Tropomyosin controls sarcomere-like contractions for rigidity sensing and suppressing growth on soft matrices

Haguy Wolfenson\textsuperscript{1,2,7,8}, Giovanni Meacci\textsuperscript{1,2,3,7}, Shuaimin Liu\textsuperscript{2,4}, Matthew R. Stachowiak\textsuperscript{3}, Thomas Iskratsch\textsuperscript{1,2}, Saba Ghassemi\textsuperscript{2,4}, Pere Roca-Cusachs\textsuperscript{5}, Ben O’Shaughnessy\textsuperscript{3}, James Hone\textsuperscript{2,4} and Michael P. Sheetz\textsuperscript{1,2,6,8}

Cells test the rigidity of the extracellular matrix by applying forces to it through integrin adhesions. Recent measurements show that these forces are applied by local micrometre-scale contractions, but how contraction force is regulated by rigidity is unknown. Here we performed high temporal- and spatial-resolution tracking of contractile forces by plating cells on sub-micrometre elastomeric pillars. We found that actomyosin-based sarcomere-like contractile units (CUs) simultaneously moved opposing pillars in net steps of \(\sim 2.5 \text{ nm}\), independent of rigidity. What correlated with rigidity was the number of steps taken to reach a force level that activated recruitment of \(\alpha\)-actinin to the CUs. When we removed actomyosin restriction by depleting tropomyosin 2.1, we observed larger steps and higher forces that resulted in aberrant rigidity sensing and growth of non-transformed cells on soft matrices. Thus, we conclude that tropomyosin 2.1 acts as a suppressor of growth on soft matrices by supporting proper rigidity sensing.

The rigidity of the extracellular matrix (ECM) plays critical roles in cell apoptosis, proliferation and differentiation\textsuperscript{1,2}. Accordingly, aberrant rigidity sensing is involved in many medical disorders\textsuperscript{3,4}. For example, the anchorage-independent growth of cancer cells\textsuperscript{5,6} indicates that their rigidity sensing machinery is malfunctioning. Importantly, whereas the effects of ECM rigidity on cell fate are observed on timescales of hours to days, rigidity sensing is a rapid and cyclic process that occurs on much shorter timescales\textsuperscript{7,8}.

According to the current model, during cell spreading and migration, when the cell edge protrudes forward, nascent integrin adhesions are built on initial contact with the matrix. This is followed by generation of traction forces on the adhesions through local actomyosin-based contractile units\textsuperscript{9} (CUs), and subsequent linkage of these adhesions to the general rearward flow of actin towards the centre of the cell (the integrin ‘clutch’ model\textsuperscript{10,11}). At its most basic sense, rigidity sensing is manifested as the decision to reinforce the adhesions during the initial period of force application\textsuperscript{12}. On stiffer substrates, stronger adhesions are built, thereby allowing them to resist the forces from actin flow\textsuperscript{12,13}. When measured at the sub-micrometre scale, cells displace matrix-coated 0.5-\(\mu\)-m-diameter flexible pillars to a constant distance irrespective of rigidity\textsuperscript{9}. This indicates that there is a well-developed mechanism to link rigidity sensing, force production, and adhesion reinforcement, through sub-micrometre contractions in a few tens of seconds.

Hence, in this study, we analysed cellular forces during rigidity sensing with a new high-resolution technology. Using arrays of polydimethylsiloxane micropillars as substrates (Supplementary Video 1), we find that mouse embryo fibroblast (MEF) CUs resemble sarcomeres and pull opposing pillars in nanometre-level myosin-II-generated stepwise contractions as verified by different analytical tools. What determines rigidity sensing is the number of steps taken before reaching a \(\sim 20\text{ pN}\) force level, which activates adhesion reinforcement. The stepwise movements are markedly altered after the knockdown of tropomyosin 2.1 (Tpm2.1, formerly known as Tm1; ref. 14), indicating that it has a critical role in controlling force production and rigidity sensing. We further link the role of Tpm2.1 in rigidity sensing to suppression of cellular growth on soft matrices.

RESULTS

Molecular organization of CUs resembles sarcomeres

When plated on 0.5-\(\mu\)-m-diameter fibronectin-coated pillars, fibroblasts use CUs at the cell edge (Fig. 1a,b) to pull on neighbouring...
Figure 1 Contractile units (CUs) at cell edges require myosin. (a) Schematic illustration of a CU at the cell edge. (b) Left: actual CUs observed at the edge of a cell spreading on 8.4 pN nm⁻¹ pillars (experiment was repeated 7 times, 45 videos taken altogether). Arrows represent pillar movement vectors; red, contractile pairs; yellow, non-paired pillars. Cell edge is marked in blue. Right: typical displacement versus time of two pillars that were part of a CU. (c) α-Actinin localizes to the cell edge during the P2 stage of spreading (∼15 min after initial attachment) but is distributed evenly during P1 (from initial attachment up to ∼15 min). Experiment was repeated three times (10 videos altogether). (d) GFP–myosin-IIA and immunolabelled myosin-IIA localize to the cell edge. Experiment was repeated twice. (e) Myosin-IIA is required for force production in CUs. Typical forces generated by myosin-IIA-knockdown (KD) cells show a significant reduction of the inward-directed forces (see also Supplementary Fig. 1b); only ∼25% of the pillars show inward movements compared with >80% in wild-type cells. CUs are rarely observed, and even in such cases they are short-lived and cause small pillar displacements (average maximum displacement = 23 ± 2 nm). Experiment was repeated twice (9 videos altogether). (f) Treatment of the cells with blebbistatin (50 μM) leads to a rapid halt in pillar displacement. Experiment was repeated twice (6 videos altogether).

To verify that the localization of the different sarcomeric proteins was not pillar dependent, we analysed cells on fibronectin-coated coverslips after 15 min of spreading and observed similar distributions relative to β₃-integrin adhesion sites. Tmod3 (Supplementary Fig. 2b) and p-MLC (Fig. 2b) appeared between nascent adhesions, Tmod3 appeared between adhesions with some overlap with β₃-integrin (Fig. 2b), and α-actinin co-localized with β₁-integrin (Fig. 2b). As the CU-generated forces were relatively low (maximum force ∼400 pN), we postulated that the p-MLC clusters corresponded to single myosin filaments that drove the local contractions. To test this, we tracked the pillar movements by GFP–α-actinin-expressing cells, fixed and then stained for p-MLC. After analysis to find contractile pillar pairs (see below), we performed super-resolution analysis (3B microscopy; ref. 24) and observed that α-actinin was concentrated on the pillar edges and that p-MLC between the contracted pillars had a dumbbell shape that resembled that of bipolar mini-filaments (Fig. 3a). The average size of these filaments, 377 ± 16 nm, matched the known size of myosin-II mini-filaments (Fig. 3b).

Overall, these results were consistent with a sarcomeric organization within CUs. In this, α-actinin anchored force-bearing, Tpm-decorated actin filaments at the pillars, and an active myosin-IIA filament was in the centre, producing contractile forces on the pillars (Fig. 3c).
Figure 2  Distribution of sarcomeric proteins in CUs. (a) Top: patches of p-MLC localize between pillars at the cell edge, whereas α-actinin is localized around the pillars. Middle: tropomyosin (Tpm) overlaps with α-actinin at the edges of the pillars (arrow in zoom-in image) and is also located between pillars (arrowhead). Experiment was repeated 3 times. Bottom: normalized average fluorescence intensities of α-actinin, p-MLC and Tpm on 0.5 μm pillars measured from line scans between two adjacent pillars (n = 20 traces from 4 cells in each case).

Figure 3  Myosin mini-filaments appear in CUs. (a) 3B super-resolution image of p-MLC (red) and α-actinin (green) in a contractile pair, where the displacement of the pillars was tracked (traces, right) and the final displacement vectors (about 35 nm) are marked (arrows). Note the dumbbell shape of p-MLC, consistent with the known shape of myosin mini-filaments. Experiment was repeated 3 times (7 videos altogether). (b) Left: additional examples of dumbbell-shaped p-MLC filaments from super-resolution fluorescence analyses. Right: the length histogram of these dumbbell shapes matches the known size of myosin mini-filaments. n = 30 patches from 5 cells. (c) Schematic of a CU with the relevant molecular components.

Pillar displacement occurs by nanometre-level steps that are constant regardless of rigidity

As myosin was known to move by nanometre-scale steps26,27, and the velocity of pillar displacement was relatively low (2.5–3.5 nm s−1), it seemed possible to analyse the contractions at the nanometre level. Videos taken at a frame rate of 100 Hz were used to analyse individual pillar positions using a cross-correlation technique that enabled nanometre-level tracking over time28. To test the accuracy of this method, we used a piezo device to move the pillar array in steps of 0.6 or 1.2 nm at frequencies of up to 4 steps per second (Fig. 4a...
Cells pull on pillars with nanometre-level steps. (a) Top: median-averaged tracking data of a single pillar displaced by a piezo device with 1.2 nm steps at 2 steps per second (blue), along with fitting data using the step-detection algorithm (black). Bottom: the frequency of detection and average step sizes detected (± s.e.m.) using the step-fitting algorithm on data obtained by piezo-controlled movements with 0.6 and 1.2 nm steps at different rates. n = 102, 96, 99 steps from 12, 10, 11 pillars for the 0.6 nm steps at 2, 3, 4 steps per second data, respectively; the mean detected step sizes were insignificantly different from 0.6 nm: P values = 0.11, 0.57 and 0.22, one-sample t-test. n = 90, 92, 94 steps from 10, 11, 11 pillars for the 1.2 nm steps at 2, 3, 4 steps per second data, respectively; the mean detected step sizes were insignificantly different from 1.2 nm: P values = 0.19, 0.27, 0.3, one-sample t-test. All piezo-driven experiments were repeated 4 times for each case. (b) Top: median-averaged displacement data of a single pillar that was part of a CU at the cell edge (green) with the raw 100 Hz measurements in grey, along with fitting data using the step-detection algorithm in black. Bottom: negative control data along with its step-fitting algorithm in black. (c) Histograms of the steps detected in the real and negative control data (n = 527 steps from 24 pillars in 7 cells; experiment was repeated 4 times).

and Supplementary Fig. 3a). With a step-fitting algorithm\textsuperscript{29} as well as pairwise-distance analysis\textsuperscript{30,31} we were able to detect the steps reliably (Fig. 4a and Supplementary Fig. 3b).

Next, as cells spread on arrays of 1.3-\(\mu\)m-high, 0.5-\(\mu\)m-diameter pillars (pillar stiffness = 8.4 pN nm\(^{-1}\)), we tracked pillar movements during P2 in the lamellipodium. In local CUs, we analysed movements of two opposing pillars as they were pulled together, defining a contractile pair (Supplementary Video 2). The high-frequency displacement curves contained abrupt transitions (steps) from one pillar position to another (Fig. 4b) and pairwise-distance analysis showed a step size of 1.1 ± 0.2 nm (Supplementary Fig. 3c). As a control for thermal vibrations, light fluctuations, and detector noise, for each pillar we analysed a ‘step-free’ curve derived from a polynomial fit to the displacement curve with added noise from a pillar outside the cell (Fig. 4b and Supplementary Fig. 3d). These negative control curves contained many fewer steps than the real ones (Fig. 4b), and pairwise-distance analysis did not detect the 1.1 nm steps seen in the real cell displacement data (Supplementary Fig. 3e). When we applied the step-detection algorithm, we found that on average, each negative control curve contained ~45% ramps, compared with ~12% in the real curves (see Supplementary Fig. 3f for definition of steps versus ramps). The steps that were found in the negative control curves were described by a single Gaussian distribution centred at 0.4 nm, with ~90% of the steps below 0.6 nm (Fig. 4c), providing a threshold below which the steps detected were attributed to noise. When we applied the step-detection algorithm to the real cell displacement curves, the distribution of detected steps was well fitted with two Gaussians, one centred at 0.4 nm and the other at 1.2 nm (Fig. 4c). When the noise-dependent steps were subtracted from the real data, the remaining steps had an average value of 1.2 ± 0.6 nm (mean ± s.d.). We also performed negative control measurements to verify that no contribution to the steps came from optical or pillar configuration effects (Supplementary Fig. 4), as well as an additional positive control experiment in which >90% of the steps were detected (188/207 steps from 16 pillars in 8 runs) when the piezo device was programmed to better mimic the real pillar displacements (1 nm steps separated by random, exponentially distributed time intervals).

Taken together, these results showed that contractions of CUs involved nanometre-scale steps at a frequency of ~2–3 steps per second and were reliably detected with our method.

Next, cells were plated on soft pillars (2.3 \(\mu\)m high, 0.5 \(\mu\)m diameter; stiffness = 1.6 pN nm\(^{-1}\)). As in vitro data showed bigger myosin step sizes under lower loads\textsuperscript{2}, we expected larger steps on the softer pillars. However, the mean step size was indistinguishable on both rigidities: 1.2 ± 0.7 nm for soft (n = 344 steps from 20 pillars) versus 1.2 ± 0.6 nm for stiff pillars (n = 307 steps from 20 pillars).
Regardless of rigidity, we postulated that rigidity sensing was related to...

Early contractile steps are simultaneous

To further characterize the CUs, we analysed the very early stages of CU displacements towards each other, during which only a few myosin molecules were expected to be involved. We observed simultaneous steps (within ~100 ms) towards the centre of the CU (inward) in both pillars (Fig. 5a and Supplementary Fig. 5a). A plot of the time between the inward and outward steps showed a normal distribution centred at zero time (Fig. 5b). The sum of simultaneous steps gave an average total displacement of 2.5 ± 0.6 nm on the stiff pillars and 2.4 ± 0.7 nm on the soft ones (Fig. 5c), approximately twofold larger than the mean step size for single pillars. Not all steps were simultaneous, but the lack of pairing for some steps was attributed to interactions of filaments with other neighbouring pillars or with the general flow of actin rearward. Simultaneous steps were not found in antiparallel displacements of neighbouring pillars that were not part of CUs (a completely random time difference between the steps was found in such pillars; Fig. 5a,b).

These results indicated that an actomyosin structure connecting the pillars contracted by steps of ~2.5 nm about every 300 ms, and displaced each pillar approximately equally, providing strong evidence that the displacements were driven by a single bipolar myosin filament.

Rigidity sensing is regulated by the level of force

As step sizes and maximal displacement of the pillars were the same regardless of rigidity, we postulated that rigidity sensing was related to...
If reinforcement of the adhesions occurred during the pause, then proteins that could strengthen the integrin–actin interaction such as α-actinin (which binds both to actin and integrin) might have been recruited. Indeed, cross-correlation analysis between pillar displacements and GFP–α-actinin intensity around pillars showed that its recruitment correlated strongly with force development, whereas the recruitment of vinculin (which binds to actin but not to integrin) did not (Supplementary Fig. 6a–c). Initial forces were developed seemingly without α-actinin, possibly mediated by talin15 (Supplementary Fig. 6d), but the peak in α-actinin fluorescence (and not talin fluorescence) preceded the peak in force development by ~7.5 s (Fig. 6c), indicating that it was required to reach peak force. Consistent with this, α-actinin recruitment typically increased markedly during pauses in displacement (Fig. 6d). We suggest that the pause at a force level of ~20 pN was for adhesion reinforcement that was needed for further force development.

**Tpm2.1 affects force, step size, step rate, and rigidity sensing**

The constant step size indicated that there was a structural restriction of motor movement, perhaps due to Tpm, which mechanically alters myosin binding to actin in muscle. To determine which Tpm isoform was present at the cell edge, we transfected the cells with YFP- or GFP-tagged Tpm2.1, Tpm1.6 or Tpm1.7, and found that the last two localized primarily to central regions of cells, whereas YFP–Tpm2.1 localized to the cell edges (Supplementary Fig. 7a). When we knocked down Tpm2.1 expression (Tpm2.1-KD; Supplementary Fig. 7b) and immunostained the cells for HMW Tpms, we observed that the edge localization was lost (Supplementary Fig. 7c). Live imaging of spreading of YFP–Tpm2.1-expressing cells showed that Tpm2.1 gradually assembled at protruding cell edges and then retracted with the membrane (Supplementary Video 4), consistent with a possible involvement of Tpm2.1 in local contractions for rigidity sensing.

To test this, we analysed the effect of Tpm2.1-KD on cell spreading on different rigidities. Whereas Tpm2.1-KD cells spread equally on both stiff and soft pillars after 1 h, control cells spread to ~1.5-fold greater area on stiff pillars compared with soft ones (Fig. 7a). Also, control cells were polarized and elongated, but Tpm2.1-KD cells were typically round (Supplementary Fig. 7d). These results indicated that Tpm2.1-KD cells were defective in their rigidity sensing. Importantly, after Tpm2.1-KD, the local contractions were highly disorganized (Supplementary Fig. 7e) and pillar displacements were significantly larger than in control cells (with even higher displacements on softer pillars; Fig. 7b). Further, the rate of pillar movement increased twofold compared with control rates (Fig. 7c and Supplementary Fig. 7f). Analysis of stepping movements in Tpm2.1-KD cells revealed step sizes of up to ~5 nm that were not observed in controls, and an average step size of 2.2 ± 0.7 nm (Fig. 7d and Supplementary Fig. 7g). In many cases, high forces caused release of pillars (similar to the ultrasoft pillars) and halted further spreading, possibly owing to impaired adhesion development. Indeed, control cells plated on fibronectin-coated coverslips for 1 h formed large, elongated adhesions, but the Tpm2.1-KD cells formed only small, round adhesions at the cell edge.
Tpm2.1 regulates force production, step size and adhesion growth. (a) Average area ± s.e.m. of cells transfected with non-targeting siRNA (control) and Tpm2.1-KD cells after 1 h of spreading on stiff and soft pillars (n=50, 52, 51, 54 cells). Experiment was repeated 3 times. (b) Mean ± s.e.m. of the maximal displacements of pillars when control and Tpm2.1-KD cells were plated on stiff and soft pillars (n=81, 79, 70, 71 pillars from at least 5 cells in each case). Experiment was repeated twice. (c) Left: typical pillar displacement by a Tpm2.1-KD cell, showing high displacement rate and large steps. Right: quantification of pillar displacement rates (mean ± s.e.m.) by Tpm2.1-KD and control cells (n=20 pillars from 3 cells in each case). Experiment was repeated three times. (d) Average step sizes for control and Tpm2.1-KD cells. Red lines are the median values, the edges of the blue boxes are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, and outliers (data greater than 3 s.d. from the median) are plotted individually as red dots; comparison between boxes is done by the overlap of the notches: if they do not overlap, the conclusion with 95% confidence is that the true medians do differ (n=344 and 316 steps from 20 and 15 pillars for control and KD cells, respectively). Experiment was repeated 3 times. (e) Adhesions are much smaller after Tpm2.1-KD. Left: micrographs showing the distribution of paxillin–GFP in Tpm2.1-KD and control cells. Right: quantification of adhesion sizes in Tpm2.1-KD and control cells (n=245 and 256 adhesions from 6 cells in each case). Experiment was repeated twice. ***P < 0.001, two-tailed, equal variance t-test.

Tpm2.1 suppresses growth on soft matrices by supporting rigidity sensing

Many cancer cells that were anchorage-independent for growth exhibited very low Tpm2.1 levels, and its re-expression reverted them back to become anchorage-dependent and unable to grow on soft matrices.\textsuperscript{37,38} Thus, we postulated that anchorage-independence was linked to an aberrant rigidity response. To test this, we analysed malignant MDA-MB-231 and non-malignant MCF-10A breast epithelial cells. Immunostaining of these cells showed that Tpm was present at the edges of MCF-10A cells but not of MDA-MB-231 cells (Fig. 8a).

CU's were observed in MCF-10A cells but not in MDA-MB-231 cells (Supplementary Fig. 7i). Forces produced by MDA-MB-231 cells were significantly higher than MCF-10A cells (maximal displacements of 1.6 pN nm\textsuperscript{−1} pillars by MDA-MB-231 and MCF-10A cells were 187 ± 53 and 49 ± 20 nm, respectively; n=39, 35 pillars), and there was considerable adhesion breakage by MDA-MB-231 but not by MCF-10A cells. High-resolution analysis of the pillar movements indicated that the average step size for MCF-10A cells was the same as wild-type MEFs, whereas for MDA-MB-231 cells it was twofold larger, similar to Tpm2.1-KD MEFs (Fig. 8b). MCF-7 cells, another malignant breast epithelial cell line with low Tpm2.1 levels,\textsuperscript{39} had a similar step size as MDA-MB-231 cells (Fig. 8b), and high maximal displacements (92 ± 21 nm, n=38 pillars). When we knocked down Tpm2.1 expression in MCF-10A cells (Supplementary Fig. 7j), the maximal displacements increased to 103 ± 15 nm (n=40 pillars) and step sizes to 1.9 ± 0.8 nm (Fig. 8b). Conversely, when we transfected MDA-MB-231 cells with YFP–Tpm2.1, the average step size decreased twofold (Fig. 8b) and the maximal displacements dropped to controls, 50 ± 18 nm (n=45 pillars). Also, YFP–Tpm2.1-expressing MDA-MB-231 cells formed larger adhesions than non-expressing cells (Fig. 8c).
Figure 8 Tpm2.1 differentiates between normal and malignant cell lines and controls growth on soft matrices. (a) Examples of MCF-10A (M-10A) and MDA-MB-231 (M-231) cells plated on pillars and immunostained for Tpm. Experiment was repeated twice. (b) Average step sizes for MCF-10A, MDA-MB-231, MCF-7 (M-7), Tpm2.1-KD MCF-10A, and MDA-MB-231 cells with YFP–Tpm2.1 expressed (n = 231, 248, 245, 311, 270 steps from at least 12 pillars in each case). Experiment was repeated twice for each case. Red lines are the median values, the edges of the blue boxes are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, and outliers (data greater than 3 s.d. from the median) are plotted individually as red dots; comparison between boxes is done by the overlap of the notches: if they do not overlap, the conclusion with 95% confidence is that the true medians do differ. (c) Adhesion sizes are larger in MDA-MB-231 cells when Tpm2.1 is expressed. Left: micrographs showing the distribution of paxillin–GFP in MDA-MB-231 and MDA-MB-231+Tpm2.1 cells. Right: quantification of adhesion sizes in Tpm2.1-KD and control cells (n = 220 and 217 adhesions from 6 cells in each case). Experiment was repeated twice. (d) Soft agar assay showing growth of Tpm2.1-KD MCF-10A cells but not of control cells. Cells were stained with crystal violet. Experiment was repeated twice. Error bars represent s.d.

To test the involvement of Tpm2.1 in anchorage-independent growth, we performed a soft agar assay and observed that control MCF-10A cells did not survive after two weeks in culture whereas Tpm2.1-KD MCF-10A cells proliferated and formed colonies (Fig. 8d), confirming that defective rigidity sensing in the absence of Tpm2.1 contributed to the ability of cells to grow on soft matrices.

DISCUSSION

Rigidity sensing is a fundamental function that is highly dynamic, occurs on small length scales, and affects many cell processes. In these studies, sarcomere-like CUs contract 500 nm pillars by nanometre-level steps for rigidity sensing. Applying our analysis method to lamellipodial CUs, we reliably find single pillar steps of about 1.2 nm at low and intermediate rigidities that differ significantly from noise. In contractile pairs of pillars, there are simultaneous, antiparallel displacements of ~2.5 nm. This step size is smaller than the working stroke size of myosin-II reported from single-molecule in vitro studies; however, it is consistent with the fact that the stroke size decreases when the velocity of actin filament sliding is low and when Tm is present. Our measurements also correlate well with the in vitro movements of actin filaments by myosin filaments that occur in steps of 2.7 nm (ref. 42).

Nanometre-level movements could theoretically originate from several different mechanisms, including conformational changes of adhesion proteins, receptor movements, subtraction of single actin monomers from filaments attached to pillars, or myosin contractions. Although we cannot completely rule out other possibilities, several observations indicate that the steps are due to myosin contraction. First, simultaneous antiparallel steps of pillar pairs indicate that the steps are due to myosin contraction. Second, the CUs contain many sarcomere proteins and a single bipolar myosin filament. Third, a bipolar myosin filament with only few myosin heads engaged can produce ~30–50 pN (ref. 32) and the pause force of 20–30 pN fits well with this stall force. Fourth, the depletion of Tpm changes the step length as expected for myosin movements controlled by Tpm.

Assuming that contractile steps are myosin dependent, there are two different mechanisms that could account for the 2.5 nm step size. The first possibility is that the steps we detect are not the full myosin working stroke; this is in line with recent X-ray interference and mechanical measurements of sarcomeres that suggest that the myosin stroke size occurs by sub-steps of ~2.75 nm (refs 40, 45). The second possible mechanism is based on recent measurements that show that Tpm limits the binding of myosin to actin. Under this model, a myosin filament effectively moves from one actin monomer on a single strand to the next on the neighbouring strand, thus displacing the actin filament by half the length of an actin monomer, that is, ~2.7 nm. This latter model is more favourable because Tpm depletion not only...
increases step size, but also the velocity. Thus, under this model, in the absence of Tpm2.1 there are more available binding locations for myosin on the actin filaments, allowing it to produce high forces rapidly (Supplementary Fig. 8a).

The speed of pillar movement, 2.5–3 nm s\(^{-1}\), is significantly lower than the stepping rates of myosin molecules found \textit{in vitro}, but is consistent with the movement velocities of actin nodes by myosin in fibroblasts\(^{46}\), as well as with measurements and computational models that show that the behaviour of single myosin molecules is vastly different from small myosin ensembles (mini-filaments; refs 44,47). In the latter case, increased load significantly reduces the movement velocities of actin filaments, to the range of several nanometres per second\(^{44,47}\). Further, when we test a wider range of pillar rigidities, we find that there is an inverse relationship between force and velocity (Supplementary Fig. 8b), in line with the known behaviour of sarcomeres, which contract slower with increased load.

In terms of the molecular mechanism of rigidity sensing, we propose that it is not the step size or rate of stepping, but rather exceeding a velocity (Supplementary Fig. 8b), in line with the known behaviour of sarcomeres. This pause is best explained by the fact that under high loads the lifetime of myosin–actin attachment increases substantially\(^{48}\) (another process that could contribute to the pause is the 'latch state' of myosin heads, which can occur when light chains of bound myosin heads are de-phosphorylated, leaving myosin bound and still generating tension\(^{49,50}\)). As the force-producing mini-filaments contain a small number of myosin heads, the ~20 pN force level puts each head under a high load where it is in a strongly actin-bound state\(^{48}\), resulting in a plateau in the displacement curve. As the step length is constant regardless of rigidity, the myosin heads need to move many more steps on softer pillars to develop the same tension. Once the threshold force is reached, there is reinforcement of the integrin adhesions that involves recruitment of α-actinin and presumably other adhesion proteins\(^{51}\). After reinforcement, forces on adhesions continue to increase, perhaps through further activation or recruitment of myosin (Supplementary Fig. 8a). On ultrasoft surfaces the force does not rise rapidly enough to induce a pause, and the adhesions dissociate without reinforcement. Conversely, adhesion breakage is observed also in the absence of Tpm2.1 owing to the very high forces that are produced rapidly.

Notably, these observations may explain the involvement of Tpm2.1 as a repressor of cancer\(^{10,52}\). When plated on soft matrices, non-transformed cells produce low forces, leaving the adhesions small\(^{33,54}\), and thus the integrin-mediated signals that promote cell-cycle progression are absent; instead, apoptotic signals are activated. Cancer cells override the requirement for stable adhesions to proliferate, and thus they can grow on soft matrices. We propose that signalling cascades would be repeatedly activated by the high forces that are being produced in the absence of Tpm2.1 (presumably through activation of mechanosensory proteins\(^{55,56}\)). Hence, the cells would not be dependent on the formation of strong adhesions. This is indeed what we observe in the case of non-malignant MCF-10A Tpm2.1-KD cells. This model suggests that there is an accumulation of mechanically activated signals over time. Thus, the connection between force and vital cellular signals should be further studied by long-term tracking of forces, protein dynamics, and activation of signalling cascades.

\[^4\]Author contributions

H.W., G.M., S.L., F.R.-C., J.H. and M.P.S. designed the study; H.W., G.M., S.L. and T.I. performed the experiments; H.W., G.M., S.L., M.R.S., T.I. and B.O’S. were involved in the data analysis; S.L. and S.G. fabricated pillar moulds; H.W. and S.L. wrote Matlab codes for analysis of pillar movements; H.W., G.M., J.H. and M.P.S. wrote the manuscript.

\[^5\]Competing financial interests

The authors declare no competing financial interests.

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\[^6\]METHODS

Methods and any associated references are available in the online version of the paper.

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\[^9\]AUTHOR CONTRIBUTIONS

H.W., G.M., S.L., F.R.-C., J.H. and M.P.S. designed the study; H.W., G.M., S.L. and T.I. performed the experiments; H.W., G.M., S.L., M.R.S., T.I. and B.O’S. were involved in the data analysis; S.L. and S.G. fabricated pillar moulds; H.W. and S.L. wrote Matlab codes for analysis of pillar movements; H.W., G.M., J.H. and M.P.S. wrote the manuscript.

\[^10\]COMPETING FINANCIAL INTERESTS

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**METHODS**

**Cell culture, transfections, and spreading experiments.** Wild-type MEFs were generated in-house by J. Sap’s laboratory and myosin-IIA-KD MEFs were generated from the wild-type MEFs as described previously. MDA-MB-231 and MCF-10A cells were obtained from J. Groves (University of Arizona, USA and Mechanobiology Institute, National University of Singapore, Singapore); MCF-7 cells were from ATCC. MCF-7 and MDA-MB-231 cells were cultured at 37 °C in a 5% CO2 incubator in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU ml−1 penicillin–streptomycin, 2 μM l-glutamine and 2 μM HEPES; MCF-10A cells were cultured at 37 °C in a 5% CO2 incubator in DMEM supplemented with 20 ng ml−1 EGF, 10 ng ml−1 bovine insulin, 500 ng ml−1 hydrocortisone, 5% horse serum albumin, and 100 IU ml−1 penicillin–streptomycin (all reagents were from Life Technologies). Cell lines were not authenticated. MEFs were tested for mycoplasma contamination and found to be negative.

Transfections were carried out 1 day before measurements using the Amaxa Nucleofector System (Lonza) according to the manufacturer’s instructions, with ~106 cells per reaction and 4–5 μg DNA.

One day before spreading experiments, cells were sparsely plated to minimize cell–cell interactions before re-plating. The following day, the cells were trypsinized using TrypLE (Life Technologies), centrifuged with growth medium, and then resuspended and pre-incubated in Ringer’s buffer (150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 20 mM Hepes and 2 g l−1 D-glucose at pH 7.4) for 30 min before the experiment. Cells were then spread on pillar arrays, or on silanized cover glasses (2 h in 20% nitric acid, followed by exposure to gaseous 1,1,1,3,3,3-hexafluoro-2-propanol (SiH2Cl6)) that were pre-coated with 10 ng ml−1 human plasma full-length pure fibronectin (Roche) or collagen I (Corning) for 1 h at 37 °C. In all cases we made sure that the cells were not clustered when plated on the substrates.

**Plasmids and siRNA oligonucleotides.** GFP- and YFP-tagged Tpm plasmids were a gift from P. Gunnung (The University of New South Wales, Australia); mCherry–tropomodulin3 was a gift from C. Gregorio (University of Arizona, USA); GFP–α-actinin was a gift from C. Oey (University of North Carolina, USA); GFP–vinulin was a gift from K. Yamada (National Institute of Health, USA); RFP–paxillin was a gift from M. Partridge (Columbia University, USA); GFP–VASP was a gift from J. Wehland (German Research Center for Biotechnology, Germany).

Knockdown of Tpm2.1 was performed with siRNA oligonucleotides (target sequence: 5′-AACGACATCGTCTGAGATCTCA-3′) labelled with Alexa-488 (Qiagen). Cells in 35 mm plates were transfected with 2,100 ng oligonucleotide duplexes using GeneSilencer’s siRNA transfection reagent (Gene Therapy Systems) according to the manufacturer’s instructions. After transfection, cells were incubated for 72 h for efficient depletion of the target protein. Specific knockdown of Tpm2.1 was then tested by western blotting using TM-311 (Sigma) as a primary antibody. Although on average the knockdown was not complete (~70–75%), single-cell analysis showed that the effects of Tpm2.1 KD were most prominent in cells that showed high fluorescence levels of the transfected siRNA.

For verification of the effect of Tpm2.1 knockdown, an mRNA plasmid (Mission siRNA, sigma) was used (target sequence: 5′-TTGGTTACCAAACCTGAAAT-3′) for transfecting the cells (transfection was carried out using the Amaxa system); experiments were performed 72 h following transfection.

Knockdown of Tpm1.7 was carried out using shRNA plasmids (Sigma; target sequence: 5′-CGGAGATCCGTCTGTAAC-3′, shRNA2–5′-CGGATCTAGCCTTFCGGACT-3′). The myosin-IIA knockdown cell line (target sequence: 5′-GGTGGAAGTGAAGGAAAGC-3′) was previously described[3]. For verification of the effect of myosin-IIA knockdown, an shRNA plasmid (Sigma; target sequence: 5′-CGGAAAACTCTCGCTATG-3′) was used. Western blotting was used to test knockdown levels using a primary antibody against myosin-IIA (Abcam, ab24762). Load controls for the western blots were done using a tubulin antibody.

High-frequency bright-field microscopy and pillar displacement measurements. Time-lapse imaging of pillars was performed with bright-field microscopy using an ORCA-Flash2.8 CMOS camera (Hamamatsu) attached to an inverted microscope (Olympus IX-81), controlled by Micromanager software[29]. Images were recorded continuously at 100 Hz using a ×100 1.4 NA, oil-immersion objective (yielding a pixel size of 36 nm pixel−1). For each cell, a video of ~5 min (corresponding to 30,000 frames) was recorded; stage drift and focus changes were minimized by using a controlled 37 °C chamber.

To minimize the noise arising from the imaging conditions, we removed the shutter from the illumination path and used a >600 nm filter that allowed us to increase the light intensity significantly to reach high pillar-to-background contrast without significant photo-damage to the cells. As we were analysing the position of the pillars 100 times per second, the noise for a 1 s period was tenfold lower than in the standard protocols where only one pillar position per frame was recorded. All of these improvements resulted in a typical noise amplitude that ranged between 0.8 and 1.2 nm, with generally lower noise for the softer pillars (theoretically, increasing the pillar length should also increase the thermal noise of the pillar tip; however, we find that the noise level depends mostly on the contrast between the pillars and background, which is higher with the 2.3 μm pillars and therefore their noise amplitude is lower).

**Tracking pillar movements.** Pillar tracking over time was performed with Imagel (National Institutes of Health) using the Nano Tracking plugin[30]. In this technique, two regions of interest (ROIs) were used: one containing an image of a pillar of interest and another containing the surrounding area through which this pillar moved during the video (typically the latter ROI was ~2-fold larger than the former even though the movements were much smaller than twice the size of a pillar). Next, calculation of the cross-correlation between the pillar image in every frame of the video was performed with an image of the same pillar (called the ‘kernel’) from the first frame of the video. The cross-correlation of the pillar image with the kernel had a peak centred at the position in the pillar image where the surrounding intensity most closely matched the intensity distribution in the kernel. Next, an intensity threshold value, T, was subtracted from the cross-correlation, and the negative pixels were discarded, isolating only those pixels that contributed to the central peak. The centroid (x, y) of these remaining pixels was then calculated according to:

$$x = \frac{\sum C(x,y) x}{\sum C(x,y)}$$

$$y = \frac{\sum C(x,y) y}{\sum C(x,y)}$$

This way, the relative position of the pillar in every frame of the video was accounted for. To account for stage drift, the average displacement of a set of pillars far from any cell was subtracted from the data. After analysis, a displacement map on each frame in the video was generated in-house by J. Sap’s laboratory (see Supplementary Video 2).

Using this technique, the overall precision of position measurements made every 10 ms (based on the standard deviation of the frame-by-frame differences in the movements of a pillar not in contact with the cells, see ref. 58)) was 0.8–1.2 nm. As the time between steps was on average 0.25–0.28 s, we could reduce this noise level 2.5–3-fold by averaging over multiple frames without blurring the steps using a 15 pt linear moving-medium filter (similar results were obtained when we used a 21 pt nonlinear Savitzky–Golay filter[31]).

In general, factors that might affect the accuracy of the measurements are changes in focus and uneven illumination of a pillar[2]. We made sure that the focus was stable by analysing a reference pillar outside the cell in each video; changes in focus would result in changes in the apparent movement of a reference pillar, and so we monitored that its movement was stable on average throughout the measurement (that is, the overall second-scale movement, not the rapid millisecond-scale fluctuations). The uneven illumination of a pillar might result in a skewed representation of the pillar’s centroid. Whereas typically the illumination was even over regions larger than a single pillar, a local effect could occur when the cell was not completely covering a pillar, resulting in one side of the pillar being brighter than the other. This resulted in some cases in an apparent outward movement of pillars on cell–pillar contact that we referred to in our previous publication as a ‘lensing effect’[25]. We estimated that this effect could account for a maximum displacement value of ~25 nm (ref. 9); therefore, in the current work we analysed the nano-steps only after the cell edge has passed the pillars completely. Another concern that we took into account was that we might see pillar movements as a result of contact between the pillar and the substrate before the experiment. Cells were then spread on pillar arrays, or on silanized cover glasses (2 h in 20% nitric acid, followed by exposure to gaseous 1,1,1,3,3,3-hexafluoro-2-propanol (SiH2Cl6)) that were pre-coated with 10 ng ml−1 human plasma full-length pure fibronectin (Roche) or collagen I (Corning) for 1 h at 37 °C. In all cases we made sure that the cells were not clustered when plated on the substrates.

**Step-fitting algorithm.** Fitting the median-filtered pillar movements data (either cell- or piezo-generated) was done using the L1-PWC algorithm described in ref. 29. This algorithm detects steps generated by molecular motors in noisy data series. The only parameter that can be changed in this algorithm is γ, which controls the smoothing of the data and should be set to at least 2σ, where σ is the standard deviation of the noise. In practice, analysis of data series of pillar movements by cells loaded with filamentous actin showed that γ values ranging from 2 to 15; therefore, we used a γ value of 10 for all of the analyses of pillar movements.

In some cases, a clear transition from one pillar position to the next was fitted by the algorithm as a large step (of ~1 nm) with a much smaller step immediately adjacent to it; thus, if we were to simply subtract the pillar position in one frame from the previous, this would have resulted in an underestimation of the step size (see Supplementary Fig. 3). Therefore, we performed a post-fitting procedure in which we defined as a step only one pillar position per frame where the transition lasts less than 5 frames, and the second pillar position is being held for...
more than 10 frames. This enabled the distinction between transitions that occurred by steps and those that occurred by ramps (see Supplementary Fig. 3f for examples).

Piezo-controlled measurements. For the piezo-device experiments, movements of pillar arrays by the piezo stage (PI, model P-517) were controlled by a Modular Piezo Controller (PI, model E-500) and a 10 MHz DDS function generator (Vavetek, model 29). Step sizes were first calibrated by verifying a linear relationship between function generator voltage and step size. In this way it was determined that 0.1 V corresponded to a step size of 0.6 nm. In a typical experiment using the piezo device, we took videos of areas of ~6 × 50 pillar arrays. Within a single sequence, there were very few pillar-to-pillar variations (see Supplementary Fig. 3a). In these experiments, the piezo device moved 9 steps (of 0.6 or 1.2 nm) in varying rates (2-4 steps per second) and at the end of this cycle it jumped back to its starting position to start another cycle with the same parameters.

As an additional positive control, to better mimic the pillar displacements, we programmed the piezo device to move by 1 nm steps with time intervals that were randomly selected from an exponential distribution ranging from 20 to 50 frames between the steps and an overall pillar movement velocity of 2.5-3 nm s⁻¹. Each run lasted 5 s and contained 13-15 steps (see main text).

Construction of negative control data. As a negative control, for each pillar that we analysed we used Matlab to construct a curve that mimicked the original data by taking the overall pillar displacement trend (a 5th degree polynomial fit of the displacement) and adding it to the noise during the time of the displacement. The noise was taken from a pillar that was not in contact with the cell. We also verified that the fluctuations of pillars that were not in contact with cells were the same as those of pillars underneath cells while the latter were not being displaced (see Supplementary Fig. 4d).

Thus, these data contained a realistic signal and were subjected to an identical data analysis as the real data. We did this to test the alternative hypothesis that the steps arose from the analysis method or from some stage/environment noise (thus considering possible systematic errors that might exist in the real data). In this alternative scenario the pillars were moving in a smooth manner and the steps that we were detecting were the result of the pillar noise. If the alternative scenario were true, there would have been no difference in the steps between our real data and the negative control data. However, a significant difference between the steps in the real and negative control data was observed, thus rejecting the alternative hypothesis.

Notably, noise alone contributed almost no steps, rather it was the combination of noise and the slope of the displacement (the 5th degree polynomial) that gave rise to the small steps (up to ~0.6 nm).

Pairwise-distance analysis. Analyses of the pairwise differences between data points in pillar displacement curves were performed for a given filtered record (filtered using a 15-point median filter) by considering all possible inter-point amounts of displacement (that is, 2 3 10 frames, where N was the final frame number of each data record) and then plotting the histogram of all the resulting pairwise-distance differences (that is, the pairwise-distance distribution function, PDF) between ~25 and ~25 nm, with a bin size of 0.1 nm. The PDFs of all records were then normalized between 0 and 1 and averaged, resulting in the PDFs presented in Supplementary Figs 3 and 7. Next, fast Fourier transform was performed using Matlab on each single PDF after removing the so-called "flat" area by subtracting a moving average of each PDF from itself (the peaks in the power spectra were observed also when applying the Fourier transform directly to the original PDFs, but they were more pronounced after the removal of the DC component). The resulting power spectra were then averaged, followed by smoothing of the average power spectrum with a 10-point moving average window, and normalization to unity at a spatial frequency of 0 nm⁻¹. An identical power spectrum was obtained when applying the Fourier transform to the averaged PDF and when applying it to each individual PDF and then averaging the resulting power spectra. To compute the error in the peak location in the Fourier transforms, we recorded the spatial frequency that corresponded to the peak in each individual curve and calculated the s.e.m. of all these values.

Fluorescence microscopy. For immunofluorescence microscopy, cells were plated for 15 min on fibronectin-coated pillars, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Immunolabelling was performed with primary antibodies overnight at 4°C, and with Cy3- or Alexa-488-conjugated secondary polyclonal antibodies (Jackson ImmunoResearch Laboratories and Invitrogen, respectively) for 1 h at room temperature. Primary antibodies used were against Thr 18/Ser 19 p-MLC (Santa Cruz Biotechnology, sc-12896), HMW Tpm antibody (model Cascade-II:512, Photometrics), and therefore another likely scenario is that each primary antibody binds to two secondary antibodies, and thus 9 secondary antibodies would label 8 p-MLC molecules in a half mini-filament. Hence, we concluded that on average between 16 and 36 p-MLC molecules (myosin heads) were present in the clusters observed between the pillars.

Imaging for 3B analysis. 3B microscopy uses Bayesian analysis of blinking and bleaching events in a sample of data with many overlapping fluorophores, to calculate the structure of the system at enhanced resolution. Imaging for 3B analysis was performed using an Olympus IX-81 microscope maintained at 37°C with a ×60/1.45 NA oil-immersion objective and an electron-multiplying CCD camera (model Cascade-II:512, Photometrics). 3B analysis was done as described. Owing to the computational cost, the 3B software was run on the Titan cluster at the Center for Computational Biology and Bioinformatics (Columbia University); http://wiki.c2b2.columbia.edu/systems/index.php/Documentation/Titan_cluster.

Pillar and soft gel fabrication. Moulds for making polydimethylsiloxane (PDMS) pillars were fabricated using electron beam lithography in hard poly(methyl methacrylate) (PMMA) substrates. PMMA was then spin-coated onto a silicon substrate and then hard-baked on a hot plate for 1 h. An electron beam lithography tool (NanoBeam nBS) was then used to pattern holes in the PMMA. The depth of holes was dependent on the thickness of the PMMA. PMMA (mixed at 10:1 with its curing agent, Sylgard 184; Dow Corning) was then poured onto the PMMA moulds, cured for 12 h at 37°C, and then hard-baked at 100°C for 2 h. The PMMA was then cooled in acetone and removed from the moulds. Pillar bending stiffness, was calculated using Euler–Bernoulli beam theory: where D and L are the diameter and length of the pillar, respectively, and E is the Young's modulus of the material (PDMS).

Fluorescent labelling of pillar tops was done by stamping highly hydrophobic quantum dots (Life Technologies) after peeling off the PDMS pillars from the moulds in the air (rather than while immersed in isopropanol).

Soft agar assay. The soft agar assay was performed using the Cell Transformation Assays, Standard Soft Agar kit from Cell Biologics according to the manufacturer’s instructions. Five thousand cells were seeded in each well of a 96-well plate. The experiment was repeated twice with triplicates of each condition in each experiment.

Statistics and reproducibility. Sample sizes were chosen to test whether the distributions of the populations were normal. Indeed, the samples test sizes and cell areas had normal distribution, which justified the use of Student’s t-test.
All pillar displacement experiments were performed at least three times on
different days. All fluorescence imaging of immunostained or transfected cells and
the soft agar assay were repeated twice on different days. Immunostainings and
western blots were performed using established antibodies that were previously used
in published manuscripts.

MCF-10A, MDA-MB-231 and MCF-7 cells that were used in this study are highly
established cell lines that are typically used for comparing malignant versus non-
malignant breast epithelial cells. The MEF cells were previously described and have
been used in the Sheetz laboratory in the past decade for cell spreading experiments.
All cells were kept at a low passage number and passed 2–3 times a week.

Analyses of pillar movements were performed only in cases where two pillars
were moving primarily towards each other, which was true in ~1/3 of the CUs that
we analysed; in most CUs there were three or more pillars that moved during the
contraction events (see also ref. 9).

Cross-correlation analysis. Pillar displacements and fluorescence traces were
obtained from time-lapse videos taken at 1 frame per second with a 60× objective
in an Olympus Fluoview FV500 laser-scanning confocal microscope, maintained
at 37°C.

For each pillar analysed, the cross-correlation function between pillar deflection
and protein localization, $c(\tau)$, was calculated:

$$c(\tau) = \frac{\langle d(t + \tau) - \langle d \rangle \rangle \langle I_{\text{max}}(t) - \langle I_{\text{max}} \rangle \rangle}{\sigma_d \sigma_I}$$

where $d$ is the pillar deflection, $I_{\text{max}}$ is the maximum intensity of GFP-α-actinin
or GFP–vinculin within distance 0.75D of the centre of the pillar, and $\langle \rangle$ indicates
a time average. $\sigma_d$ and $\sigma_I$ are the standard deviations of $d$ and $I_{\text{max}}$ over time. Time
traces were low-pass-filtered with a cutoff frequency of 0.15 Hz. For each offset time,
$\tau$, the cross-correlation takes a value between −1 and 1. Cross-correlation functions
from different pillars were averaged, and then the time delay of protein localization
compared with pillar deflection was defined to be the offset time of the maximum of
the averaged cross-correlation function. As this quantity is affected also by negative
(correlation) contributions coming from secondary displacement peaks we also used
the temporal distance between the first peak in pillar displacement and the first peak
in protein intensity to further characterize the relation between these two events.

Code availability. The plugin for pillar tracking and the code for pillar movement
analyses are available on request.

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