorophores bound to phalloidin, we did not detect significant colocalization between the cytoplasmic F-actin and vimentin networks within control cells relative to cells treated with latrunculin A (actin inhibition [inh.]) to depolymerize F-actin or blebbistatin (NMII inh.) to disrupt the piston machinery (Petrie et al., 2014). To more precisely identify where these networks interact, we used a proximity ligation assay (PLA) that generates a fluorescent signal when antibodies bound to the proteins of interest are within 40 nm (Alam, 2022). In control HFFs probed for actin–vimentin interactions with PLA, there were abundant fluorescent puncta throughout the cytoplasm of the cell, while extensive negative controls, including PLA probes alone, ®-actin plus an nonimmune antibody, and the individual actin and vimentin antibodies yielded a negligible signal (Figure 1, C and D). Importantly, NMII-inhibition reduced the actin–vimentin PLA signal, consistent with the disruption of the nuclear piston machinery by blebbistatin treatment (Petrie et al., 2014).

Next, we used an intermolecular fluorescence resonance energy transfer (FRET)–based approach to confirm these initial findings and extend them to cells migrating through 3D CDM. To calibrate our FRET acquisition settings, we plotted the corrected sensitized emission FRET signal (Fc) against donor intensity in cells transfected with a single plasmid expressing mTurquoise (FRET donor) directly connected with mNeongreen (FRET acceptor) by a 10-amino acid spacer (T-10-NG) or cotransfected with plasmids separately expressing mTurquoise or mNeongreen (Figure 2A). Sensitized emission FRET imaging of HFFs expressing mTurquoise–actin and mNeongreen–vimentin confirmed their robust interaction in the cytoplasm of cells migrating on 2D surfaces and through 3D CDM (Figure 2, B and C). Critically, treatment with the Rho-associated protein kinase (ROCK) inhibitor Y-27632 reduced the interaction of mTurquoise–actin and mNeongreen–vimentin in HFFs migrating through 3D CDM. Together, these data suggest that actin and vimentin interact closely throughout the cytoplasm in cells migrating on 2D surfaces and in 3D matrices and this interaction is partially dependent on actomyosin contractility.

Plectin cross-links actin and vimentin networks in HFFs

Next, we tested the hypothesis that plectin cross-links F-actin and vimentin networks within our HFFs. To determine the relative localization of plectin and vimentin within HFFs, we next quantified their colocalization using structured illumination microscopy (Figure 3, A and B). While filaments of plectin and vimentin filled the cytoplasm near the nuclei in these cells and ~40% of the plectin network colocalized with vimentin, there was no significant change in colocalization following NMII inhibition to disrupt the piston machinery, consistent with the relative insensitivity of F-actin and vimentin colocalization to NMII inhibition (Figure 1, A and B). To verify that these networks are interacting in HFFs, we used a plectin–vimentin proximity ligation assay (PLA) with extensive negative controls. In control HFFs probed for plectin–vimentin interactions with PLA, there was a strong, nonpunctate signal throughout the cytoplasm that was significantly more intense than all of the negative controls (Figure 3, C and D). We speculate that this PLA signal pattern is a result of plectin proteins binding along the length of the vimentin filaments, rather than more discrete connections that could result in individual puncta (Svitkina et al., 1996).

Transfection of HFFs with pan-plectin siRNA significantly reduced plectin expression (by ~62%, *p < 0.0001) compared with that in control cells (Figure 4, A and B). Importantly, the efficacy of plectin knockdown was confirmed with an independent set of pooled siRNAs (Supplemental Figure S1). Next, we compared actin–vimentin interactions in control and plectin siRNA-transfected cells. Control cells had abundant PLA puncta throughout the cytoplasm, whereas plectin knockdown cells had significantly fewer PLA puncta, without any significant change to cell spread area (ns, p = 0.6241) (Figure 4, C–E). We speculate that the residual interactions between actin and vimentin following plectin knockdown may be mediated by the tail domain of vimentin (Esue et al., 2006) or a result of the partial plectin knockdown. Thus, we find that plectin helps to cross-link actin and vimentin and these interactions occur primarily in the cytoplasm. Taken together, these data suggest that
the actin and vimentin networks interact in both a NMII- and plectin-dependent manner in 2D HFFs and these connections are assembled before the activation of the nuclear piston mechanism in 3D matrices.

Plectin–vimentin interactions require NMII activity and are governed by two-dimensional substrate rigidity

Because we found that NMII activity and plectin expression both facilitate actin–vimentin interactions, we next wanted to test whether NMII activity is required for plectin-dependent cytoskeletal cross-linking. A PLA to probe for plectin–vimentin interactions in control siRNA, control siRNA + NMII-inhibited, and plectin siRNA-treated cells revealed bright, filamentous PLA puncta in control cells as compared with both control siRNA + NMII-inhibited and plectin siRNA-treated cells (Figure 4, F and G). These data suggest that NMII activity is needed to maintain cross-links between the F-actin and vimentin networks, as well as the interaction of plectin and vimentin.

We next determined whether the assembly of this cytoskeletal machinery was governed by the classical rigidity-sensing machinery, which similarly relies on contractility (Plotnikov et al., 2012). To test the hypothesis that plectin binds vimentin in response to substrate stiffness, we plated HFFs on soft (0.2 kPa) or stiff (64 kPa) 2D substrates and performed a PLA to measure plectin–vimentin interactions. Interestingly, we found a significant decrease in PLA signal in cells on stiff plates from that in cells on soft plates (Figure 4, H and I). Taken together, these data illustrate that plectin is a mechanosensitive protein that can bind vimentin in response to NMII activity and substrate rigidity.

Plectin expression is required for two-dimensional mechanotransduction and slows two-dimensional motility

Next, we determined how plectin expression affects the 2D migration and mechanotransduction of HFFs. Specifically, we assessed how plectin expression contributes to cell motility (Doyle et al., 2021),
We started by plating control and plectin knockdown HFFs on 2D glass and compared their cellular velocity. We found that plectin expression slows cell movement on 2D, as plectin knockdown cells had a significantly increased velocity over that of control cells (Figure 5A). In addition, we determined that plectin expression does not contribute significantly to intracellular pressure within cells on 2D surfaces (Figure 5B). Next, we immunostained HFFs that had been transfected with control and plectin siRNAs on 2D glass for YAP, where a higher nuclear-to-cytoplasmic YAP ratio would indicate increased mechanotransduction associated with substrate rigidity (Dupont et al., 2011). Interestingly, we found that plectin knockdown cells on 2D glass had significantly lower nuclear-to-cytoplasmic YAP ratios than 2D control cells (Figure 5, C and D). Together, these data illustrate that plectin expression slows 2D migration and that plectin also facilitates the nuclear translocation of YAP, suggesting that both plectin and NMII activity are needed for cells to respond to 2D substrate rigidity.

Plectin expression is required for the generation of compartmentalized pressure and rapid three-dimensional migration

Having established that plectin is required for mechanotransduction on 2D surfaces, we next assessed whether plectin linkages play a role in 3D migration by helping to pull the nucleus forward to generate compartmentalized pressure (Petrie et al., 2014). We transfected control siRNA- and plectin siRNA-transfected cells with a GFP-nuclear localization signal (NLS) in 3D CDM and directly compared the nuclear velocity of the two cell populations. There was a significant decrease in nuclear velocity in the plectin knockdown cells compared with control cells (Figure 6A), indicating that plectin expression could facilitate nuclear movement through 3D CDM. To confirm whether plectin expression is required to help pull the nucleus forward and generate compartmentalized pressure we compared the front–back pressures within control and plectin siRNA treated cells. We measured a significantly higher pressure in the front of the nucleus compared with behind the nucleus in control HFFs using the nuclear piston to migrate through 3D CDM, as expected (Petrie et al., 2014). In contrast, plectin knockdown cells had lower, equalized intracellular pressure between the front and back of the cell, consistent with the nucleus no longer being pulled forward to generate compartmentalized pressure (Figure 6B). Critically, both of these phenotypes were recapitulated by knocking down plectin with a second, independent set of pan-plectin siRNAs (Supplemental Figure 1, C and D). Next, we
FIGURE 4: Plectin helps to connect the actin and vimentin networks and responds to substrate stiffness in HFFs. Plectin expression facilitates interactions between the actin and vimentin networks. (A) A representative Western blot showing the significant decrease in plectin expression in plectin siRNA-transfected cells compared with control siRNA-transfected cells ($N = 9$). * $p < 0.0001$ versus control. Quantified in (B). (C) Representative maximally projected confocal stacks of actin–vimentin PLA assays performed in control and plectin siRNA-transfected cells. The PLA puncta (green) and DAPI (magenta) demonstrate that plectin knockdown cells have significantly fewer PLA puncta than control cells, suggesting that plectin can mediate actin–vimentin interactions ($n \geq 30$, $N = 3$) * $p = 0.0025$. Bars = 15 µm. Quantified in (D). (E) Plectin siRNA transfection did not significantly affect cell spread area compared with control cells. ns, $p = 0.6241$ versus control cells. (F) Representative maximally projected confocal stacks of PLA assays performed in control cells and control cells treated with the NMII inhibitor blebbistatin, as well as plectin siRNA-transfected cells. The PLA signal (green) and DAPI (magenta) show that NMII activity facilitates the interactions of plectin with vimentin. Plectin knockdown also reduces the plectin–vimentin PLA signal compared with untreated control cells ($n = 30$, $N = 3$) * $p = 0.0127$ versus control cells. Bars = 15 µm. Quantified in (G). (H) Maximally projected confocal stacks of plectin–vimentin PLA (green) and DAPI (magenta) show that cells plated on soft (0.2-kPa) substrates have a significantly brighter PLA signal than cells plated on stiffer (64-kPa) substrates ($n \geq 20$, $N = 3$) * $p = 0.0006$ versus soft. Bars = 15 µm. Quantified in (I).
determined whether plectin expression is similarly required for low-pressure lamellipodia-based migration through 3D collagen. We placed control and plectin knockdown cells in 3D collagen and compared the velocity of plectin knockdown and control cells. Interestingly, plectin knockdown also significantly reduced the velocity of cells using a nuclear piston-independent mode of 3D cell migration (Figure 6C). These data suggest that there is a global requirement for plectin expression during 3D cell migration, along with a specific function in helping to pull the nucleus forward to generate compartmentalized pressure in cross-linked 3D matrices (Petrie et al., 2012, 2014).

Because plectin expression was required for YAP translocation into the nucleus on 2D glass (Figure 5, C and D), we hypothesized that plectin similarly plays a role in nuclear YAP translocation in HFFs in 3D CDM. When we plated control and plectin knockdown cells in 3D CDM, we found that plectin knockdown cells had significantly reduced YAP enrichment in the nucleus compared with control cells (n ≥ 30, N = 3) * p < 0.0001. Bars = 15 µm. Quantified in (D).

3D CDM, we found that plectin knockdown cells had significantly reduced YAP enrichment in the nucleus compared with control cells (Figure 6D). Taken together, these data illustrate that plectin linkages are necessary for both high-pressure, lobopodial 3D migration and YAP-linked mechanotransduction.

Plectin organizes vimentin and polarizes NMII in three-dimensional lobopodial HFFs

Finally, we determined how plectin organizes components of the cytoskeleton during nuclear piston-driven migration through 3D CDM. We started by comparing vimentin and actin localization in control and plectin siRNA-transfected fibroblasts migrating in 3D...
In lobopodial fibroblasts migrating through 3D CDM, we investigated how plectin expression organizes NMII in cells using the nuclear piston to migrate through 3D CDM. In 3D control cells, NMIIA was polarized in front of the nucleus of 3D control fibroblasts, as expected (Petrie et al., 2014). However, in 3D plectin knockdown cells, NMIIA was nonpolarized (Figure 7, F and G), which indicates the loss of compartmentalized NMIIA which is critical for lobopodial migration (Petrie et al., 2014). Altogether, these data illustrate that plectin plays an important role in organizing vimentin filaments and polarizing NMII to the anterior cytoplasmic compartment of 3D lobopodial HFFs.

**DISCUSSION**

We sought to identify the cytoskeleton cross-talk that facilitates force transmission from actomyosin contractility to the nucleus in lobopodial fibroblasts migrating through 3D CDM. We found that the cytolinker protein plectin cross-links actin and vimentin networks in a NMII-dependent manner in 2D fibroblasts. This plectin complex is mechanosensitive, since plectin binds vimentin in response to NMII contractility and substrate stiffness (Figure 8A). These plectin linkages slow down migration and facilitate mechanotransduction on 2D surfaces by helping YAP translocate into the nucleus. Plectin expression is required for the efficient migration of both low-pressure lamellipodial cells through 3D collagen and high-pressure lobopodial cells through 3D CDM. Critically, plectin helps to pull the nucleus forward to generate the compartmentalized pressure necessary for lobopodia formation (Figure 8B; Petrie et al., 2014), as well as playing a more global role in 3D mechanotransduction. Specifically, plectin also facilitates the translocation of the mechanosensitive transcriptional regulator YAP into the nucleus. Finally, we find that plectin expression organizes vimentin filaments around the nucleus and polarizes both focal adhesions and NMIIA towards the front of lobopodial fibroblasts. These cytoskeletal components are critical to the nuclear piston machinery (Petrie et al., 2014; Petrie et al., 2017) and their redistribution following plectin knockdown suggests that plectin helps to organize these components to facilitate pulling the nucleus through 3D matrices.

It is of note that our plectin knockdown data phenocopy many outcomes of NMII inhibition in migrating fibroblasts. For example, NMII activity slows 2D motility and is required for rapid migration in both 3D collagen (Petrie et al., 2017; Rai et al., 2017; Wang et al., 2019) and CDM (Petrie et al., 2017). Further, both plectin knockdown and NMII inhibition dissociate the nuclear piston machinery by reducing actin–vimentin interactions, slowing nuclear translocation, and preventing the generation of compartmentalized pressure (Petrie et al., 2014). These data suggest that NMII activity governs plectin-dependent linkages and that NMII activity and plectin binding vimentin and actomyosin networks (Svitkina et al., 1996; Jiu et al., 2015) in response to NMII activity (Figure 4, C and D), both of which are critical to 3D CDM. The vimentin network in control cells is uniformly localized around the nucleus and throughout the cytoplasm. However, in plectin knockdown cells, vimentin filaments were concentrated in the front of the cell (Figure 7, A and B). This redistribution was specific to the vimentin network because the distribution of the F-actin (Figure 7, A and C) and microtubule (Figure 7, D and E) networks were not significantly affected by plectin knockdown. Taken together, these data suggest that plectin expression helps to organize vimentin filaments around the nucleus and in the back of the cell but does not significantly affect F-actin or microtubule organization within lobopodial HFFs using the nuclear piston to migrate through 3D CDM.

Because it is known that plectin can bind vimentin and actomyosin networks (Svitkina et al., 1996; Jiu et al., 2015) in response to NMII activity (Figure 4, C and D), both of which are critical to 3D lobopodial migration (Petrie et al., 2014; Petrie et al., 2017), we investigated how plectin expression organizes NMII in cells using the nuclear piston to migrate through 3D CDM. Altogether, these data illustrate that plectin plays an important role in organizing vimentin filaments and polarizing NMII to the anterior cytoplasmic compartment of 3D lobopodial HFFs.

**FIGURE 7:** Plectin organizes cytoskeleton in 3D lobopodial fibroblasts. (A, Top) Immunofluorescence images of vimentin (gray) and DAPI (magenta) show that plectin knockdown causes the vimentin network to collapse and bundle towards to front of 3D fibroblasts, as evidenced by decreased back:front ratio \( n = 27, N = 3 \). * \( p = 0.0003 \). Quantified in (B). (A, Bottom) Immunofluorescence images show that there is no significant difference in the back:front ratio of the F-actin network (green) in control versus plectin knockdown cells \( n = 27, N = 3 \). n.s., \( p = 0.4809 \). Bars = 15 µm. Quantified in (C). (D) Immunofluorescence images show that plectin knockdown does not significantly alter the back:front ratio of microtubules (gray) in fibroblasts in 3D CDM \( N = 27, N = 3 \). n.s., \( p = 0.4378 \). Bars = 15 µm. Quantified in (E). (F) Immunofluorescence images of NMIIA (gray) and DAPI (magenta) show that myosin II is localized more at the cell front in control 3D fibroblasts; however, it is nonpolarized in plectin knockdown 3D fibroblasts \( n = 27, N = 3 \). * \( p = 0.005 \). Bars = 15 µm. Quantified in (G).
expression generate force and transmit it to the nucleus, respectively, to facilitate 3D nuclear movement. Further, we speculate that the similarity of phenotypes between NMII inhibition and plectin knockdown in 2D and 3D environments reflects that plectin-mediated cross-talk between distinct cytoskeletal filaments and the nucleus is an essential component in connecting actomyosin contractility to cell migration and adhesion.

Our data further suggest that plectin plays a unique role in polarizing both vimentin and NMII in 3D lobopodial HFFs. It has been established that lobopodial fibroblasts do not establish polarity through canonical PI3K-Cdc42-based polarization (Haugh et al., 2000; Nalbant et al., 2004; Petrie et al., 2012). Our data show that plectin expression can help to polarize high-pressure lobopodial cells by helping organize vimentin and NMII in the anterior cytoplasmic compartment in front of the nucleus. Interestingly, we find that plectin knockdown does not significantly affect microtubule organization in this context, despite the clear patterning of microtubules by vimentin in other cell types and environments (Gan et al., 2016; Leduc and Etienne-Manneville, 2017; Li et al., 2019; Schaeedel et al., 2021). Based on these results, plectin emerges as a novel candidate protein that may help polarize 3D lobopodial cells.

Last, it is of particular note that plectin has many splice isoforms that can cross-link vimentin to focal adhesions (Burgstaller et al., 2010), the nuclear envelope (Wilhelmsen et al., 2005; Postel et al., 2011; Ketema et al., 2013; Staszewska et al., 2015), and actin (Andra et al., 1998; Sutoh Yoneyama et al., 2014; Jiu et al., 2015). Because we utilized pan-plectin siRNAs in our studies, further work is needed to uncover the specific roles that each of these isoforms play in 3D nuclear translocation. For example, it is possible that plectin 1f, which docks vimentin filaments into focal adhesions (Burgstaller et al., 2010), is upstream of plectin-mediated nuclear YAP translocation whereas plectin 1 or 1c, which cross-links vimentin with actin and the nuclear envelope (Abrahamsberg et al., 2005; Wilhelmsen et al., 2005), plays a direct role in pulling the nucleus forward by linking actomyosin force to the nucleus. Understanding the specific contributions of these individual plectin isoforms will be essential to understanding how cytoskeletal cross-talk enables 2D and 3D migration, as well as the diversity of NMII function in moving cells.

**FIGURE 8:** A pre-assembled, mechanosensitive actin–plectin–vimentin network is required for the nuclear piston mechanism of 3D cell migration. (A) Our data suggest that the assembly of the connections between the actin, plectin, and vimentin networks occurs independent of activation of the nuclear piston mechanism of 3D cell migration. Fibroblasts on soft 2D surfaces (Right) have increased plectin binding to vimentin compared with fibroblasts on surfaces of intermediate stiffness (Left). This preassembled actin–plectin–vimentin network also requires NMII contractility to prevent its disassembly, both on 2D surfaces and in 3D matrices. (B) This preassembled actin–plectin–vimentin network is required for actomyosin contractility to be able to transmit its pulling force to the nucleus to generate compartmentalized pressure via the nuclear piston mechanism of 3D cell movement (Right), as well as low-pressure lamellipodia-based migration through 3D collagen (Left).

**MATERIALS AND METHODS**

Request a protocol through Bio-protocol.

**Reagents and cell culture**

Primary human foreskin fibroblasts (HFFs) were procured from the American Type Culture Collection and were used at passages 5–14. Cells were maintained in DMEM (HyClone) containing 7.5% fetal bovine serum (MilliporeSigma), 100 U/ml penicillin, 100 mg/ml streptomycin, and 4.5 g/L glucose at 37°C and 10% CO₂. The myosin II inhibitor blebbistatin (Thermo Fisher Scientific) was used at 25 µM, the ROCK inhibitor Y-27632 (MilliporeSigma) was used at 10 µM, and the F-actin inhibitor Latrunculin A (Thermo Fisher Scientific) was used at 0.05 µg/ml. Cells were treated with these inhibitors for 1 h at 37°C and 10% CO₂.

**siRNA sequences, cDNA constructs, cell transfection, and electroporation**

Primary HFFs were transiently transfected with the siRNA sequence (Ambion) Silencer Select Plectin siRNA (5’-AGC UCA AGA UCU CCU AAA T-3’) or a control siRNA sequence purchased from Thermo Scientific. A second, independent set of pooled siRNAs were used to confirm the key findings with the single siRNA: plectin (5’-GCA CUC AUC UUG CGU GAC A-3’, 5’-UCG AGG GCC GGU GUC GAA GAA-3’, 5’-GGC AAG ACG GUC ACC AUU U-3’, and 5’-GAA GAG GAC CAG AUC GAC A-3’) ON-TARGET plus SMART pool siRNAs, along with SMART pool control siRNA, was purchased from Dharmacon (now Horizon Discovery). Cells were transiently transfected using Lipofectamine 2000 (Thermo Fischer Scientific) per the manufacturer’s instructions. To make control and plectin siRNA–transfected cells express GFP-NLS for cell migration assays, they were electroporated utilizing an electroporation kit and 4-D-Nucleofector Core Unit electroporator (Lonza) according to the manufacturer’s instructions. These cells were then plated into 35-mm glass-bottomed dishes (WPI) coated with either collagen or CDM.
Plasmids encoding mTurquoise, mNeongreen, mT-10-mNG, and GFP-NLS were described previously (Petrie et al., 2014; Case et al., 2015). All vectors used for fluorescent protein expression were constructed using the C1 or N1 Clontech vector backbone. To generate mTurquoise–actin, the C terminus of mTurquoise was attached to the N-terminus of actin using BspEI and BamHI restriction enzyme sites and separated by an 18–amino acid linker (SGLRSGGGGASGGSG). To generate mNeongreen–vimentin, the C terminus of vimentin was attached to the N terminus of mNeongreen using XhoI and BamHI and separated by a seven–amino acid linker (GDPVVAT).

Antibodies and immunofluorescence imaging
Cells were fixed with either 4% paraformaldehyde (Electron Microscopy Science) or ice-cold 99% methanol (Sigma Millipore). Cells were permeabilized using 0.25% Triton X-100 and blocked with 0.2% BSA solution in 1× PBS. All antibodies were diluted at either 1:250 or 1:500 concentration in 0.2% BSA solution. The antibodies used for immunofluorescence were rabbit anti-plectin (Abcam), mouse anti-vinculin (Sigma Millipore), mouse anti-vimentin (Sigma Millipore), goat anti-mouse 488 (Invitrogen), goat anti-rabbit 488 (Invitrogen), goat anti-rabbit 647 (Invitrogen), Acti-stain 488 phalloidin (Cytoskeleton Inc.), rhodamine phalloidin (Invitrogen), and DAPI (Thermo Scientific). Fixed-cell imaging was done using an LSM 700-Karl Zeiss scanning confocal microscope with a 63×, 1.4 NA oil objective. Analysis of immunofluorescence images was done using Fiji/ImageJ (National Institutes of Health; Schindelin et al., 2012).

For structured illumination microscopy (SIM) imaging, 5 × 10^4 cells were seeded in 35-mm glass-bottomed dishes (World Precision Instrument) with a glass thickness of 170 µm. Cells were labeled with Acti-Stain 488 phalloidin (Cytoskeleton) + rhodamine phalloidin (Thermo Fisher Scientific) or anti-vimentin (see above) + rhodamine phalloidin. All cells were fixed, permeabilized, and stained with DAPI as above. Cells were imaged using a DeltaVision OMX V4 inverted microscope (GE Healthcare) equipped with 60 ×/1.42 NA SIM oil immersion objective lens (Olympus), 405-, 488-, and 568-nm laser lines, and a sCMOS pco.edge camera (PCO). Images were acquired through OMX Master and then reconstructed and aligned using softWoRx software (Version 6.1.1). Co-localization analysis was performed using the FIJI (National Institutes of Health) Coloc. 2 function. The two channels of interest were compared by autothresholding the 488 and 568 channels and then generating the Mander’s correlation coefficient (Singan et al., 2011). This quantifies the percentage of the total signal from the first channel which overlaps with signal from the second channel. The value used was tM2, where actin was the first channel and vimentin was the second. For plectin and vimentin staining, vimentin was the first channel and plectin was the second, and tM2 was also used.

Fluorescence resonance energy transfer imaging and quantification
HFFs were transfected as indicated using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. The next day, FRET images of the live cells were captured and the Fc image was calculated as described below. The binding of mTurquoise–actin and mNeongreen–vimentin was detected by visualizing the FRET-dependent sensitized emission of the mNeongreen acceptor fluorophore in the presence of the mTurquoise donor fluorophore (Case et al., 2015). Optimal FRET acquisition settings were determined by imaging the positive FRET control (T-10-NG; a single construct with mTurquoise directly connected to mNeongreen by a 10–amino acid spacer) and negative FRET control (T + NG; mTurquoise and mNeongreen freely diffusing within the cell) and strictly maintaining the established acquisition settings during all subsequent FRET imaging. The spectral bleed-through ratios were established by imaging cells expressing either the mTurquoise donor or mNeongreen acceptor alone using the established sensitized emission FRET acquisition settings. All FRET images were processed using the LSM FRET Tool macro (Carl Zeiss). The corrected FRET image (Fc) was generated from the raw FRET image as described previously (Petrie et al., 2012). Each channel image was thresholded before processing, and saturated and zero-intensity pixels were excluded from the calculations. Maximum projections of the Fc confocal z-stacks were generated using Zen software. The magnitude of the Fc signal was measured in raw Fc images with the LSM FRET Tool macro (Carl Zeiss).

Cell migration assays
Cells were plated onto 35-mm glass-bottomed dishes (WPI) with either 1.7 mg/ml rat tail collagen (Corning) or CDM, or just onto glass. CDM was grown on 35-mm glass-bottomed dishes (WPI) according to the following instructions (Petrie et al., 2012). Collagen dishes were made by mixing reconstitution buffer, 10× DMEM (Invitrogen), and 10.6 mg/ml rat-tail collagen (BD) at a ratio of 1:1:8. The next day, live-cell imaging was done on either a SpinSR10 spinning disk confocal (Olympus) or a 510 NLO mETA AxioObserver Z1 (Zeiss) in a chamber maintained at 10% CO_2 and 37°C; spinning disk movies were captured using a 60× oil objective and brightfield movies were captured using a 32× objective. Cells were tracked using the manual tracking plugin (F. Cordelieres, Institute Curie, Paris, France) and chemotaxis plugin (Ibidi) on ImageJ (National Institutes of Health).

Measuring intracellular pressure
Intracellular pressure was measured directly using the micropressure measurement technique (Petrie and Koo, 2014). We utilized the 900A micropressure system (WPI) with a 0.5-µm micropipette (WPI) filled with 1-M KCl solution that was calibrated in a chamber with 0.1-M KCl. Once the system was calibrated, the micropipette was injected into cells using an MPC-325 micromanipulator (Sutter Instruments). Cells were visualized using an LSM 700 microscope (Zeiss) and were kept in a chamber where conditions were maintained at 37°C and 10% CO_2. Using the calibrated micropipette, cells were poked at a slight angle and the needle was held in place for 2–5 s and then removed. The pressure readings that were generated during this window of time were averaged as the positive intracellular pressure within the cell.

Proximity ligation assay
From 25,000 to 50,000 cells were seeded on 35-mm glass-bottomed dishes (WPI). For substrate stiffness experiments, Cytosoft Imaging 24-well plates of 0.2 kPa and 64 kPa stiffness (Advanced Biomatrix) were used. The Duolink PLA Kit (Millipore Sigma) was used per manufacturer’s instructions. Specifically, the mouse minus, rabbit plus, and orange detection kit were used together. Cells were initially fixed with ice-cold 99% methanol. After the cells were treated per the PLA protocol, they were imaged within 24 h using an LSM 700 scanning confocal microscope (Carl Zeiss) with a 63×, 1.4 NA objective. Images were analyzed using Fiji through the “Analyze Particles” function or by measuring the raw integrated density of the entire cell, as indicated within the individual figures, and normalized to cell spread area as indicated in the figures.
Western blotting
Cells were lysed using RIPA lysis buffer that was supplemented with 1X protease inhibitor (Roche) and the lysates were collected using cell scrapers. The lysates were mixed with 2X reducing sample buffer (made with 0.125M Tris, 4% SDS, 10% 2-ME, 20% glycerol, and 0.05% bromophenol blue) and heated to 95°C for 5 min on a heating block. These samples were then loaded onto a 4–12% Tris-glycine polyacrylamide gel (Thermo Fisher Scientific) and then transferred to a nitrocellulose membrane (Thermo Fisher Scientific). The primary antibodies used for Western blotting were rabbit anti-plectin (Abcam) and mouse anti-GAPDH (Fitzgerald). The secondary antibodies used were goat anti-rabbit IgG Alexa 680 or 800 and goat anti-mouse 680 or 800 (Thermo Fisher Scientific). All Western blots were imaged using a LI-COR scanner. All blots were analyzed by comparing the intensity of the control GAPDH to the intensity of plectin.

Statistical Methods and illustrations
All statistical analysis was done using Prism 7 (Graphpad Software, Inc.). All data comparing two variables were analyzed using t tests. All data comparing three or more variables was analyzed through one-way analysis of variance with Tukey’s post hoc test. Any differences between variables were considered significant if p < 0.05. All graphical data are represented as mean ± SEM.

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