The dynamic crosstalk between cytoskeletal filaments regulates the cytoplasmic mechanics across the apicobasal axis

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Abstract

The cytoplasm exhibits viscoelastic properties, displaying both solid and liquid-like behavior, and can actively regulate its mechanical attributes. The cytoskeleton is a major regulator among the numerous factors influencing cytoplasmic mechanics. We explore the interdependence of various cytoskeletal filaments and the impact of their density on cytoplasmic viscoelasticity. The heterogeneous distribution of these filaments give rise to polarised mechanical properties of the cytoplasm along the apicobasal axis. Actin filament (F-actin) disassembly softens the basal cytoplasm

while stiffening the mid-cytoplasm due to increased vimentin filament assembly. Disruption of microtubules (MT) or depletion of vimentin softens both the basal and mid-cytoplasm. Cyto D treatment results in localised increase of vimentin assembly in the mid cytoplasm which is dependent on the cytolinker plectin. Nocodazole treatment has a negligible effect on F-actin distribution but significantly alters vimentin's spatial arrangement. We demonstrate that Cyto D treatment upregulates vimentin expression via ROS-mediated activation of NF-κB. This manuscript investigates how different cytoskeletal filaments influence the rheological characteristics of various cytoplasmic regions.

Introduction

The cytoplasm is a gel-like substance enclosed within the cell membrane that plays a critical role in maintaining cellular structure and facilitating various biochemical processes (Fels et al., 2009; Pollard, 1976; Trevors, 2011). It exhibits viscoelastic properties, possessing both viscous and elastic characteristics that enable it to function effectively (Moeendarbary et al., 2013). This unique nature allows the cytoplasm to behave like a fluid under slow deformations, permitting the smooth movement and distribution of organelles and molecules (Hu et al., 2017). Conversely, it acts like an elastic solid when subjected to rapid stress (Munder et al., 2016). This viscoelasticity is crucial for cellular processes such as motility (Bray, 2000), cell division (Xie et al., 2022), and intracellular transport (Guigas et al., 2007), providing the necessary resilience and flexibility. The viscoelastic properties of the cytosol are influenced by several factors, including the composition and organization of the cytoskeleton (Bausch et al., 1998), the concentration of cytoplasmic proteins (Ellis, 2001; Zimmerman & Minton, 1993), and the presence of cellular organelles (Najafi et al., 2023). The cytoskeleton, consisting of actin, microtubules, and intermediate

filaments, provides structural support that enhances the cytoplasm's elasticity and its capacity to withstand mechanical stress (Pegoraro et al., 2017). The density and cross-linking of these filaments directly affect the cytoplasm's mechanical properties (Pegoraro et al., 2017). The density and distribution of cytoskeletal filaments are heterogeneous across the cytoplasm. For example, actin filaments typically form a dense meshwork at the cell periphery, offering structural support and aiding in cell shape maintenance (Svitkina, 2020). In contrast, in the cell interior, actin filaments may organize into stress fibers, facilitating cell contraction and motility (Tojkander et al., 2012). Similarly, microtubule filaments exhibit a dynamic distribution pattern throughout the cytoplasm, extending from the microtubule organizing centre (MTOC) towards the cell periphery (Sanchez & Feldman, 2017; Schuh & Ellenberg, 2007). Vimentin, a type III intermediate filament protein, is essential for maintaining cellular integrity, providing mechanical strength, and supporting cellular structures (Liu et al., 2015). Vimentin filaments form a network that resists mechanical stress (Mendez et al., 2014). These filaments create a dynamic network within the cytoplasm, enveloping the nucleus and extending throughout the cell to provide structural support and organization (Zhang et al., 2021). Studies have shown that de novo assembly of vimentin filaments throughout the cytoplasm significantly contributes to the cytoskeletal architecture (Sarria et al., 1990). These cytoskeletal components can interact either directly or via cytoskeletal cross-linkers such as plectin (Wiche, 1998). The interactions between these filaments create a dynamic, meshwork-like pattern within the cytoplasm, which can reorganise in response to various internal and external stimuli (Hohmann & Dehghani, 2019). The distribution of these cytoskeletal filaments varies across different regions of a cell, leading to significant heterogeneity in the cytoplasmic mechanical properties. Several studies have been conducted on the

mechanical properties of the cytoplasm, but most provide information about the overall mechanical behaviour without offering much detail about the localised viscoelastic properties of different cytoplasmic regions. The rheology of cytoskeletal filaments has been extensively characterised in various in vitro setups; however, their behaviour inside the cytoplasm is not well understood and may differ significantly. Additionally, how the density and crosstalk between the cytoskeletal filaments affect cytoplasmic mechanics across the apicobasal axis remains elusive. This study reports that the density of actin, microtubule, and vimentin filaments is anisotropic along the apicobasal axis of NIH 3T3 fibroblasts. Using particle tracking microrheology (PTM), we demonstrate that the mechanical properties of the cytoplasm along the apicobasal axis are directly influenced by the density of these cytoskeletal filaments. This manuscript establishes the role of individual cytoskeletal filaments in determining the local mechanical properties of the basal and mid-cytoplasm. Furthermore, our findings indicate that crosstalk between cytoskeletal components is crucial for maintaining the mechanical integrity of the cytoplasm. Additionally, we report that the actin-disrupting agent Cyto D enhances the expression of vimentin via ROS-dependent activation of NF-κB.

Results

Mechanical properties of the cytoplasm are polarised along the apicobasal axis

The cytoplasm contains various cytoskeletal components, such as actin microfilaments, microtubules (MT) and intermediate filaments (IF) (Hohmann & Dehghani, 2019). However, their distribution and abundance are not uniform and vary along the apicobasal axis. **Figure 1A** illustrates the distribution of these three major cytoskeletal filaments: actin, microtubules, and vimentin (a type III intermediate filament). This heterogeneous distribution results in locally variable mechanical properties of the cytosol (Fletcher & Mullins, 2010). To evaluate these local mechanical properties, we analysed the trajectories (r(t)) of fluorescently labelled microspheres, each with a diameter of 200 nm, scattered throughout the cytoplasm (**Figure 1B**). The trajectories were derived by monitoring the random, spontaneous movement of the microsphere centroids across 1000 frames over a 20-second duration, corresponding to one frame every 20 milliseconds. These microspheres serve as probes for the local viscoelastic properties of the cytoplasm. The mean squared displacement (MSD) values obtained from the trajectories of these microspheres provide insight into the local viscoelastic properties of the cytoplasm. The time-scale-dependent compliance values derived from MSDs describe the local deformability of the cytoplasm in response to thermal forces ~ kBT/a. Here, kB, T, and a represent Boltzmann's constant, the absolute temperature of the medium, and the radius of the microsphere, respectively. These forces arise from the thermal fluctuations of the probe microspheres (Wirtz, 2009). **Figure 1C** compares MSDs and compliance of the most and least compliant cytoplasmic regions at ten different locations within a given cell. Consistent with previous findings (Tseng et al., 2002), our results suggest that local deformability varies significantly throughout the cytoplasm of a cell. It is presumed that

116 the average density of cytoskeletal filaments varies along the apicobasal axis of the
117 cytoplasm (**Figure 1A**). Therefore, we hypothesised that these variations in
118 cytoskeletal filaments contributed to the polarisation of the locally heterogeneous
119 mechanical properties of the cytoplasm along the apicobasal axis. To resolve the
120 mechanical properties along the apicobasal axis, regions within $0 < z < 2$ microns and
121 $z > 2$ microns are designated as the basal and mid / apical regions of the cytoplasm,
122 respectively (**Figure 1A**). We designated the first sharply focused DIC image of the
123 edge of the cell as the basal plane during particle tracking microrheology (PTM), while
124 when calculating the fractal dimension (FD) values, the basal plane was determined
125 based on the presence of well-focused actin stress fibres (Maninová & Vomastek,
126 2016). To minimise the ambiguity that arises from height variations between different
127 cells, we selected cells that had comparable heights (~4±1μm). A histogram of the cell
128 heights used for analysis is presented in **Figure S1-A**. By analysing the trajectories
129 of at least 60 microspheres embedded in the basal or mid-cytoplasmic regions of at
130 least 10 different cells (**Figure S1-B** and **S1-C**), we determined the mechanical
131 properties of the cytoplasm along the apicobasal axis. **Figure 1D** compares the
132 average MSDs and compliance values of microspheres distributed in the mid and
133 basal cytoplasmic regions of at least 10 different cells. The mean MSDs indicated that
134 the mid cytoplasm demonstrated higher compliance compared to the basal cytoplasm.
135 The frequency-dependent viscoelastic moduli are algebraically derived from these
136 MSD values (Mason, 2000). The dynamic viscosity, η, is calculated by dividing the
137 viscous modulus, G'', by the corresponding angular frequency, ω. Given the frequency
138 dependence of mechanical properties, we analysed the elastic modulus (G') and
139 dynamic viscosity (η) at 1 Hz and 10 Hz frequencies to ensure uniformity in
140 comparisons across varying conditions. The elastic modulus (G') of the mid

141 cytoplasmic region was found to be significantly lower than that of the basal plane at
142 1Hz and 10Hz, indicating a softer cytoplasm at least 4-5 times (**Figure 1F-i, ii**). The
143 dynamic viscosity (η) of the mid cytoplasmic region remained at least 3 times lower
144 than the basal cytoplasmic region at both frequencies (**Figure 1F-iii, iv**). In
145 conclusion, the mechanical properties of the cytoplasm exhibited polarity along the
146 apicobasal axis, leading to a decrease in both stiffness and viscosity with increasing
147 cell height. When an elastic material undergoes deformation, it stores energy that can
148 be recovered upon stress release. However, in viscoelastic materials like cytoplasm,
149 some of this energy is also dissipated due to microstructural rearrangement, which
150 manifests as the viscous flow of the material. This dissipation of energy is indicated by
151 the dimensionless quantity known as the loss tangent, tan δ, which is the ratio between
152 viscous (G'') and elastic modulus (G'). A higher value of tan δ indicates that a greater
153 proportion of energy associated with deformation is dissipated as heat. The tan δ also
154 delineates viscoelastic solids from liquids. At the crossover frequency (ω_c), where the
155 elastic modulus (G'(ω)) intersects with the viscous modulus (G″(ω)), the mechanical
156 properties of the material undergo a transition from a viscoelastic solid (tan δ < 1) to a
157 viscoelastic liquid (tan δ > 1). The reciprocal of ω_c, designated as the relaxation time
158 (Schwarzl, 1971), represents the timescale over which the stored elastic stresses
159 dissipate due to spontaneous microstructural rearrangements. Given that the loss
160 tangent (tan δ) of the basal and mid cytoplasm remained above 1 across the frequency
161 spectrum of 1-10Hz (**Figure 1E**), it can be inferred that the cytoplasm exhibits
162 mechanical characteristics of a viscoelastic liquid. Furthermore, the decay of tan δ with
163 frequency and an apparent plateau near 10 Hz indicate that the relaxation time for
164 microstructural rearrangements of the cytoplasm is slower than 0.1 sec for both the
165 basal and mid regions of the cytoplasm. Next, we examined the relative density of

166 actin (**Figure 1G-i**), microtubule (**Figure 1G-ii**), and vimentin (**Figure 1G-iii**) filaments
167 across the basal ($0 < z < 2\mu m$), mid ($2\mu m < z < 4\mu m$) and apical ($z > 4\mu m$) cytoplasmic
168 regions. To quantify the density of cytoskeletal filaments, we computed their fractal
169 dimension (FD) values. The representative binary images of actin, vimentin, and
170 microtubule filaments across the apicobasal axis used for the FD analysis are
171 presented in **Figure S1-D**. **Figure 1H** compares the FD values of different cytoskeletal
172 filaments across the apicobasal axis. The relative abundance of these filaments varies
173 significantly in the basal cytoplasm; however, in the mid and apical regions, the
174 differences in their densities are negligible. Actin and vimentin filaments were
175 predominantly concentrated in the basal cytoplasm, with their densities gradually
176 decreasing towards the mid and apical regions. In contrast, microtubule (MT) filaments
177 showed relatively higher concentration around the mid cytoplasmic region compared
178 to the basal and apical regions. Based on **Figure 1H**, we deduced that disrupting actin
179 filaments would lead to distinct changes in the mechanical properties of the basal and
180 mid cytoplasm. Specifically, we anticipated that both regions would become softer due
181 to actin disassembly.

182 ***Disruption of actin filaments has contrasting effects on the mechanical***
183 ***properties of the basal and mid cytoplasm.***

184 Next, using cytochalasin D (Cyto D), we investigated the impact of actin filament
185 disruption on the mechanical properties of the basal and mid cytoplasmic regions. Cyto
186 D inhibits the polymerization of actin by binding to its barbed ends (Brown & Spudich,
187 1981; Casella et al., 1981; Cooper, 1987; Masiello et al., 1982). **Figure 2A** compares
188 the mechanical compliance, $\Gamma(t)$ and the MSD, between control and Cyto D treated
189 cells. Disruption of actin filaments by Cyto D reversed the mechanical polarity of the
190 cytoplasm along the apicobasal axis by making the mid cytoplasm less compliant than

the basal cytoplasm (**Figure 2A-i**). Comparison with control cells reveals that Cyto D treatment increased the compliance of the basal cytoplasm significantly (**Figure 2A-ii**), while the mid cytoplasm became slightly stiffer (**Figure 2A-iii**). Considering that the basal cytoplasm contained a higher concentration of actin filaments compared to the mid cytoplasm, we anticipated a significantly larger increase in the compliance of the basal plane than the mid cytoplasm due to actin disassembly. However, contrary to the anticipation we observed that actin disassembly caused a relatively smaller decrease of compliance in the mid cytoplasmic region (**Figure 2A-iii**). As anticipated, the disassembly of actin led to greater fluidization of the basal cytoplasm compared to the mid cytoplasm (**Figure S2-A**). Next, we examined the effect of actin disassembly on the elastic modulus (G’) and dynamic viscosity (η) of the basal and mid cytoplasm. A study by Rotsch & Radmacher (2000) reported that disassembly of actin filaments led to a significant decrease in the cell's elastic modulus, highlighting the critical role of the actin network in maintaining cellular mechanical stability. Similar to it, we found that the median elastic modulus (G') of the basal cytoplasm decreased significantly, by 8-10 fold at both 1 Hz and 10 Hz frequencies, due to the disassembly of actin filaments by cytochalasin D (**Figure 2B-i,ii**). Similarly, the dynamic viscosity (η) of the basal cytoplasm decreased 7-9 fold as a result of Cyto D treatment (**Figure 2B-iii,iv**). However, the effect of actin disruption on the mechanical properties (G' and η) of the mid cytoplasm is contrary to expectations (**Figure 2C**). Cyto D treatment caused a significant increase in both the elastic modulus (G') (**Figure 2C-i,ii**) and the dynamic viscosity (η) (**Figure 2C-iii,iv**) in the mid cytoplasm. To understand the reason behind the stiffening of the mid cytoplasm, we compared the density of actin filaments between control and Cyto D treated cells. Cyto D treatment (2 μM, 1 hour) resulted in the fragmentation of longer actin filaments into smaller segments (**Figure 2E-i**). Cyto

216 D mediated disassembly of actin caused significant loss of actin filaments in the basal,
217 mid and apical cytoplasm (**Figure 2D-i**). Therefore, we hypothesised that the observed
218 elevation in stiffness, as depicted in **Figure 2C**, despite the loss of actin filaments, was
219 due to the increased assembly of other cytoskeletal filaments within the mid
220 cytoplasmic region. The frequency dependence of the mid cytoplasm's loss tangent
221 further indicates that there are additional changes in the microstructure of the
222 cytoplasm other than the loss of actin filaments (**Figure S2-A** and **S2-C**). Next, we
223 explored the impact of actin disassembly on the density of microtubule and vimentin
224 filaments. Unlike actin filaments, Cyto D treatment did not have a significant effect on
225 the density of MT filaments (**Figure 2E-ii**). Analysis of FD values indicate that there
226 was no significant change in the density of MT filaments across the apicobasal axis
227 due to Cyto D treatment (**Figure 2D-ii**). Hence, MT filaments cannot be attributed to
228 the stiffening of the mid cytoplasm following Cyto D-induced disruption of actin
229 filaments. **Figure 1H** shows that the density of vimentin filaments was highest at the
230 basal cytoplasm and decreased gradually at the mid and apical cytoplasmic regions.
231 Treatment with Cyto D altered the distribution of vimentin filaments across the
232 apicobasal axis **(Figure 2D-iii)**. While the density of vimentin filaments showed no
233 significant changes in the basal and apical cytoplasm, the mid cytoplasm exhibited a
234 significant increase in the density of vimentin filaments in response to Cyto D
235 treatment. Confocal images clearly show the increased assembly of vimentin filaments
236 in the mid cytoplasm (**Figure 2E-iii**) of Cyto D treated cells. These observations
237 aligned with the observed increase in the elastic modulus (G') values of the mid
238 cytoplasm of Cyto D treated cells (**Figure 2C-i, ii**). Hence, we hypothesised that the
239 elevated assembly of vimentin filaments was responsible for the increased stiffness of
240 the mid cytoplasm in Cyto D treated cells.

Nocodazole-induced depolymerization of microtubules results in significant softening of the cytoplasm across the apicobasal axis.

Microtubules, like actin filaments, form intricate networks within the cytoplasm; however, their spatial arrangement significantly differs from that of actin filaments along the apicobasal axis (**Figure 1G-ii**, **Figure 1H**). Consequently, microtubule disassembly is expected to affect the mechanical properties of the cytoplasm differently from the disruption of actin filaments. We investigated the impact of microtubule disassembly on the mechanical properties of the basal and mid cytoplasmic regions. We used nocodazole to disrupt microtubules, which acts by interfering with their polymerisation (Xu et al., 2002). The effect of microtubule disassembly on the compliance of these cytoplasmic regions differed notably from that of actin filament perturbation. Unlike Cyto D treatment, the nocodazole treated cells retained the viscoelastic polarity along the apicobasal axis. The mid cytoplasm of nocodazole treated cells exhibited higher compliance compared to the basal cytoplasm (**Figure 3A-i**). A comparison between **Figure 3A-i** and **Figure 1D** demonstrated that the difference in compliance along the apicobasal axis increased by approximately 7-fold at 1 Hz and 9-fold at 10 Hz due to microtubule disassembly (**Figure S3-A**). The disassembly of microtubules led to a significant increase in compliance in both the basal (**Figure 3A-ii**) and mid cytoplasmic (**Figure 3A-iii**) regions compared to their counterparts in control cells. According to our observations, microtubule disruption had a pronounced effect on the compliance of the mid-cytoplasm compared to the basal cytoplasm. A 4 to 7 fold greater increase in compliance in the mid cytoplasm (**Figure S3-B**) further validated the higher density of microtubules within the mid cytoplasmic region compared to the basal region, as shown in **Figure 1H**. Interestingly the disassembly of microtubule makes the viscosity

266 and G' of the cytoplasm more polarised along the apicobasal axis (**Figure S3-E**). In
267 the mid cytoplasm, the disassembly of microtubules caused an intriguing change in its
268 mechanical properties. The mid-cytoplasm became more fluid-like at 10 Hz, while it
269 exhibited more gel-like characteristics at lower frequencies (**Figure S3-C**). The
270 frequency dependence of the mid-cytoplasm's loss tangent further indicated that there
271 were additional changes in the cytoplasm's microstructure other than the loss of
272 microtubule (**Figure S3-D**). Next, we probed the effect of Nocodazole treatment on
273 the other cytoskeletal filaments. Nocodazole treatment had an insignificant effect on
274 the FD of the actin filaments (**Figure 3D**, **Figure 3E-i**). As expected nocodazole
275 treatment (10uM, 1hr) severely altered the MT organization throughout the apicobasal
276 axis by reducing the density of the MT filaments (**Figure 3D-ii**, **Figure 3E-ii**). The
277 global loss of MT filaments upon nocodazole treatment justified the reduction in
278 cytoplasmic stiffness along the entire apicobasal axis. The MT disassembly had a
279 pronounced impact on the vimentin filament network (**Figure 3-Diii, Figure 3E-iii**). We
280 observed a significant reduction of vimentin filaments across the cytoplasm (**Figure**
281 **3D-iii**). Most of the vimentin filaments were aggregated as thick strands localised in
282 the perinuclear region (**Figure 3D-iii**). Nocodazole treatment altered the relative
283 spatial distribution of vimentin filaments within the cell cytoplasm. In control cells, we
284 observed a decreasing vimentin filament density along the apicobasal axis (**Figure**
285 1H). Conversely, in nocodazole-treated cells, the vimentin filament density increased
286 along the apicobasal axis (**Figure 3D-iii**). It is difficult to separate the contribution of
287 individual cytoskeletal filaments in governing the mechanical behaviour of the
288 cytoplasm. The basal cytoplasm of the nocodazole treated cells provides ideal system
289 to explore the mechanical characteristics of actin filaments in cells. The global loss of

290 vimentin filaments upon MT disassembly further contributed to the softening of the
291 basal and mid cytoplasmic regions.

292 ***Localised vimentin assembly stiffens mid cytoplasm in cytochalasin D treated***
293 ***cells***

294 The disruption of F-actin resulted in the rise of cytoplasmic stiffness in the mid
295 cytoplasmic region, with a concurrent increase in the density of vimentin filaments
296 (**Figure 2A-i** and **Figure 2D-ii**). Therefore, we hypothesised that the elevated levels
297 of vimentin filaments were responsible for the stiffening of the mid cytoplasm. To test
298 this hypothesis, we knocked out vimentin using CRISPR-Cas9 and investigated the
299 effect of Cyto D treatment on the mechanical properties of the mid cytoplasm in
300 vimentin knockout ($Vim^{-/-}$) cells. **Figure S4-A** validates the knockdown of vimentin.
301 The $Vim^{-/-}$ cells maintained the viscoelastic polarity of the cytoplasm along the
302 apicobasal axis (**Figure 4A-i**). A comparison of **Figure 4A-i** and **Figure 1D**
303 demonstrates that the polarity of compliance along the apicobasal axis slightly
304 decreased at 1 Hz and increased at 10 Hz due to the reduction of vimentin filaments
305 (**Figure S4-B**). The knockout of vimentin filaments made the basal and mid cytoplasm
306 more compliant than the control cells (**Figure 4A-ii, iii**). Further, we observed a larger
307 softening of the basal cytoplasm (75%) than the mid-cytoplasm (52%) at 1Hz due to
308 vimentin knockout (**Figure S4-C**). However, at 10Hz, mid-cytoplasm had a larger
309 softening than the basal cytoplasm (**Figure S4-C**). Cyto D treatment of $Vim^{-/-}$ cells
310 increased the compliance of the basal cytoplasm by an additional 5-fold at 1 Hz and
311 4-fold at 10 Hz (**Figure S4-D**). In contrast to the behaviour observed in wild-type cells,
312 $Vim^{-/-}$ cells did not exhibit reduced compliance or increased stiffness in the mid-
313 cytoplasm when subjected to Cyto D treatment. This established the role of vimentin
314 in the stiffening of the mid-cytoplasm upon Cyto D treatment as depicted in **Figure 2C**.

315 As anticipated, vimentin knockout caused a larger G' reduction in the basal (55%)
316 cytoplasm than in the mid (11%) cytoplasm (**Figure 4B**, **Figure 4C** and **Figure S4-E**).
317 The knockout of vimentin increased the polarity of G' and η along the apicobasal axis
318 (**Figure S4-F**). Additionally, vimentin depletion increased the fluidity of the mid-
319 cytoplasm at 1Hz (**Figure S4-G**). At 1 Hz, Cyto D treatment to Vim$^{-/-}$ cells reduced the
320 loss tangent (tan δ) of both the basal and mid cytoplasm. However, at 10 Hz, Cyto D
321 treatment increased the tan δ of the basal cytoplasm and had a negligible impact on
322 the tan δ of the mid cytoplasm. (**Figure S4-H**). Next, we investigated whether the
323 knockout of vimentin affected the spatial arrangement of actin and microtubule
324 filaments. The depletion of vimentin had an insignificant effect on the fractal dimension
325 (FD) of the actin filament and microtubule networks (**Figure 4D**). **Figure 4E** compares
326 the immunofluorescence images of the F-actin and microtubules at the basal, mid, and
327 apical cytoplasm in wild-type (WT) cells with Vim$^{-/-}$ cells. We observed no noticeable
328 changes in the spatial organisation of actin and microtubule filaments due to vimentin
329 knockout. The FD analysis (**Figure 4D**) supported this observation, suggesting that
330 the knockout of vimentin had an insignificant effect on actin and microtubule filament
331 networks in both the basal and mid-cytoplasm. Therefore, the altered mechanical
332 properties of Vim$^{-/-}$ cells can be attributed to the lack of vimentin filaments.

333 ***Plectin directs the localised assembly of vimentin filaments in the mid-***
334 ***cytoplasm of cytochalasin D treated cells***

335 The localised increase of vimentin filaments in Cyto D treated cells (**Figure 2D-iii** and
336 **2E-iii**) suggested that the formation of new filaments is controlled spatially within the
337 cytoplasm. To gain mechanistic insight into the localised growth of vimentin filaments
338 in the mid-cytoplasm, we investigated whether the existing vimentin molecules within
339 the filaments spatially reorganize or if the overexpression of vimentin in Cyto D-treated

340 cells forms new filaments localised in the mid-cytoplasm. While the levels of actin
341 (**Figure 5A**) and tubulin (**Figure 5B**) proteins were unaffected by Cyto D treatment,
342 the expression levels of vimentin showed a significant increase (**Figure 5C**).
343 Additionally, we observed a significant increase in the levels of vimentin mRNA due to
344 Cyto D treatment (**Figure 5D**). This indicates that the disassembly of actin by Cyto D
345 triggered the synthesis of additional vimentin proteins. Further, the newly formed
346 vimentin molecules preferentially assembled into filaments in the mid cytoplasm. We
347 speculated that crosslinkers such as plectin, which enable mechanosensitive
348 crosslinking of vimentin with F-actin (Marks et al., 2022) and microtubules (Svitkina et
349 al., 1996) in cells may control the localised growth of new vimentin filaments. To
350 validate our assumption, we depleted plectin using plectin siRNA (**Figure 5E**) and
351 investigated the effect of Cyto D treatment on the assembly of vimentin filaments in
352 transiently plectin-depleted cells. Treatment of Cyto D increased the levels of vimentin
353 protein (**Figure 5F**) and the mRNA (**Figure 5G**) in plectin-depleted cells. Unlike control
354 cells, the treatment of Cyto D led to an increase of vimentin density in both the basal
355 and mid cytoplasmic regions of plectin deficient cells (**Figure 5I and S5-D**). This
356 established the role of plectin in facilitating the localised assembly of vimentin in the
357 mid-cytoplasm after Cyto D treatment. Interestingly, we observed an increase in the
358 density of actin filaments in the form of stress fibers in the basal cytoplasm of plectin-
359 deficient cells (**Figure 5H and S5-D**). However, there was no observable change in
360 the total protein level of actin in plectin-depleted cells (**Figure S5-A**). This suggests
361 that the increase in actin filaments in the basal cytoplasm of plectin-depleted cells was
362 orchestrated by the spatial reorganisation of filamentous actin rather than an overall
363 increase in the level of total actin. Hence, we conclude that plectin helps maintain the
364 relative abundance of actin filaments and is essential for the localised increase in the

365 density of vimentin filaments in the mid-cytoplasmic region of Cyto D-treated cells. The
366 reorganisation of actin and vimentin filaments in plectin-depleted cells resulted in a
367 more gel-like consistency of the basal and mid cytoplasm at higher frequencies
368 (**Figure S5-B** and **S5-C**).

369 ***Cyto D induced assembly of vimentin is dependent on ROS-mediated activation***
370 ***of NF-κB pathway***

371 We observed that the disassembly of actin filaments by Cyto D caused an
372 overexpression of vimentin via transcriptional upregulation (**Figure 5D**). The newly
373 synthesized vimentin then assembled into filaments in the mid-cytoplasm (**Figure 2D-**
374 **iii**). The treatment of Cyto D produces ROS (Kustermans et al., 2005), which is known
375 to activate the transcription factor NF-κB (Morgan & Liu, 2011). Vimentin is a well
376 reported target of NF-κB (Marsigliante et al., 2016; Zhou et al., 2019). Therefore, we
377 speculated that Cyto D treatment activated NF-κB in NIH 3T3 cells through the
378 generation of ROS, subsequently triggering the transcription of vimentin. Additionally,
379 it remained unclear whether the elevation of ROS alone was sufficient or if the
380 disassembly of actin filaments was also required to trigger the transcription and
381 assembly of vimentin. Using the transient expression of p65-EGFP in NIH 3T3 cells,
382 we established that Cyto D treatment activated NF-κB within 15 minutes (**Figure 6A-i**
383 and **6B-i**). We used an analogue of resveratrol, widely used as an NF-κB inhibitor, to
384 establish that Cyto D activated NF-κB via the canonical pathway (**Figure 6A-ii**).
385 Comparing the nuclear fraction of p65 in control cells with Cyto D-treated cells (**Figure**
386 **6C**) established that the Cyto D treatment activated the nuclear translocation of
387 endogenous p65. Further, in the presence of the NF-κB inhibitor, Cyto D treatment
388 failed to activate the transcription of vimentin (**Figure 6D-i**). This established that Cyto
389 D activated the transcription of vimentin through NF-κB. The negligible change in the

density of vimentin along the apicobasal axis post Cyto D treatment in cells pre-treated with the NF-κB inhibitor (**Figure 6E**, **Figure S6-B**) suggests that the increased filaments in the mid-cytoplasm arose from newly translated vimentin. Next, we explored whether Cyto D treatment led to an increase in the levels of intracellular ROS and further, whether the ROS was necessary to activate NF-κB. As expected, Cyto D treatment increased the intracellular ROS in NIH 3T3 cells (**Figure 6F**), which could be prevented by pre-treatment with vitamin C. Treatment of vitamin C alone neither altered the levels of intracellular ROS (**Figure 6F**) nor did it activate nuclear translocation of NF-κB (**Figure 6G-i and G-ii**). Vitamin C had a negligible effect on the density of vimentin filaments (Figure **S6-C**). Following that, we examined whether treatment of Cyto D in vitamin C pre-treated cells affected the transcription of vimentin. Despite the disassembly of actin filaments, the presence of vitamin C prevented Cyto D-mediated elevated expression of vimentin (**Figure 6H**). As expected, the presence of vitamin C prevented Cyto D-mediated increased assembly of vimentin filaments in the mid-cytoplasm (**Figure 6I**). This suggests that generation of ROS following the disruption of actin filaments is essential for the upregulation of vimentin in Cyto D treated cells. Interestingly, the elevation of ROS with H_2O_2 treatment led to a transcriptional (**Figure 6J**), as well as a translational (**Figure S6-G**) upregulation of vimentin (**Figure 6J**) and elevated density of the vimentin filaments across the apicobasal axis of cytoplasm (**Figure 6K and Figure S6-H**).

414

415 **Discussion**

416 The components of the eukaryotic cytoskeleton, including actin, microtubules, and
417 intermediate filaments, are interconnected, forming a complex meshwork of
418 biopolymers (Hohmann & Dehghani, 2019). This cytoskeletal meshwork is one of the
419 major regulators of the cytoplasmic mechanics (Fletcher & Mullins, 2010). The extent
420 to which the assembly and distribution of different cytoskeletal components are
421 interdependent, and their impact on the mechanical properties of the cytoplasm, is
422 incompletely understood and requires further investigation. Vimentin can control the
423 organisation of cortical actin in mitotic cells (Serres et al., 2020). Another study also
424 emphasizes the importance of the interaction between vimentin filaments and cortical
425 actin for normal cell division (Duarte et al., 2019). In our study, we observed that the
426 organisation of actin and microtubule filaments in interphase fibroblasts remained
427 unaffected by the knockout of vimentin (**Figure 4D** and **4E**). While the disassembly of
428 actin filaments by Cyto D did not affect the density of microtubules, it resulted in an
429 increased density of vimentin filaments in the mid-cytoplasm (**Figure 2D**), mediated
430 by ROS-dependent activation of NF-κB. Cyto D treatment of cells pre-treated with
431 either an NF-κB inhibitor (**Figure 6E-ii**) or a ROS quencher (**Figure 6I-ii**) prevented
432 the increase in vimentin filaments in the mid-cytoplasm. This suggests that the
433 disassembly of actin filaments alone does not trigger the assembly of vimentin
434 filaments and requires ROS as an essential component for the activation of NF-kB.
435 Nocodazole-induced disruption of microtubules did not affect the density of F-actin
436 (**Figure 3D** and **3E-i**). However, intriguingly, nocodazole treatment reduced the density
437 of vimentin filaments along the apicobasal axis (**Figure 3D** and **3E-iii**). Several reports
438 suggest the importance of microtubules in the proper assembly and dynamics of

439 vimentin filaments (Hookway et al., 2015; Prahlad et al., 1998). Therefore, we
440 conclude that nocodazole-induced microtubule disruption impairs the proper assembly
441 of vimentin filaments in NIH 3T3 fibroblasts. In U2OS cells, depletion of vimentin leads
442 to an increase in stress fibers in the basal cytoplasm, along with a mild increase in
443 actin monomers (Jiu et al., 2017). In contrast, depletion of vimentin in NIH 3T3
444 fibroblasts had a negligible effect on the stress fibres (**Figure 4D** and **4E-i**). This
445 suggests that the interdependence of the relative distribution of different cytoskeletal
446 components is cell type-dependent. Disassembly of actin by Cyto D caused a localised
447 increase in vimentin filaments in the mid-cytoplasm (**Figure 2D** and **2E-iii**). We show
448 that the localised assembly of vimentin in Cyto D-treated cells is dependent on the
449 cytoskeletal crosslinker protein plectin (**Figure 5I** and **S5-D**), suggesting that plectin
450 regulates the relative abundance of different cytoskeletal components in NIH 3T3
451 fibroblasts.

452 The rheological properties of the cytoplasm, specifically its stiffness (G') and fluidity
453 (η), are crucial for understanding cellular mechanics. In this manuscript, we investigate
454 the polarity of these rheological properties along the apicobasal axis. Cells situated
455 within a physically anisotropic extracellular microenvironment may exhibit distinct
456 behaviors in their basal and apical regions. By examining the polarity of cytoplasmic
457 stiffness and viscosity, we gain insights into how the mechanical properties of the
458 cytoplasm influence cellular sensing and respond to extracellular mechanical cues in
459 a heterogeneous 2D microenvironment. Furthermore, polarity in cytoplasmic stiffness
460 is expected to influence the organization and migration of cells within tissues. Previous
461 research has shown that fibroblast cells exhibit variable cytoplasmic stiffness at the
462 leading and trailing edges during migration (Ford & Rajagopalan, 2018). Cortical F-
463 actin is reported to enhance the stiffness of the cell cortex (Patteson et al., 2019). The

464 distribution of actin, vimentin, and microtubules in different regions of the cytoplasm is
465 inhomogeneous at macroscopic scales (**Figure 1H**). Thus, we speculate that the
466 rheological properties of different cytoplasmic regions are differentially affected by
467 various cytoskeletal filaments. Our study demonstrates that the anisotropic
468 organisation of these cytoskeletal filaments results in polarised cytoplasmic stiffness
469 and viscosity along the apicobasal axis in fibroblast cells cultured on glass substrate.
470 The significantly higher relative abundance of actin and vimentin filaments in the basal
471 cytoplasm (**Figure 1H**) suggests that these filaments likely govern the rheological
472 properties of the basal cytoplasm. Cyto D treatment reduced the stiffness (**Figure 2B**)
473 and increased the fluidity of the basal cytoplasm (**Figure S2A**) without altering the
474 abundance of microtubules and vimentin (**Figure 2D**). Interestingly, depletion of
475 vimentin (**Figure S4-A**) did not affect the abundance of actin or microtubule filaments
476 in the basal cytoplasm (**Figure 4D**). Additionally, treatment with nocodazole, which
477 disrupted both vimentin and microtubules in the basal cytoplasm (**Figure 3D**),
478 significantly decreased the elastic modulus, G' of the basal cytoplasm. This confirms
479 that actin and vimentin filaments jointly dictate the rheological properties of the basal
480 cytoplasm.

481 Longer actin filaments are absent in the mid-cytoplasm (**Figure 1G-i**). While we
482 anticipate a substantial decrease in the density of actin filaments in the mid-cytoplasm
483 compared to the basal cytoplasm in control cells, the FD of actin in the mid-cytoplasm
484 (1.58) shows only a slight reduction compared to that in the basal cytoplasm (1.72).
485 This smaller decrease in FD (**Figure 1H**) can be attributed to the presence of denser
486 actin filaments in the cell cortex. Consequently, the FD values do not accurately reflect
487 the actual density of F-actin in the perinuclear mid-cytoplasmic region. Therefore, we
488 expect the rheological properties of the mid-cytoplasm to be less dependent on actin

filaments and more influenced by vimentin and microtubules due to the absence of longer actin filaments. However, in Vim$^{-/-}$ cells, Cyto D treatment altered the rheological properties of the mid-cytoplasm without affecting microtubules (**Figure 4B** and **4C**). We speculate that these changes in the rheological properties of the mid-cytoplasm in Vim$^{-/-}$ cells following Cyto D treatment are due to alterations in the packing fraction of organelles (Gurel et al., 2014).

In cytoplasm where molecular motion of the cytoskeletal network is constrained by strong crosslinking and high filler content, a decrease in loss tangent with increasing frequency is expected, indicating reduced energy dissipation at higher frequencies. In contrast, an increase in loss tangent with frequency suggests a complex, time-dependent relaxation process, where energy dissipation mechanisms become more active at shorter time scales (higher frequencies). We infer that this behavior reflects the entanglement and weak crosslinking of cytoskeletal filaments. Since nocodazole treatment results in the loss of microtubules and a significant reduction in vimentin filaments while maintaining the density of actin filaments, we assume that the mechanical properties of the basal cytoplasm in nocodazole-treated cells are primarily governed by the actin filament network. The loss tangent of the basal cytoplasm in these cells resembles that of control cells, supporting our assumption. However, the loss tangent exhibits significantly different characteristics in the mid-cytoplasm (**Figure S3-C**). Therefore, we speculate that in nocodazole-treated cells, the mechanical properties of the mid-cytoplasm are not predominantly determined by cytoskeletal filaments but rather by the distribution of organelles. The comparable increase in compliance of the basal cytoplasm in control cells, as well as in vim$^{-/-}$ cells following Cyto D treatment (**Figure S4-D**), suggests that vimentin and actin filaments independently govern the mechanical characteristics of the basal cytoplasm.

515 **Materials and methods**

516 ***Cell culture***

517 NIH 3T3 fibroblast cells were obtained from NCCS, Pune, India. The cells were
518 cultured in DMEM media (Himedia, #AL007G) supplemented with 10% FBS (Himedia,
519 #RM10434) and 5% penicillin-streptomycin (Himedia, A001A) antibiotic cocktail
520 solution. The cells were consistently maintained at 37°C and 5% CO_2 inside a standard
521 cell culture incubator. Throughout the experiments, the cells were continuously
522 screened for mycoplasma contamination. For imaging purposes, cells were plated on
523 confocal dishes coated with poly-L-lysine (Sigma, #P8920) for maximum adherence,
524 ensuring that cell density never exceeded 60% confluency.

525 ***Drug treatment***

526 Cytochalasin D (Sigma, #C8273) was used at a concentration of 2 μM for 1 hour.
527 Nocodazole (Merck, #487928) was used at a concentration of 10 μM for 1 hour. NF-
528 κB inhibitor (CAY10512) was used at a concentration of 250 μM, 1 hour prior to the
529 addition of cytochalasin D. Vitamin C (Merck, A92902) was used at a concentration of
530 200 μM for 1 hour before treating with cytochalasin D. Cells were incubated with two
531 different concentrations (100 and 200 μM) of hydrogen peroxide (Merck, 107209)
532 solution for 15 minutes. Thereafter, the H_2O_2-containing media was replaced with
533 DMEM and the cells were further incubated for 40-60 minutes before carrying out
534 experiments.

535 ***Transfection***

536 The EGFP-p65 plasmid (Addgene plasmid #111190) was a gift from Johannes A.
537 Schmid (Schmid et al., 2000). Transfection was performed with FuGENE HD
538 (Promega, #E2311) transfection reagent according to the manufacturer's instructions.

539 Cells were kept in serum-free DMEM for 12 hours prior to transfection. On the day of
540 transfection, the confluency of the cells was around 60-80%.

541 ***Knockdown of plectin***

542 Knockdown of plectin was performed using predesigned Dicer-substrate siRNA
543 (DsiRNA) from IDT (# mm.Ri.Plec.13.1). IDT negative control DsiRNA (# 51-01-14-03)
544 was used as a control siRNA. siRNAs were transfected into cells using FuGENE HD
545 (Promega, #E2311) transfection reagent according to the manufacturer's instructions.
546 Cells were incubated in siRNA-added media for a minimum of 72 hours to ensure
547 efficient depletion of plectin. The silencing of plectin was validated by western blot
548 analysis.

549 ***Knockout of vimentin using CRISPR-Cas9***

550 The vimentin knockout ($Vim^{-/-}$) NIH 3T3 cell line was generated using the CRISPR-
551 Cas9 technique. The guidelines for performing CRISPR-Cas9-mediated knockout
552 were adopted from the work published by Ran et al., 2013.(Ran et al., 2013). The
553 guide RNAs for vimentin were designed using the web-based CHOPCHOP tool
554 (Montague et al., 2014). The following oligos were used to construct the dual-stranded
555 guide RNA (gRNA) for vimentin:

Top strand (5'-3')	**CACC**GCGGAGCCACCGAACATCCTG
Bottom strand (5'-3')	**AAAC**CAGGATGTTCGGTGGCTCCGC

556

557 The bold letters denote the 5' overhangs required for ligation into the Cas9 vector
558 plasmid. The remaining 21 nucleotides of the top strand constitute the actual sequence
559 of the gRNA: 5'-GCGGAGCCACCGAACATCCTG-3'. The top and bottom strands

560 were annealed together to create the dual-stranded gRNA, which was then cloned into
561 the pSpCas9(BB)-2A-Puro V2.0 vector plasmid (Addgene ID #62988). The 5'
562 overhangs of these oligos were phosphorylated using T4 Polynucleotide Kinase
563 (Thermo Scientific, #EK0031) to facilitate efficient ligation. The annealing of these two
564 oligos was performed in a thermocycler, gradually reducing the temperature from 95°C
565 to 25°C at a rate of 5°C per minute. Next, the gRNA construct was cloned into the
566 pSpCas9(BB)-2A-Puro V2.0 vector for co-expression with Cas9. Prior to ligation, the
567 pSpCas9(BB)-2A-Puro V2.0 vector was digested with BpiI restriction endonuclease
568 enzyme (Thermo Scientific, #ER1011) at 37°C for 1 hour. There are two BpiI restriction
569 sites in the pSpCas9(BB)-2A-Puro V2.0 vector. After digestion, a fragment between
570 these two sites is excised, and the gRNA construct is inserted and ligated into the
571 vector between these two sites. The ligation reaction was performed by incubating the
572 gRNA insert and the pSpCas9(BB)-2A-Puro V2.0 vector at a ratio of 7:1 in the
573 presence of T7 DNA ligase (New England Biolabs, # M0318S) enzyme at 25°C for 1
574 hour. After successful cloning of the gRNA into the pSpCas9(BB)-2A-Puro V2.0 vector,
575 the entire construct was introduced into DH5α strain of competent bacteria using the
576 standard bacterial transformation protocol. Following transformation, the bacteria were
577 plated on Luria agar plates containing ampicillin. Bacteria which were successfully
578 transformed with pSpCas9(BB)-2A-Puro V2.0 become resistant to ampicillin and gave
579 rise to individual colonies. A few of these colonies were picked for colony PCR to
580 confirm the presence of the cloned pSpCas9(BB)-2A-Puro V2.0 vector. Details of the
581 primers used in the colony PCR is given below,

Forward primer (5'-3') (U6 forward primer)	GAGGGCCTATTTCCCATGATT

Reverse primer (5'-3') (Bottom strand of the gRNA construct)	AAACCAGGATGTTCGGTGGCTCCGC

582

583 Following colony PCR, positive colonies were then plated onto another ampicillin-
584 containing agar plate. One or two colonies from this plate were then picked up and
585 grown in a liquid Luria broth culture medium at 37°C for overnight. The cloned gRNA
586 + pSpCas9(BB)-2A-Puro V2.0 constructs were then isolated from the bacterial culture
587 using a plasmid isolation kit (Macherey-Nagel, #740588.50). Following isolation, the
588 cloned constructs were transfected into NIH 3T3 cells using FuGENE HD (Promega,
589 #E2311) transfection reagent. pSpCas9(BB)-2A-Puro V2.0 plasmid contains
590 puromycin resistance gene. Next, the transfected cells were subjected to puromycin
591 (Sigma, #P8833) antibiotic treatment to isolate puromycin-resistant single-cell
592 colonies. These colonies were then screened for the presence of vimentin using
593 immunostaining and dot blot methods. Colonies with no vimentin expression were
594 isolated and subsequently used in the later experiments.

595 ***Western blotting***

596 For Western blot experiments, protein lysate of each sample was prepared from
597 approximately 3.2×10^6 cells seeded on a 60 mm cell culture dish. Cells were washed
598 three times with ice-cold PBS, then scraped and lysed in RIPA lysis buffer solution
599 supplemented with 1X protease and phosphatase inhibitor cocktail (Merck, PPC1010).
600 Protein concentrations were quantified using Bradford reagent. Blocking was
601 performed using 5% BSA solution (1 hour at room temperature) and the washes were
602 done using TBST buffer (Tris-buffered saline with 0.1% Tween 20). The antibodies
603 were diluted in 5% BSA solution. The following primary antibodies were used: vimentin

604 rabbit monoclonal antibody (dilution 1:1000; CST, #5741), β-tubulin rabbit polyclonal
605 antibody (dilution 1:1000, Abclonal, #AC008), β-actin mouse monoclonal antibody
606 (dilution 1:1000, SCBT, #sc-47778), GAPDH mouse monoclonal antibody (dilution
607 1:2000, Abclonal, #AC002), NF-kB p65 (L8F6) mouse monoclonal antibody (dilution
608 1:1000; CST, #6956), plectin mouse monoclonal antibody (dilution 1:500; SCBT, #sc-
609 33469), histone H3 rabbit polyclonal antibody (dilution 1:1000; Invitrogen, # PA5-
610 16183). The following horseradish peroxidase (HRP)-tagged secondary antibodies
611 were used: anti-mouse secondary antibody (dilution 1:10000; Invitrogen, #31430) and
612 anti-rabbit secondary antibody (dilution 1:10000; Invitrogen, #31460).
613 Chemiluminescent signals from were developed by incubating the blots in a substrate
614 solution containing luminol, coumaric acid, and H_2O_2, prepared in 1.5M Tris-HCl buffer
615 (pH 8.8), for 30-40 seconds. The band intensities were quantified using Fiji. The
616 intensity ratios of the total protein to the loading control GAPDH were calculated.

617 ***Protein isolation from nuclear and cytoplasmic fractions***

618 Cells were harvested using a scraper after three washes with ice-cold PBS. The cell
619 pellet was then resuspended in approximately 500 μl of cytoplasmic extraction buffer,
620 supplemented with 0.05% NP-40 detergent. The samples were vortexed frequently to
621 separate the nuclear fraction from the cytoplasmic fraction. Subsequently, the samples
622 were centrifuged at 10,000 × g and 4°C for 15 minutes, and the supernatant,
623 containing the cytoplasmic fraction, was collected. The remaining pellet was
624 resuspended in 400 μl of nuclear extraction buffer and vortexed every few minutes for
625 40 minutes to lyse the nuclear membrane. Finally, the samples were centrifuged at
626 15,000 × g and 4°C for 15 minutes, and the supernatant, containing the nuclear protein
627 fraction, was collected.

628 ***Cell fixation and immunostaining***

629 For immunostaining, cells were seeded on glass-bottom confocal dishes coated with
630 either poly-L-lysine or fibronectin solution for optimal adhesion. The confluency of the
631 seeded cells was kept below 70%. Following various drug treatments, cells were fixed
632 in 4% PFA solution and permeabilized using 0.1% Triton X-100 in PBS. Blocking was
633 performed using 2% BSA solution in PBS for 1 hour at room temperature. Cells were
634 incubated with the following primary antibody solutions for 16 hours at 4°C: vimentin
635 anti-rabbit monoclonal antibody (dilution 1:100; Cell Signalling Technology, #5741) and
636 β-tubulin rabbit polyclonal antibody (dilution 1:200; Abclonal, #AC008). Alexa Fluor
637 488 and 594-tagged anti-rabbit secondary antibodies (CST) were used at a 1:500
638 dilution. F-actin was visualised using Alexa Fluor 488 conjugated phalloidin (dilution
639 1:200, Invitrogen, # A12379).

640 qPCR analysis

641 The total RNA from NIH 3T3 cells was extracted using RNeasy mini kit (Qiagen,
642 #74104). cDNA was synthesised from the RNA samples using Verso cDNA synthesis
643 kit (Thermo Scientific, #AB1453A). qPCR was performed using Genious 2X SYBR
644 Green Fast qPCR Mix (Abclonal, #RK21204) in an Applied Biosystems 7500 Fast
645 Real-Time PCR system. The sequences of the primers used for the qPCR are
646 provided in the table below,

Vimentin	Forward (5'-3')	ATGCGGCTGCGAGAGAAATTG
	Reverse (5'-3')	TCAAGACGTGCCAGAGAAGCA
GAPDH	Forward (5'-3')	CAGGAGAGTGTTTCCTCGTCC
	Reverse (5'-3')	ATGAAGGGGTCGTTGATGGC

647

648 GAPDH was used as the housekeeping gene to normalise the expression levels of
649 vimentin.

650 ***Measurement of ROS***

651 Intracellular ROS levels under various conditions were quantified using DCFDA
652 (Merck) solution. Cells were seeded on a 96-well plate at a density of approximately
653 25000 cells per well and allowed to adhere properly for overnight. Thereafter, the cells
654 in each well were incubated with 100μL of 20 μM DCFDA solution prepared in serum-
655 free DMEM media for 1 hour. Next, the DCFDA solution was washed out, and the cells
656 were treated with the desired drug solutions (prepared in DMEM) for the appropriate
657 duration. Subsequently, the fluorescence intensity of DCFDA was measured using a
658 fluorescence plate reader (Ex/Em = 485/535 nm). The fluorescent intensity correlates
659 with ROS levels.

660 ***Microscopy and image analysis***

661 Immunofluorescent images of actin, vimentin, and microtubule filaments were
662 captured using a Zeiss LSM 880 confocal microscope with a 63X (NA 1.4) oil
663 immersion objective (Zeiss, Thornwood, NY). Z-stack images were captured with an
664 interval of 0.45 μm between each slice. To minimize cell-to-cell variation, cells with
665 similar heights were selected, maintaining a consistent number of z-slices (8-10) for
666 each cell. Fluorescent images of cells expressing EGFP-p65 were captured using a
667 Zeiss AXIO Observer.Z1 wide-field epifluorescence microscope with a 63X oil
668 immersion objective. Live cell imaging was performed on a heated microscope stage
669 (+37°C) in presence of 5% CO_2. Images were acquired using either Zen 2.0 or
670 μManager software. Calculation of nuclear-to-cytoplasmic (N/C) intensity ratios of

671 EGFP-p65 was carried out by selecting regions of interest (ROIs) from cytoplasmic
672 and nuclear regions, and their mean intensity values were calculated.

673 ***Fractal dimension analysis***

674 Fractal dimension (FD) values of the actin, vimentin, and microtubule filaments were
675 calculated using the FracLac (v2.5) plugin (Karperien, 1999) for ImageJ. 16-bit
676 confocal images were converted into 8-bit binary images. Thresholding was performed
677 using the Otsu method. During thresholding, efforts were made to ensure maximum
678 visibility of cytoskeletal filaments while minimizing background noise.

679 ***Particle tracking microrheology (PTM)***

680 The entire procedure of PTM was conducted as described in the standard protocol
681 (Tseng et al., 2002; Wirtz, 2009). Fluorescently tagged carboxylate modified
682 microspheres (Invitrogen, #F8811) having diameter of 200nm were ballistically shot
683 inside the cell using a gene gun system (Bio-Rad, Helios® Gene gun system). After
684 the shooting, the cells were washed with 1X PBS solution to wash away unbound
685 beads and detached cells. The cells were then kept in a cell culture incubator under
686 standard conditions (37°C, 5% CO_2) for 16 hours in serum-free DMEM medium to
687 allow healing from the injuries caused by the shooting of the microspheres. The cells
688 containing fluorescent microspheres were subsequently positioned on the microscope
689 stage at 37°C. The movements of the microspheres were recorded in the form of
690 movies using a high-speed Hamamatsu Orca Flash 4.0 CMOS camera and μManager
691 software (A. Edelstein et al., 2010; A. D. Edelstein et al., 2014). Each movie comprised
692 1000 frames, spanning a total duration of 20 seconds, with an exposure time of 20
693 milliseconds. The trajectories of the microspheres were extracted by tracking their
694 movements using the MOSAICsuite plugin (Sbalzarini & Koumoutsakos, 2005) of Fiji.

At least 5-10 beads from each cell were tracked to obtain the rheological properties of the cytoplasm. The Mean Squared Displacement (MSD) and compliance (Γ) values were computed from these trajectories using a MATLAB script. MSDs having slopes greater than 1 were filtered out. While the movement of the microspheres was recorded for 1000 frames, only the first 250 frames were considered for calculating the MSDs to avoid potential errors from later data points in the trajectories. Subsequently, the complex shear modulus (G*(ω)), viscous modulus (G''), and elastic modulus (G') were derived from the MSDs using another MATLAB script.

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Author contributions

DKS: Conceptualisation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – reviewing and editing. **DR**: Conceptualisation, performed experiments, data analysis, data interpretation, wrote MATLAB codes, Writing - reviewing and editing, preparation of figures.

Competing interests

The authors declare no competing or financial interests.

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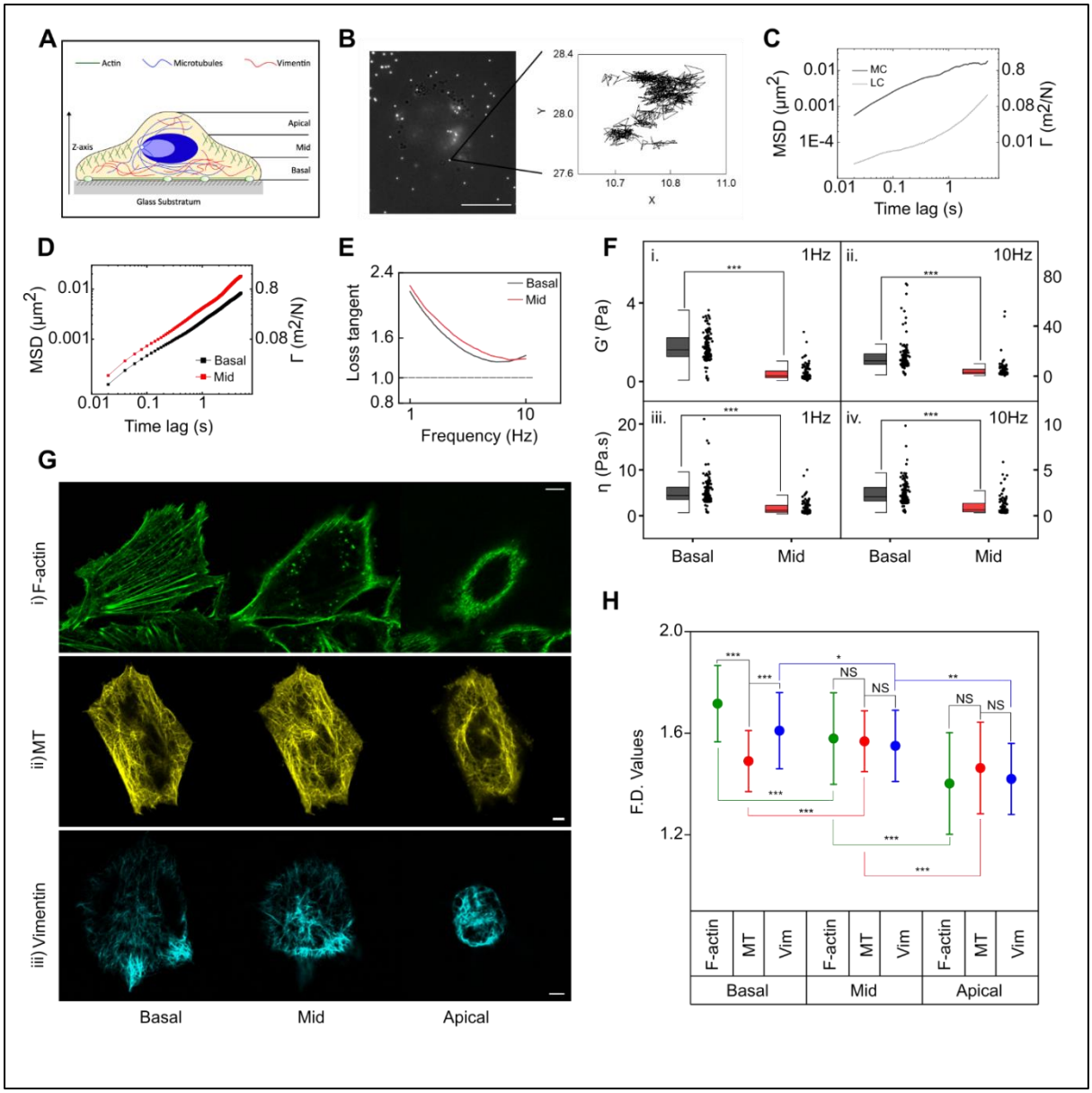
906 **Figure 1**



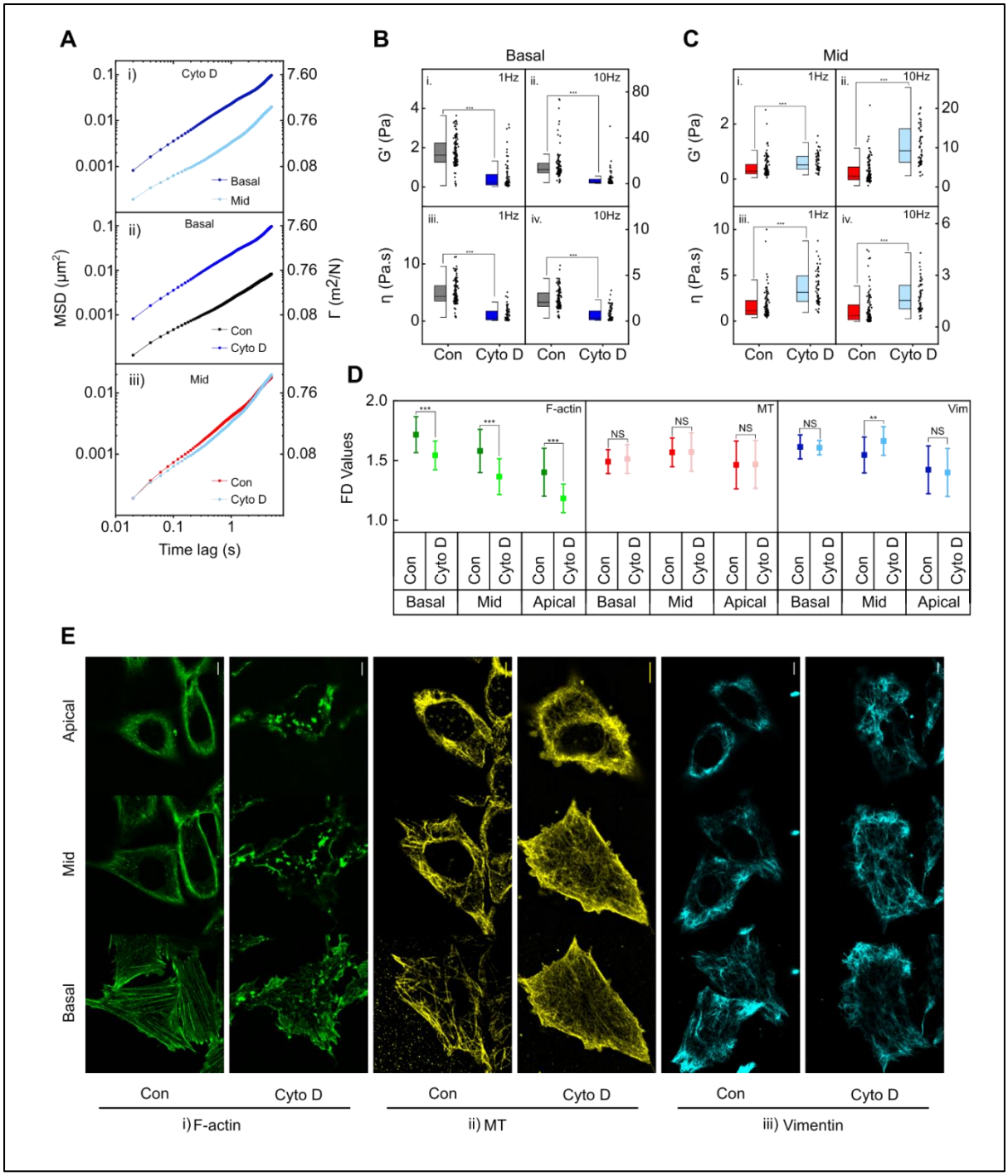
907

908 **Figure 1: Mechanical properties of the cytoplasm are polarised along the**
909 **apicobasal axis.** (**A**) Cartoon of a cell illustrating the distribution of actin, microtubule,
910 and vimentin filaments, along with segmentation of the cytoplasm into basal, mid, and
911 apical regions along the z-axis. (**B**) Fluorescently labelled microspheres with a
912 diameter of 200 nm dispersed throughout the cytoplasm of an NIH 3T3 fibroblast
913 (Scale bar, 5 μm). (Inset) Trajectory of one such microsphere tracked over a 20-second
914 period. (**C**) MSD (left axis) and compliance, Γ (right axis) of the most compliant (MC)

915 and least compliant (LC) regions within the cytoplasm of a specific cell. (**D**) Mean MSD
916 (left axis) and compliance, Γ (right axis) of the basal and mid cytoplasmic regions.
917 (N_{beads} > 80, N_{cells} > 15) (**E**) Loss tangent profiles of the basal and mid cytoplasmic
918 regions. (**F**) Comparison of elastic modulus (G') values between the basal and mid
919 cytoplasmic regions at 1 Hz (i) and 10 Hz (ii) frequencies. Comparison of dynamic
920 viscosity (η) values between the basal and mid cytoplasm at 1 Hz (iii) and 10 Hz (iv)
921 frequencies. (N_{beads} > 80, N_{cells} > 15; ***p<0.001, Mann-Whitney U test) (**G**)
922 Representative confocal images of F-actin (i), microtubule, MT (ii) and vimentin
923 filaments (iii) across the basal, mid and apical cytoplasm. (Scale bar, 5 μm) (**H**) Fractal
924 dimension (FD) values of actin, microtubule (MT) and vimentin (Vim) filaments across
925 the apicobasal axis. (Mean ± SD, N_{cells} > 100; *p<0.05, **p<0.01, ***p<0.001, NS, non-
926 significant, two sample T-test)

927

928 **Figure 2**



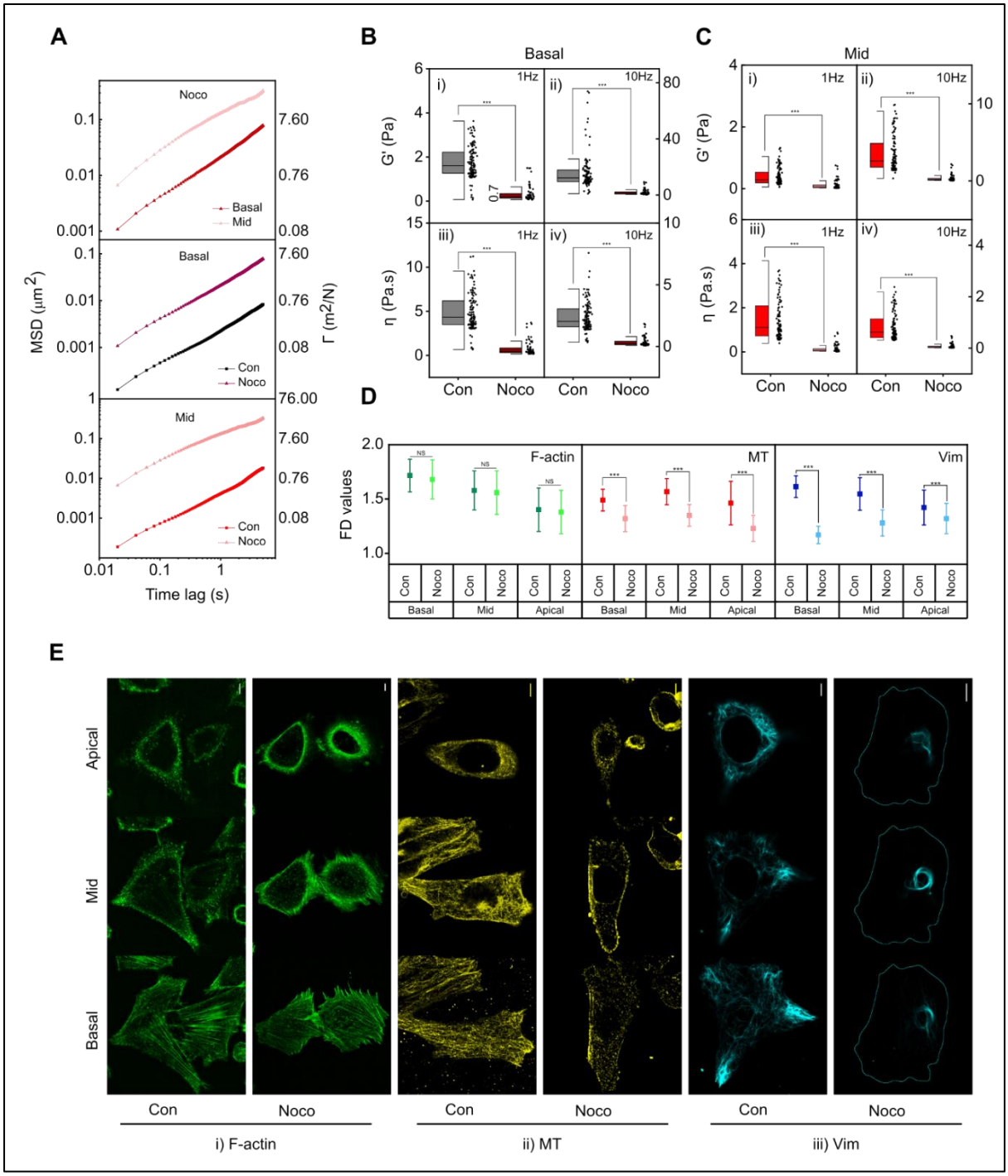
929

930 **Figure 2: Disruption of actin filaments has contrasting effects on the mechanical**
931 **properties of the basal and mid cytoplasm.** (**A**) Mean MSD (left axis) and
932 compliance, Γ (right axis) of the basal and mid cytoplasmic regions of Cyto D treated
933 cells (i), comparison of Mean MSDs (left axis) and compliance, Γ (right axis) between

934 the control and Cyto D treated cells across the basal (ii) and mid (iii) cytoplasmic
935 regions. (N_{beads} > 80, N_{cells} > 15) (**B**) Comparison of elastic moduli (G') between the
936 control and Cyto D treated cells across the basal cytoplasmic region at 1 Hz (i) and 10
937 Hz (ii) frequencies. Comparison of dynamic viscosity (η) values between the control
938 and Cyto D treated cells across the basal cytoplasmic region at 1 Hz (iii) and 10 Hz
939 (iv) frequencies. (N_{beads} > 80, N_{cells} > 15; ***p<0.001, Mann-Whitney U test) (**C**)
940 Comparison of elastic moduli (G') between the control and Cyto D treated cells across
941 the mid cytoplasmic region at 1 Hz (i) and 10 Hz (ii) frequencies. Comparison of
942 dynamic viscosity (η) values between the control and Cyto D treated cells across the
943 mid cytoplasmic region at 1 Hz (iii) and 10 Hz (iv) frequencies. (N_{beads} > 80, N_{cells} > 15;
944 ***p<0.001, Mann-Whitney U test) (**D**) Fractal dimension (FD) values of actin,
945 microtubule (MT) and vimentin filaments in control and Cyto D treated cells across the
946 apicobasal axis. (Mean ± SD, N_{cells} > 100; **p<0.01, ***p<0.001, NS, non-significant,
947 two sample T-test) (**E**) Representative confocal images of F-actin, microtubules (MT)
948 and vimentin filaments in the control and Cyto D treated cells across the apicobasal
949 axis. (Scale bar, 5 μm)

950

951 **Figure 3**



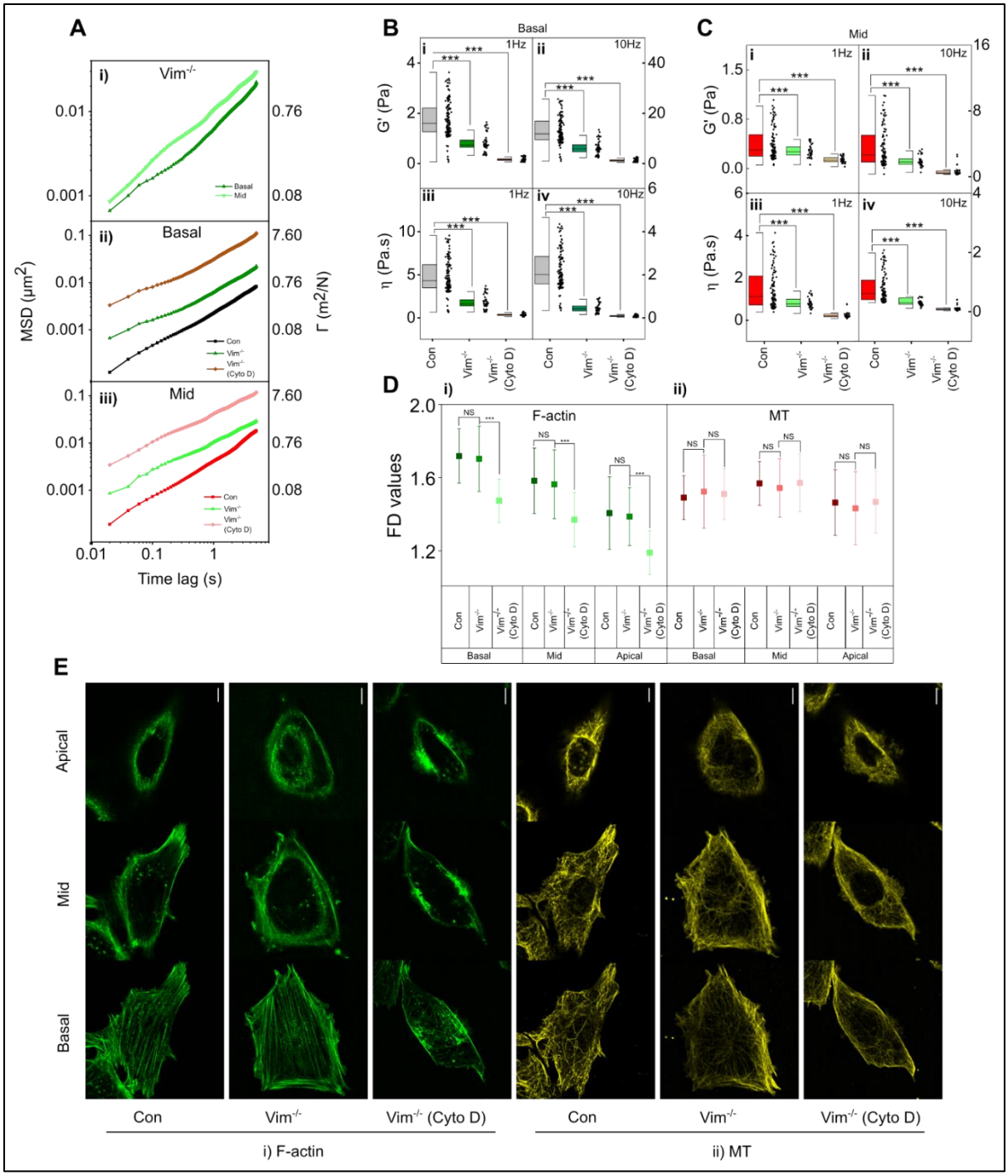
952

953 **Figure 3: Nocodazole-induced depolymerization of microtubules results in**
954 **significant softening of the cytoplasm across the apicobasal axis.** (A) Mean MSD
955 (left axis) and compliance, Γ (right axis) of the basal and mid cytoplasmic regions of
956 nocodazole (noco) treated cells (i), comparison of Mean MSDs (left axis) and

957 compliance, Γ (right axis) between the control and nocodazole treated cells across the
958 basal (ii) and mid (iii) cytoplasmic regions. (N_{beads} > 60, N_{cells} > 10) (B) Comparison of
959 elastic moduli (G') between the control and nocodazole treated cells across the basal
960 cytoplasmic region at 1 Hz (i) and 10 Hz (ii) frequencies. Comparison of dynamic
961 viscosity (η) values between the control and nocodazole treated cells across the basal
962 cytoplasmic region at 1 Hz (iii) and 10 Hz (iv) frequencies. (N_{beads} > 60, N_{cells} > 10;
963 ***p<0.001, Mann-Whitney U test) (C) Comparison of elastic moduli (G') between the
964 control and nocodazole treated cells across the mid cytoplasmic region at 1 Hz (i) and
965 10 Hz (ii) frequencies. Comparison of dynamic viscosity (η) values between the control
966 and nocodazole treated cells across the mid cytoplasmic region at 1 Hz (iii) and 10 Hz
967 (iv) frequencies. (N_{beads} > 60, N_{cells} > 10; ***p<0.001, Mann-Whitney U test) (D) Fractal
968 dimension (FD) values of actin, microtubule (MT) and vimentin filaments in control and
969 nocodazole treated cells across the apicobasal axis. (Mean ± SD, N_{cells} > 100;
970 ***p<0.001, NS, non-significant, two sample T-test) (E) Representative confocal
971 images of F-actin, microtubules (MT) and vimentin filaments in the control and
972 nocodazole treated cells across the apicobasal axis. (Scale bar, 5 μm)

973

974 **Figure 4**



975

976 **Figure 4: Localised vimentin assembly stiffens mid cytoplasm in cytochalasin D**

977 **treated cells.** (**A**) Mean MSD (left axis) and compliance, Γ (right axis) of the basal and

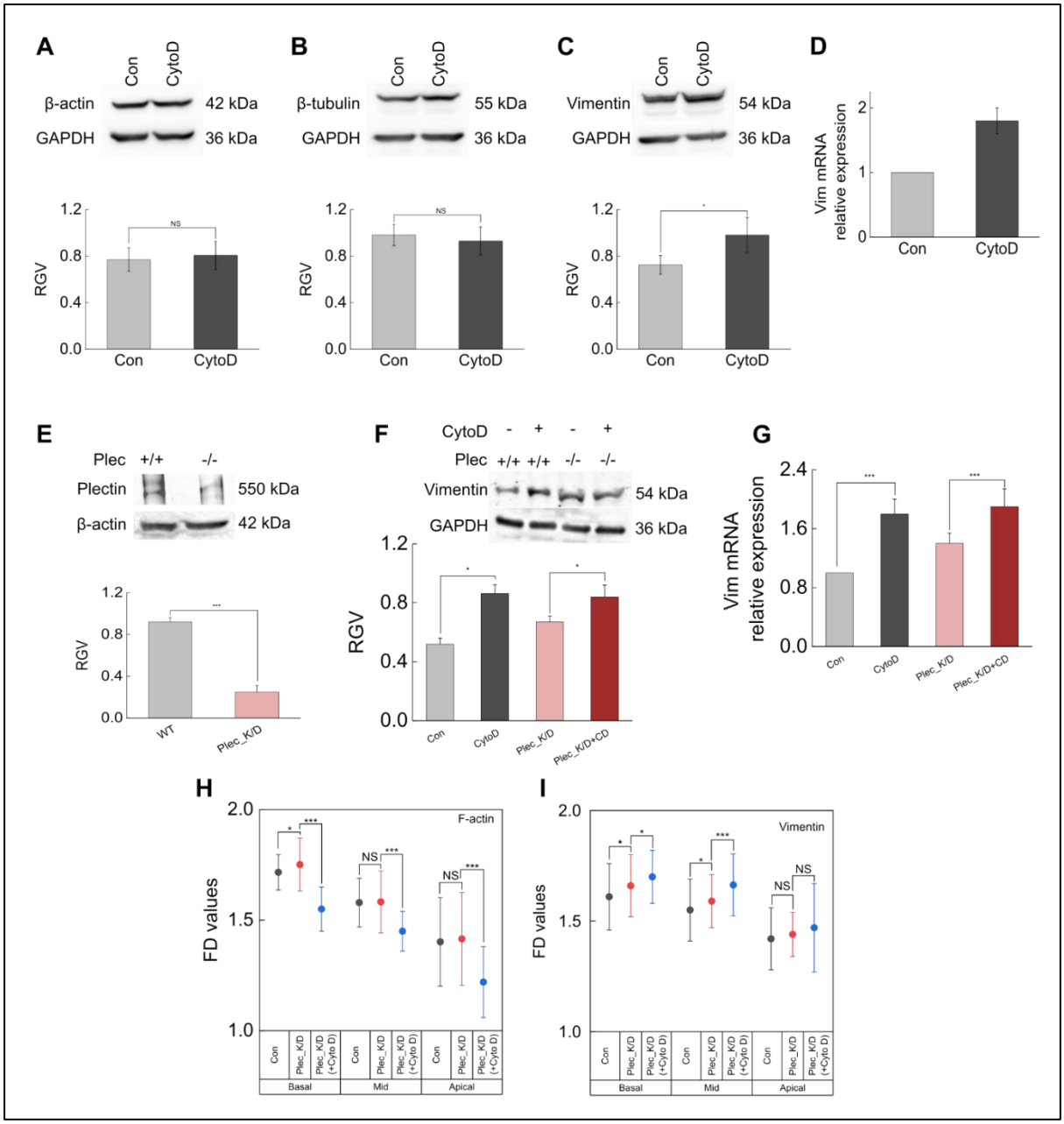
978 mid cytoplasmic regions of Vim$^{-/-}$ cells (i), comparison of Mean MSDs (left axis) and

979 compliance, Γ (right axis) between the control, Vim$^{-/-}$ and Cyto D treated Vim$^{-/-}$ cells

980 across the basal (ii) and mid (iii) cytoplasmic regions. (N_{beads} > 50, N_{cells} > 10) (**B**)
981 Comparison of elastic moduli (G') between the control, $Vim^{-/-}$ and Cyto D treated Vim^{-}
982 $^{/-}$ cells across the basal cytoplasmic region at 1 Hz (i) and 10 Hz (ii) frequencies.
983 Comparison of dynamic viscosity (η) values between the control, $Vim^{-/-}$ and Cyto D
984 treated $Vim^{-/-}$ cells across the basal cytoplasmic region at 1 Hz (iii) and 10 Hz (iv)
985 frequencies. (N_{beads} > 50, N_{cells} > 10; ***p<0.001, Mann-Whitney U test) (**C**)
986 Comparison of elastic moduli (G') between the control, $Vim^{-/-}$ and Cyto D treated Vim^{-}
987 $^{/-}$ cells across the mid cytoplasmic region at 1 Hz (i) and 10 Hz (ii) frequencies.
988 Comparison of dynamic viscosity (η) values between the control, $Vim^{-/-}$ and Cyto D
989 treated $Vim^{-/-}$ cells across the mid cytoplasmic region at 1 Hz (iii) and 10 Hz (iv)
990 frequencies. (N_{beads} > 50, N_{cells} > 10; ***p<0.001, Mann-Whitney U test) (**D**) Fractal
991 dimension (FD) values of actin and microtubule (MT) filaments in control, $Vim^{-/-}$ and
992 Cyto D treated $Vim^{-/-}$ cells across the apicobasal axis. (Mean ± SD, N_{cells} > 80;
993 ***p<0.001, NS, non-significant, two sample T-test) (**E**) Representative confocal
994 images of F-actin and microtubules (MT) filaments in the control, $Vim^{-/-}$ and Cyto D
995 treated $Vim^{-/-}$ cells across the apicobasal axis. (Scale bar, 5 μm)

996

997 **Figure 5**



998

999 **Figure 5: Plectin directs the localised assembly of vimentin filaments in the mid-**

1000 **cytoplasm of cytochalasin D treated cells** (**A**) Western blot analysis for β-actin

1001 protein levels post Cyto D (2μM, 1hr) treatment. (n=3, mean ± SD, NS, non-significant,

1002 two sample T test). (**B**) Western blot analysis for β-tubulin protein levels post Cyto D

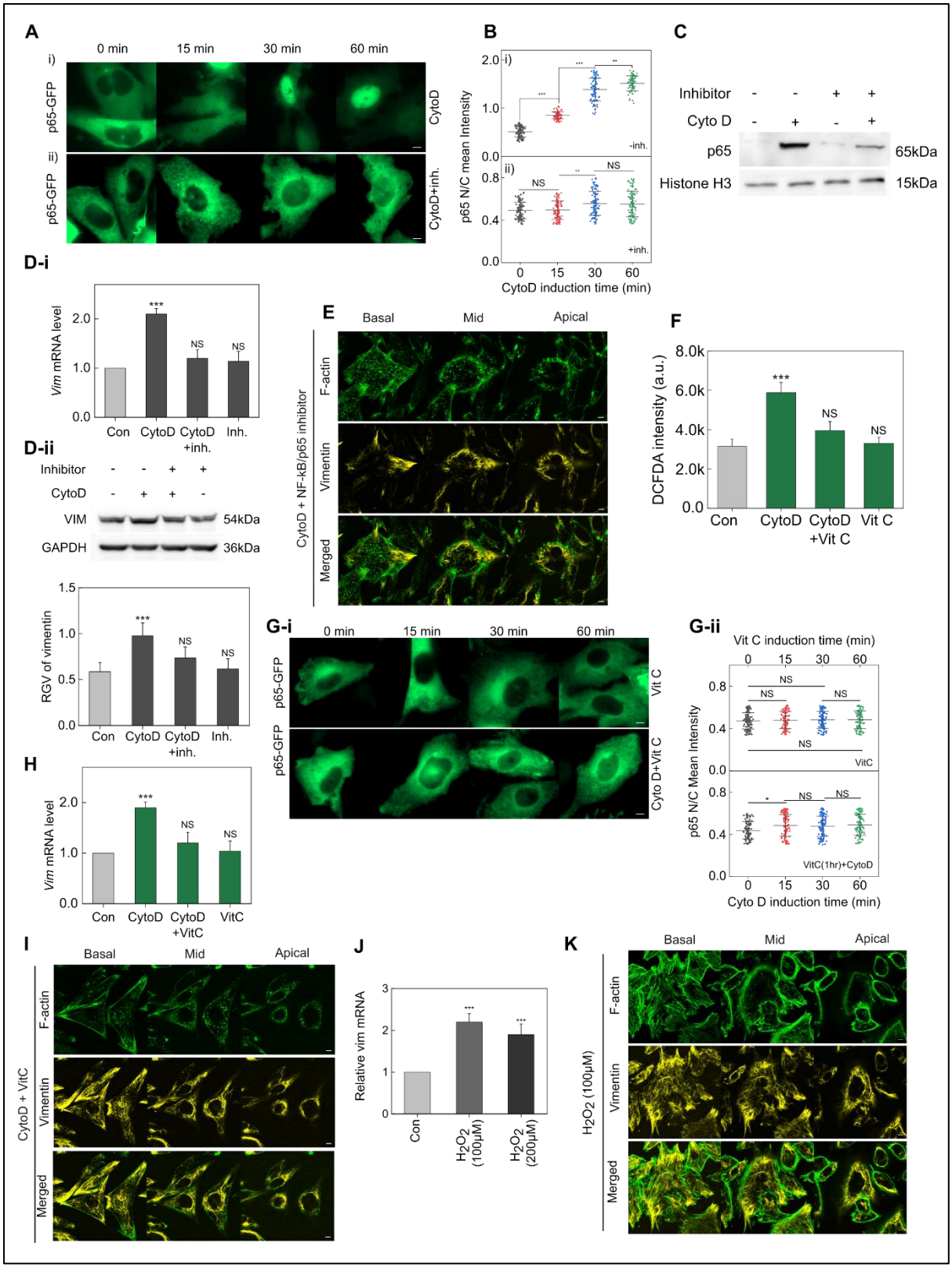
1003 (2μM, 1hr) treatment. (n=3, mean ± SD, NS, non-significant, two sample T test). (**C**)

1004 Western blot analysis for vimentin protein levels post Cyto D (2μM, 1hr) treatment.

1005 (n=3, mean ± SD, *p<0.05, two sample T test). (**D**) Relative fold change of vimentin
1006 mRNA levels (as quantified by qPCR) post Cyto D treatment (2μM, 1hr). (n=3, mean
1007 ± SD,) (**E**) Western blotting analysis of plectin for confirmation of knockdown. (n=3,
1008 mean ± SD, ***p<0.001, two sample T test) (**F**) Western blot analysis of vimentin
1009 expression in wild type and plectin knockdown cells with and without Cyto D (2μM,
1010 1hr) treatment. (n=3, mean ± SD, *p<0.05, two sample T test) (**G**) Relative fold change
1011 of vimentin mRNA levels (as quantified by qPCR) of wild type and plectin knockdown
1012 cells post Cyto D treatment (2μM, 1hr). (n=3, mean ± SD) (*p < 0.05, **p < 0.01, *** p
1013 <0.001; NS, non significant, two sample T-test). Fractal dimension (FD) values of actin
1014 (**H**) and vimentin (**I**) filaments in control, plectin depleted and Cyto D treated plectin
1015 depleted cells across the apicobasal axis. (Mean ± SD, N_{cells} > 80; *p<0.05,
1016 ***p<0.001, NS, non-significant, two sample T-test)

1017

1018 **Figure 6**



1019

1020 **Figure 6: Cyto D induced assembly of vimentin is dependent on ROS-mediated**

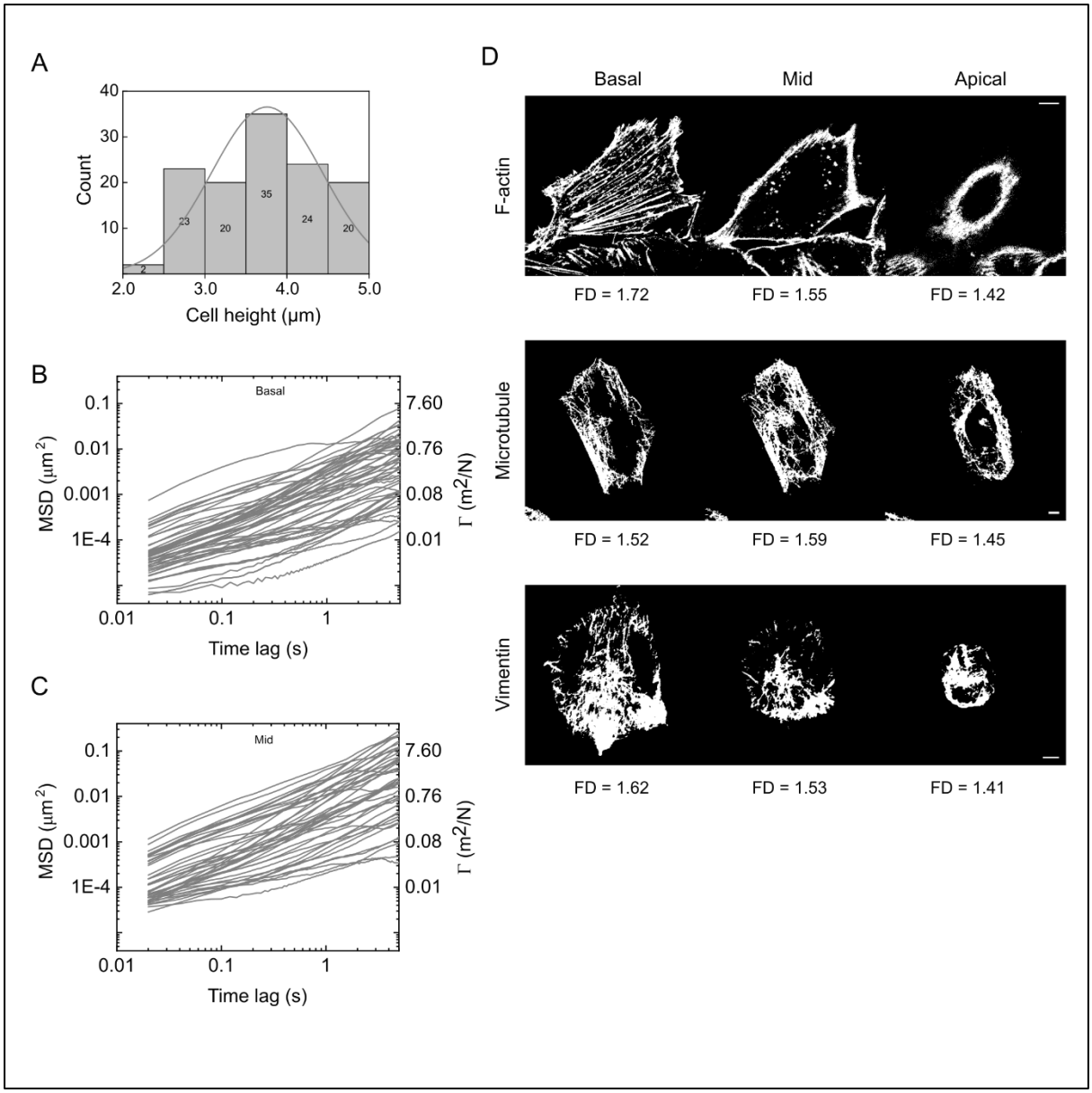
1021 **activation of NF-κB pathway**

1022 (**A**) Representative confocal images of nuclear translocation of p65-GFP at different
1023 time points post Cyto D (2μM) treatment. (Scale bar, 5μm) (**B**) Nuclear to cytoplasmic
1024 (N/C) mean intensity ratio of p65 at different time points post Cyto D (2μM) treatment
1025 with and without NF-kB inhibitor. (N_{Cells} = 80-110 per condition, experimental
1026 replicates, n = 3, mean ± SD, **p<0.01, ***p<0.001, two sample T test) (**C**) Western
1027 blotting analysis of the nuclear fraction of p65 after Cyto D (2μM, 1hr) treatment in the
1028 presence and absence of the NF-kB inhibitor. (**D-i**) Relative fold changes of vimentin
1029 mRNA levels (as quantified by qPCR) post-Cyto D (2μM, 1hr) treatment with and
1030 without NF-kB inhibitor pretreatment. (n = 3, mean ± SD, ***p<0.001, NS, non-
1031 significant, two sample T test) (**D-ii**) Western blot analysis for vimentin protein levels
1032 post-Cyto D (2μM, 1hr) treatment with and without NF-kB inhibitor pretreatment. (n =
1033 3, mean ± SD, ***p<0.001, NS, non-significant, two sample T test) (**E**) Representative
1034 confocal images of F-actin and vimentin at the basal, mid and apical regions after Cyto
1035 D treatment (2μM, 1hr) in the presence of NF-kB inhibitor. (Scale bar, 5μm) (**F**) Mean
1036 DCFDA fluorescence levels of cells after Cyto D treatment (2μM, 1hr) pre-incubated
1037 with vitamin C (Vit C) (n = 3, mean ± SD, ***p<0.001, NS, non-significant, two sample
1038 T test). (**G-i**) Representative images of nuclear translocation of p65-GFP at different
1039 time spans of Vit C incubation (upper row) and p65-GFP nuclear translocation in cells
1040 after Cyto D (2μM) treatment for various durations following a 1-hour pre-treatment
1041 with Vit C (lower row). (Scale bar, 5μm). (**G-ii**) Nuclear to cytoplasmic (N/C) mean
1042 intensity ratio of p65 at different Vit C incubation periods (top) and N/C mean intensity
1043 ratio of p65 of cells after Cyto D (2μM) treatment for various durations following a 1hour
1044 pre-treatment with Vit C (bottom). (N_{cells} = 80-120 per condition, n = 3, mean ± SD,
1045 *p<0.05, NS, non-significant, two sample T test). (**H**) Relative fold change of vimentin
1046 mRNA levels (as quantified by qPCR) post Cyto D treatment (2μM, 1hr) with and

1047 without Vit C pre-incubation. (n=3, mean ± SD, ***p<0.001, NS, non-significant, two
1048 sample T test). (**I**) Representative confocal images of F-actin and vimentin at the basal,
1049 mid and apical regions after Cyto D treatment (2μM, 1hr) in the presence of Vit C.
1050 (Scale bar, 5μm) (**J**) Relative fold change of vimentin mRNA levels (as quantified by
1051 qPCR) post H_2O_2 incubation (100μM and 200μM). (n=3, mean ± SD, ***p<0.001, two
1052 sample T test) (**K**) Representative confocal images of F-actin and vimentin at the
1053 basal, mid and apical regions after H_2O_2 incubation (100μM). (Scale bar, 5μm)

1054

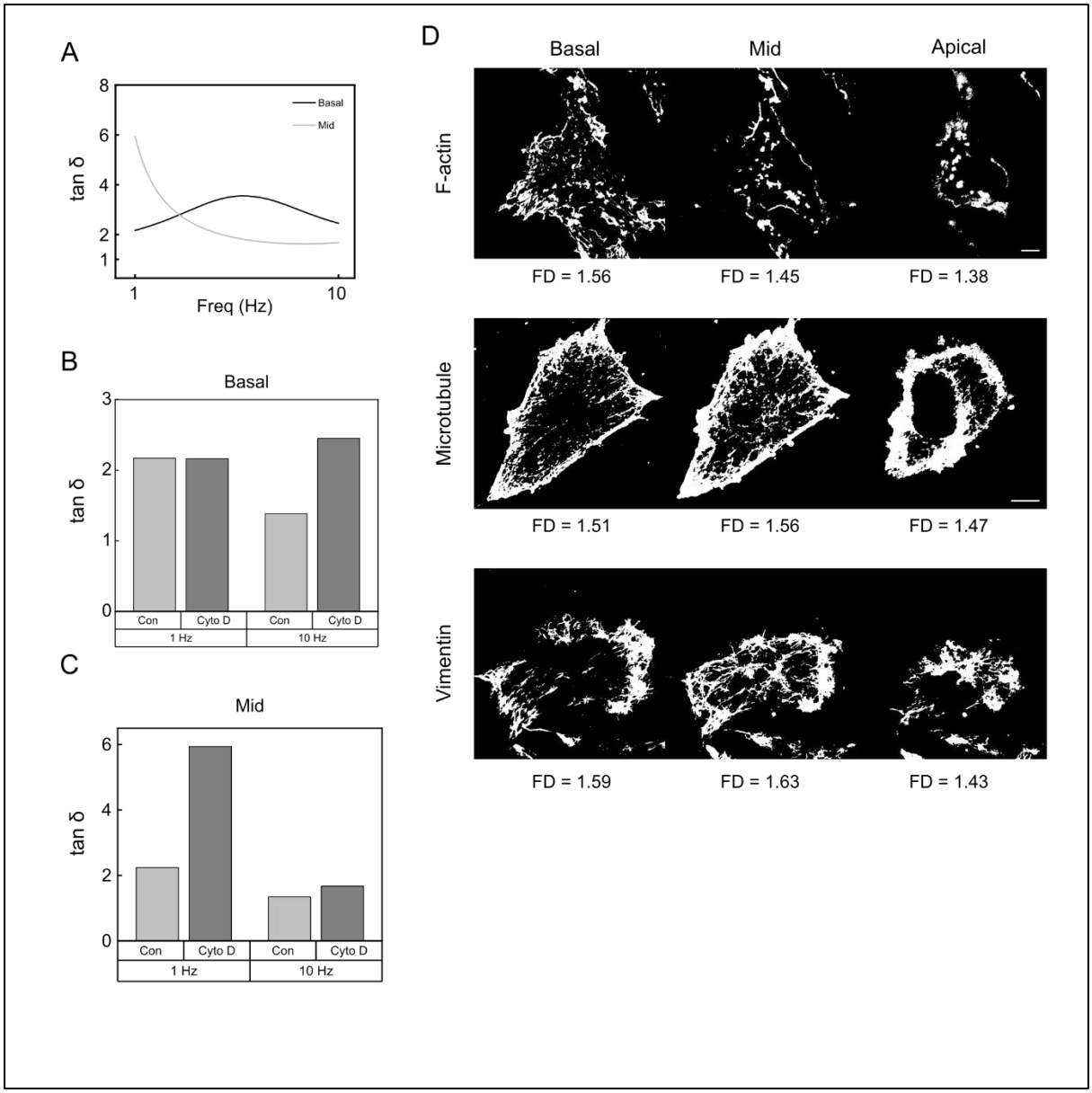
1055 **Supplementary figure S1**



1056

1057 **Figure S1:** (A) Histogram profile of the height of NIH 3T3 cells (N_{cells} > 100). MSDs
1058 (left axis) and compliance, Γ (right axis) values of individual microspheres dispersed
1059 in the basal (B) and mid (C) cytoplasm of control NIH 3T3 cells. (N_{beads} > 60, N_{cells} >
1060 10). (D) Representative binary images of actin, vimentin and microtubule filaments
1061 across the apicobasal axis of control NIH 3T3 cells and their corresponding fractal
1062 dimension (FD) values.

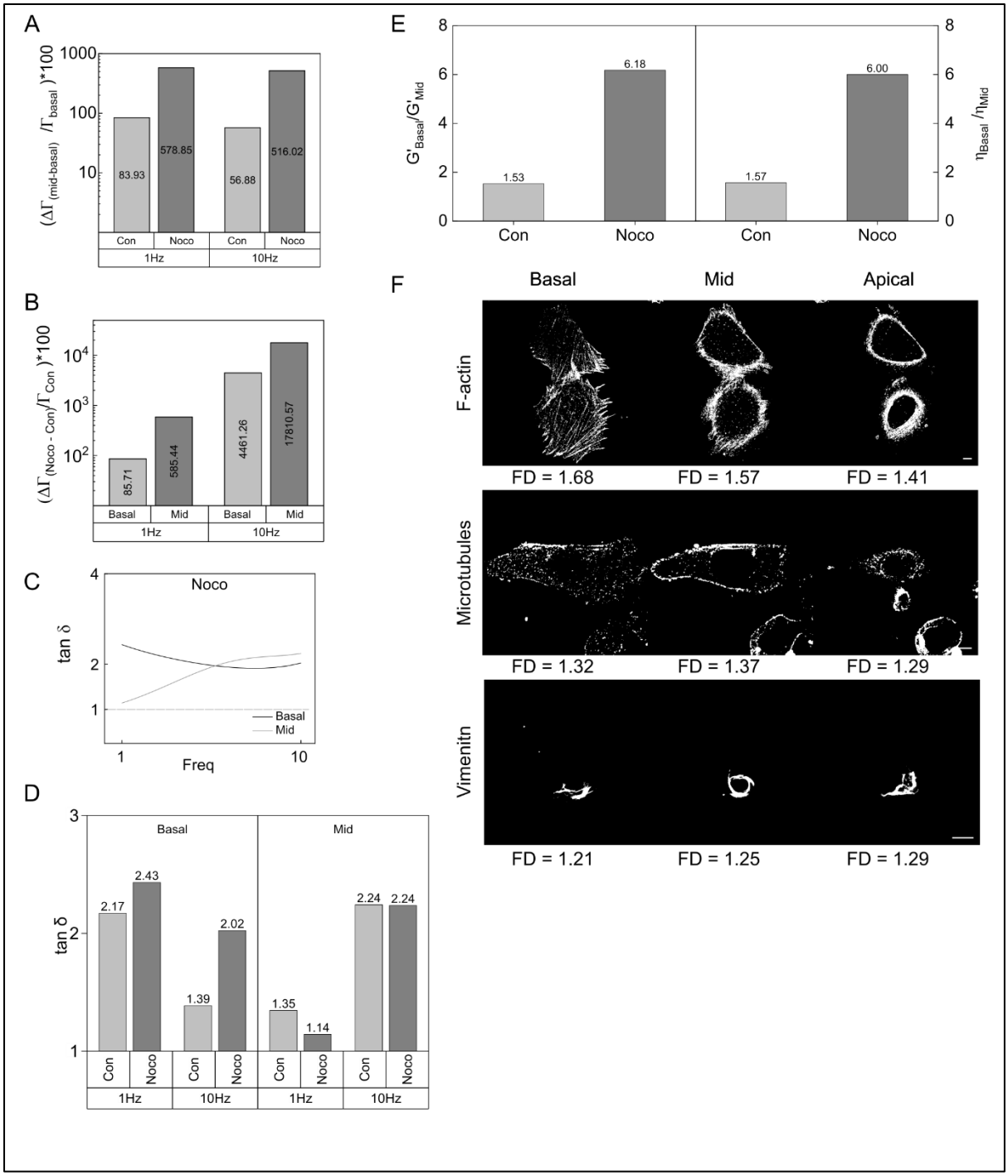
1063 **Supplementary figure S2**



1064

1065 **Figure S2**: (**A**) Mean loss tangent values of the basal and mid cytoplasm of cyto D
1066 treated cells. (**B**) Comparison of tan δ values between control and cyto D treated cells
1067 at the basal cytoplasmic region at 1Hz and 10Hz. (**C**) Comparison of tan δ values
1068 between control and cyto D treated cells at the mid cytoplasmic region at 1Hz and
1069 10Hz frequencies. (**D**) Representative binary images of actin, vimentin and
1070 microtubule filaments across the apicobasal axis of cyto D treated NIH 3T3 cells and
1071 their corresponding fractal dimension (FD) values.

1072 **Supplementary figure S3**



1073

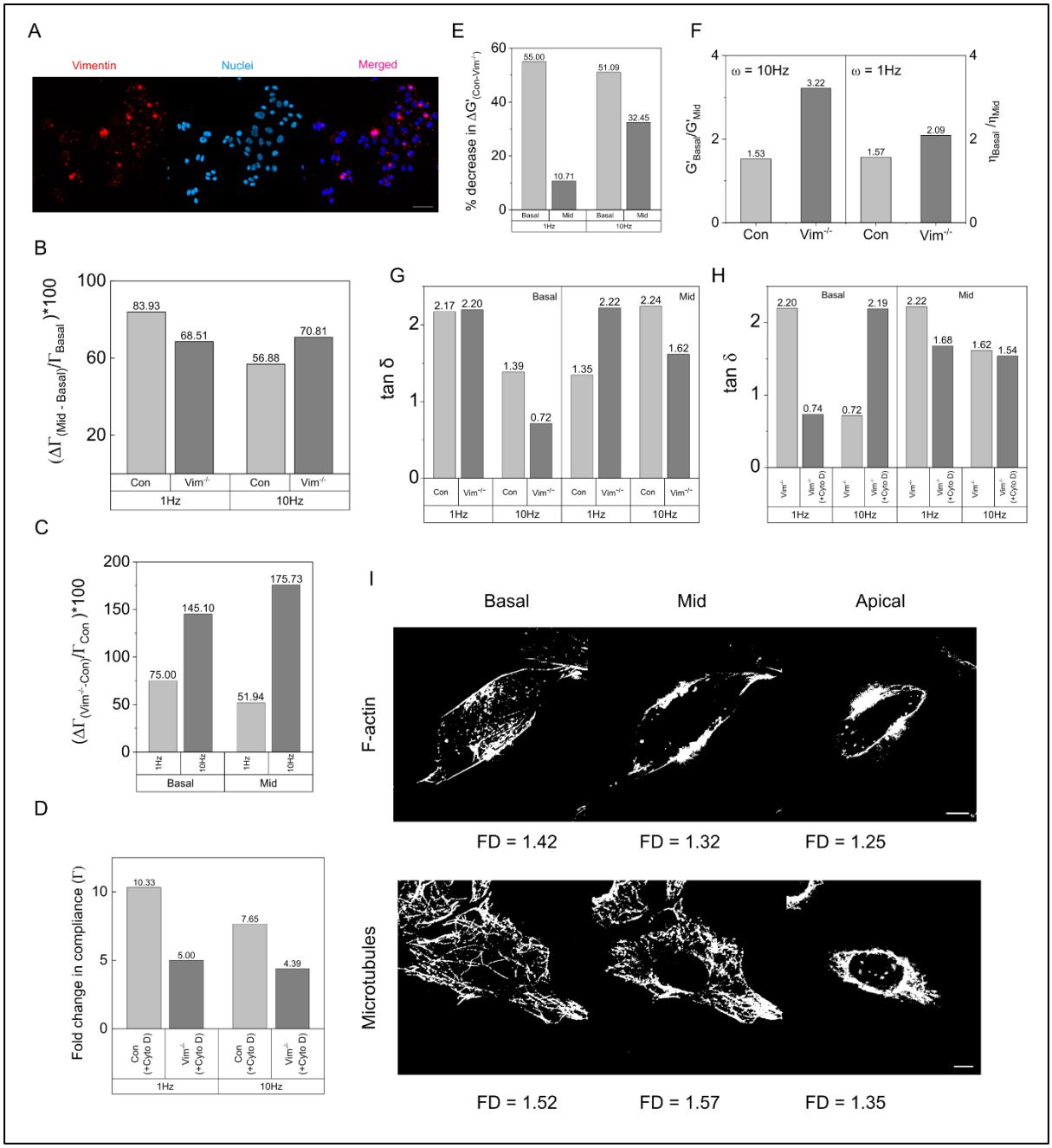
1074 **Figure S3**: (**A**) Relative fold change of the compliance, Γ of the mid cytoplasm
1075 compared to the basal cytoplasm of control and nocodazole treated cells at 1Hz and
1076 10Hz. (**B**) Relative fold change of the compliance, Γ of the nocodazole treated cells
1077 compared to the control cells in the basal and mid cytoplasm at 1Hz and 10Hz. (**C**)

1078 Loss tangent (tan δ) values of the basal and mid cytoplasmic regions of nocodazole
1079 treated cells. (**D**) Comparison of tan δ values between the control and nocodazole
1080 treated cells across the basal and mid cytoplasm at 1Hz and 10Hz. (**E**) Basal to mid
1081 cytoplasmic ratio of elastic modulus (G') and dynamic viscosity (η) in control and
1082 nocodazole treated cells. (**F**) Representative binary images of actin, vimentin and
1083 microtubule filaments across the apicobasal axis of nocodazole treated NIH 3T3 cells
1084 and their corresponding fractal dimension (FD) values.

1085

1086

1087 **Supplementary figure S4**

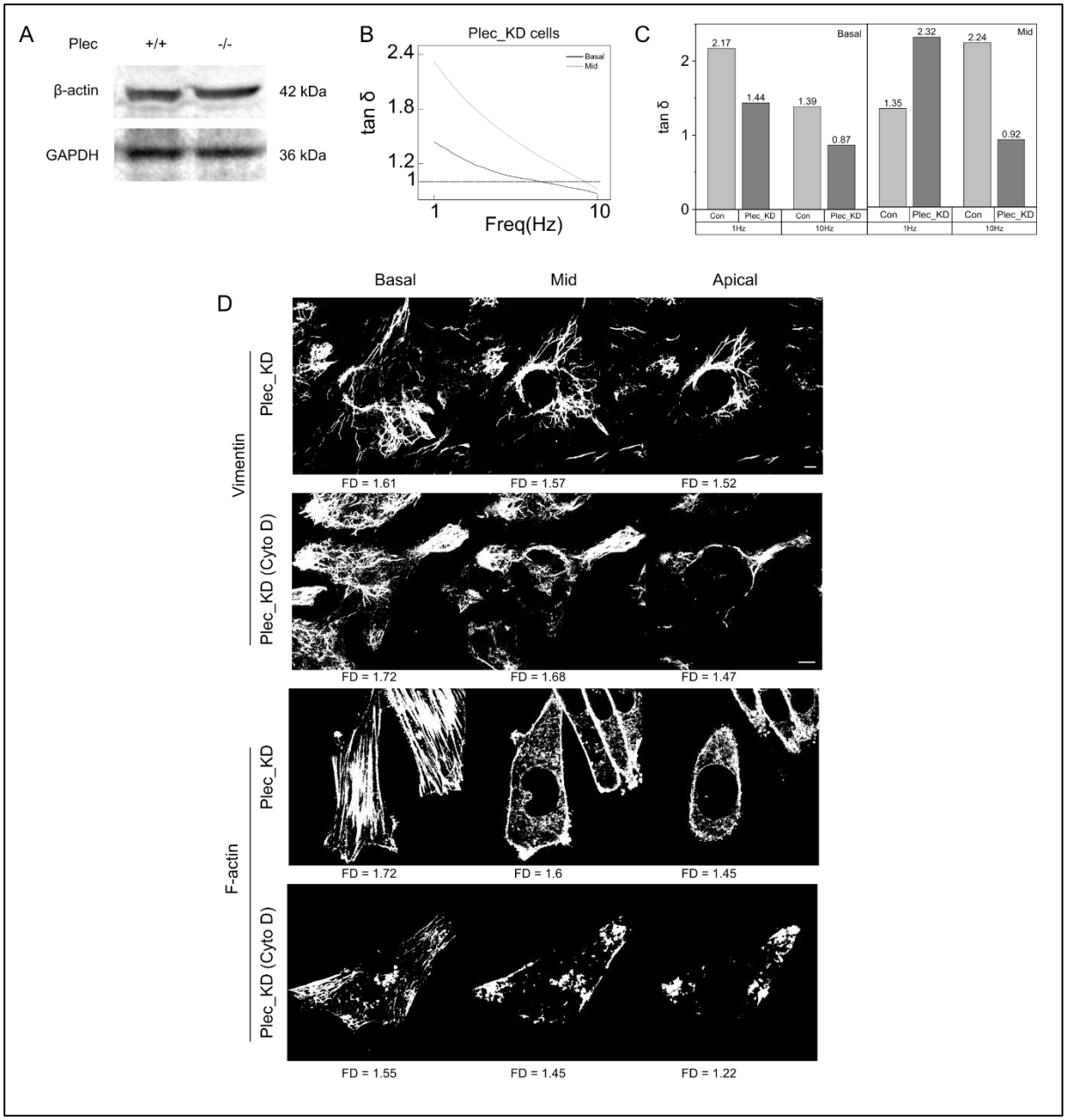


1088

1089 **Figure S4**: (**A**) Representative confocal image validating the knockout of vimentin from
1090 NIH 3T3. (Scale bar, 50μm) (**B**) Relative fold change of the compliance, Γ of the mid
1091 cytoplasm compared to the basal cytoplasm of control and Vim$^{-/-}$ cells at 1Hz and
1092 10Hz. (**C**) Relative fold change of the compliance, Γ of the Vim$^{-/-}$ cells compared to the
1093 control cells in the basal and mid cytoplasm at 1Hz and 10Hz. (**D**) Fold change in the

compliance, Γ of the basal region of control and Vim$^{-/-}$ cells after cyto D treatment at 1Hz and 10Hz. (**E**) Percentage decrease in elastic modulus (G') values upon knockout of vimentin across the basal and mid cytoplasm at 1Hz and 10Hz. (**F**) Basal to mid cytoplasmic ratio of elastic modulus (G') and dynamic viscosity (η) in the control and Vim$^{-/-}$ cells. (**G**) Comparison of tan δ values between the control and Vim$^{-/-}$ cells across the basal and mid cytoplasm at 1Hz and 10Hz. (**H**) Comparison of tan δ values between the Vim$^{-/-}$ and cyto D treated Vim$^{-/-}$ cells across the basal and mid cytoplasm at 1Hz and 10Hz. (**I**) Representative binary images of actin and microtubule filaments across the apicobasal axis of cyto D treated Vim$^{-/-}$ cells and their corresponding fractal dimension (FD) values.

1105 **Supplementary figure S5**



1106

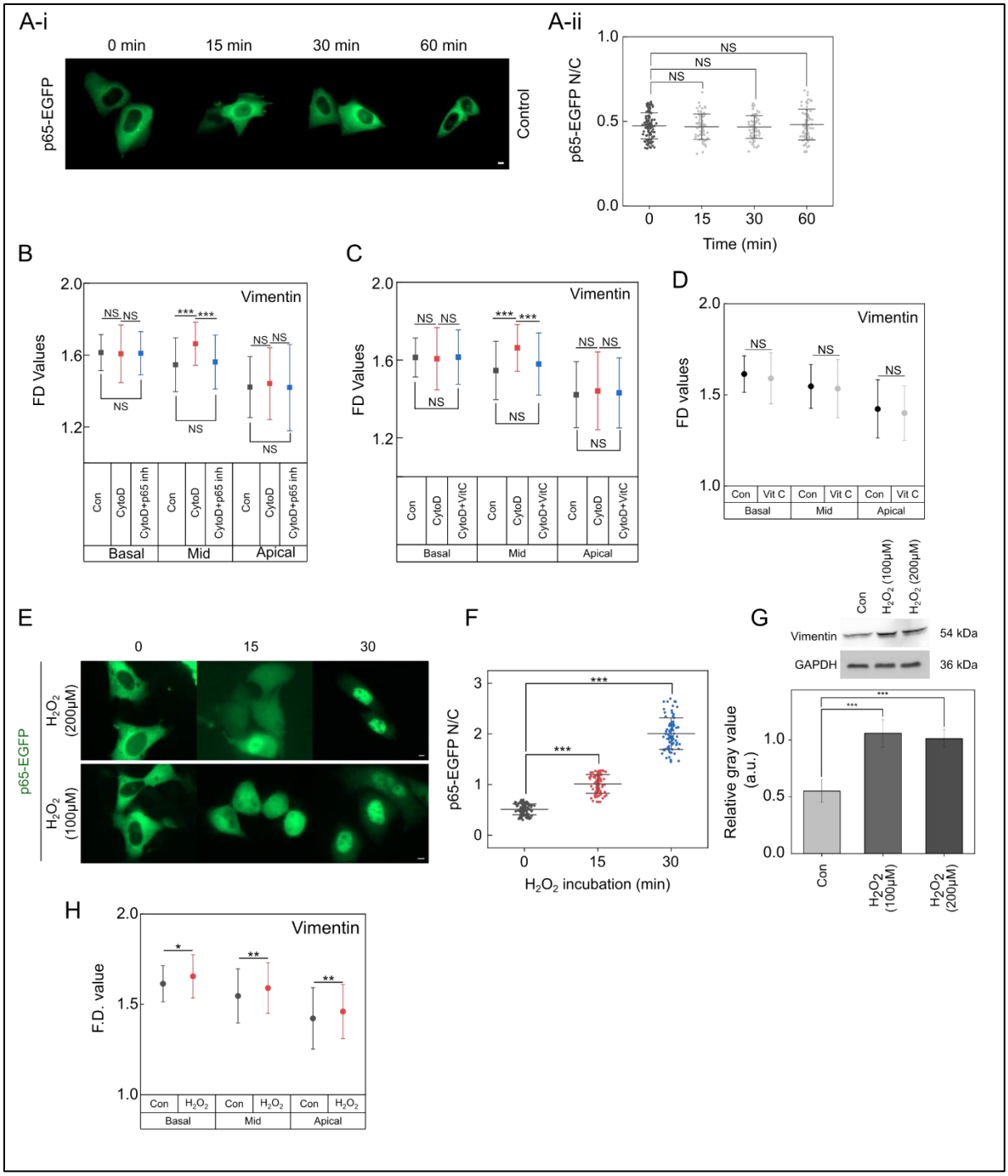
1107 **Figure S5:** (**A**) Western blot analysis of beta-actin in control and plectin depleted NIH
1108 3T3 cells. (**B**) Mean loss tangent values (tan δ) of the basal and mid cytoplasm of
1109 plectin depleted NIH 3T3 cells. (**C**) Comparison of tan δ values between the control
1110 and plectin depleted cells across the basal and mid cytoplasm at 1Hz and 10Hz. (**D**)
1111 Representative binary images of vimentin and actin filaments across the apicobasal

1112 axis of plectin-depleted and cyto D-treated plectin-depleted NIH 3T3 cells, along with

1113 their corresponding fractal dimension (FD) values.

1114

1115 **Supplementary figure S6**



1116

1117 **Figure S6:** (**A**) Tracking of p65-EGFP localization across different time points (i) (Scale
1118 bar, 5μm) and the corresponding nuclear to cytoplasmic (N/C) mean intensity ratio
1119 values (ii) (Mean ± SD, N_{cells} > 50, NS, non-significant, two sample T test). (**B**)
1120 Quantification of FD values of vimentin in control, cyto D treated and cyto D with p65

1121 inhibitor pretreated cells across the basal, mid and apical cytoplasm. (N_{cells} = 100 -140
1122 per condition, n = 3, mean ± SD, ***p<0.001, NS, non-significant, two sample T test)
1123 (**C**) Quantification of FD values of vimentin in control, cyto D treated and cyto D with
1124 vitamin C pretreated cells across the basal, mid and apical cytoplasm. (N_{cells} > 80, n =
1125 3, mean ± SD, ***p<0.001, NS, non-significant, two sample T test) (**D**) FD values of
1126 control and vitamin C treated cells across the apicobasal axis. (N_{cells} > 100, n = 3,
1127 mean ± SD, NS, non-significant, two sample T test). (**E**) Nuclear translocation of p65-
1128 EGFP at different time points following H_2O_2 incubation at two different concentrations.
1129 (Scale bar, 5μm) (**F**) Nuclear to cytoplasmic (N/C) mean intensity ratio of p65-EGFP
1130 at different time points following H_2O_2 incubation. (N_{cells} > 60, ***p<0.001, two sample
1131 T test) (**G**) Western blot analysis of vimentin following H_2O_2 incubation at two different
1132 concentrations. (n = 3, mean ± SD, ***p<0.001, two sample T test) (**H**) FD analysis of
1133 vimentin in control and H_2O_2 treated cells across the apicobasal axis. (N_{cells} > 80,
1134 *p<0.05, **p<0.01, two sample T test)

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