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Article

E-cadherin Variants Affecting Distinct Protein Domains Impact Differently on Tensional Homeostasis of Gastric Cancer Cells

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Simple Summary: Tensional homeostasis describes the ability of cells and tissues to maintain their internal mechanical tension stable at a set point value. A breakdown of tensional homeostasis is the hallmark of disease progression, including cancers. In cancers from the epithelia origin, this phenomenon is closely associated with dysfunction of E-cadherin. In this study, we investigated how E-cadherin mutations identified in the cancer context affect tensional homeostasis. Our results show that mutations affecting the juxtamembrane and intracellular domains of E-cadherin are detrimental for tensional homeostasis of gastric cancer cells.

Abstract: In epithelia, breakdown of tensional homeostasis is closely associated with E-cadherin dysfunction and disruption of tissue function and integrity. In this study, we investigated the effect of E-cadherin mutations affecting distinct protein domains on tensional homeostasis of gastric cancer cells. We used micropattern traction microscopy to measure temporal fluctuations of cellular traction forces in AGS cells transfected with the wild-type E-cadherin or with variants affecting the extracellular, the juxtamembrane, and the intracellular domains of the protein. We focused on the dynamic aspect of tensional homeostasis, namely the ability of cells to maintain a consistent level of tension, with low temporal variability around a set point. Cells were cultured on hydrogels micropatterned with different extracellular matrix (ECM) proteins to test whether the ECM adhesion impacts cell behavior. A combination of Fibronectin and Vitronectin was used as a substrate that promotes the adhesive ability of E-cadherin dysfunctional cells, whereas Collagen VI was used to test an unfavorable ECM condition. Our results showed that mutations affecting distinct E-cadherin domains influenced differently cell tensional homeostasis, and pinpointed the juxtamembrane and intracellular regions of E-cadherin as the key players in this process. Furthermore, Fibronectin and Vitronectin might modulate cancer cell behavior towards tensional homeostasis.

Keywords: Tensional homeostasis; Traction microscopy; Gastric cancer cells; E-cadherin mutations; Extracellular matrix proteins

1. Introduction

Tensional homeostasis is defined as the ability of cells to maintain their endogenous mechanical tension stable, at a preferred set point value [cf. 1-3]. A breakdown in tensional homeostasis is the hallmark of several diseases, including cancer [cf. 4,5]. In malignant epithelial cells, breakdown of tensional homeostasis is closely associated with E-cadherin dysfunction and disruption of tissue function and integrity [6,7].

E-cadherin is a main adhesion molecule that coordinates a mechanical circuit of cell-cell linkages, contractile forces and biochemical signals to sustain a functional epithelial barrier [8]. The E-cadherin extracellular domain is responsible for the homophilic binding of E-cadherin molecules on neighboring cells, assuring cohesion and force transmission

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across the epithelia. On the other hand, the intracellular portion of E-cadherin molecules is coupled with the cytoskeleton, increasing cytoskeletal stiffness and stress resistance during cell rearrangements, such as those occurring in cell division [9,10]. Therefore, it is commonly accepted that E-cadherin is a potent tumor suppressor and is involved in limiting tumor cell migration. Accordingly, genetic and epigenetic alterations of E-cadherin are observed in 70% of carcinomas and are associated with invasion and metastasis [11].

Recent studies suggest that along with loss of cell-cell adhesion, cancer cells may undergo an excessive deposition of extracellular matrix (ECM) proteins, such as collagen [cf. 12,13], in an attempt to stabilize their cytoskeletal tension through cell-matrix force transmission. This enhancement of matrix deposition creates a scaffold that contributes for cancer development by forming a physical barrier to anticancer drugs, providing growth factor and cytokines reserves, and promoting cell-ECM adhesion for successful invasion and proliferation [4,14-16]. However, it remains to be unraveled how E-cadherin dysfunction impacts mechanical forces dictating an abnormal cell-ECM dynamics.

During the last decade, it has been demonstrated that tensional homeostasis is tightly regulated by cytoskeletal tension and by traction forces occurring in isolated cells or in cellular clusters [17-21]. Furthermore, we found that clusters of endothelial cells exhibit decreased temporal fluctuations of traction forces, when compared to single cells, suggesting intercellular adhesions as relevant factors for tensional homeostasis and multicellular contexts as favorable mechanical environments [18,20]. However, in gastric cancer cells transfected with the wild-type E-cadherin clustering did not cause as significant attenuation of temporal fluctuations of traction forces as in the case of endothelial cells [20].

In the present study, we investigated the effect of cancer-associated variants of E-cadherin in intracellular force transmission and in tensional homeostasis of gastric cancer cells, taking into account cell's interplay with the ECM. For that purpose, gastric cancer cells transfected with the wild-type (WT) E-cadherin or with mutants affecting the extracellular, the juxtamembrane, and the intracellular domains of the protein were assayed in specific ECMs and subsequently, evaluated for traction forces, as well as their temporal variability. In particular, we focused on the dynamic aspect of tensional homeostasis, namely the ability of cells to maintain a consistent level of tension, with a low variability around a set point [3,21]. Our results showed that variants located in distinct protein domains yield different cell mechanic profiles, and pinpointed the juxtamembrane and the intracellular regions of E-cadherin as the key players in this process. Ultimately, our data indicated that ECM components such as Fibronectin and Vitronectin might modulate cancer cell behavior towards tensional homeostasis.

2. Materials and Methods

2.1. Cell culture and transfections.

Cells were cultured as previously described [22]. Briefly, AGS cell line (gastric adenocarcinoma, ATCC number CRL-1739) was maintained in RPMI medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Perbio) and 1% penicillin/streptomycin (Gibco, Invitrogen). Cells were incubated at 37°C under 5% CO_2 humidified air. Transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's recommendations. For transfections, we have used 1µg of DNA of vectors encoding the WT E-cadherin or the A634V (extracellular domain mutant), R749W (juxtamembrane domain mutant), and V832M (intracellular domain mutant) variants, as well as the empty vector (Mock). These E-cadherin variants are described as causative of hereditary diffuse gastric cancer syndrome (HDGC) [23-26]. Transfected cells were selected by antibiotic resistance to blasticidin (5 µg/ml; Gibco, Invitrogen). At the end of each transfection, putative cytotoxic effects were evaluated by analysing cell viability.

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2.2. Micropattern traction microscopy.

An indirect patterning method was used to create polyacrylamide (PAA) gels with a grid of covalently bound dots of 250 μg/ml AlexaFluor-488 tagged Collagen VI (Col VI, Thermo Fisher) or of a protein mix composed by 125 μg/ml AlexaFluor-488 tagged Fibronectin (Fn) and 125 μg/ml Vitronectin (Vt), as described previously [20,22]. The Fn+Vt combination was used to test an ECM substrate that promotes the adhesive ability of E-cadherin dysfunctional cells, whereas the patterning with Col VI was used to test an unfavorable ECM condition [22]. The patterns were made up of 2-μm diameter dots at 6 μm center-to-center separation. The PAA gels had an elastic modulus of $E \approx 6.7$ kPa and a poisons ratio of $\nu = 0.445$, as determined previously [27,28]. A suspension of $3\text{-}5\times10^4$ cells/ml was seeded on micropatterned gels, which were then incubated for 24 h to allow the establishment of focal adhesions (FAs) at the micropatterned dots.

Cells were subsequently imaged with an Olympus IX881 microscope and a Hamamatsu Orca R2 camera. Images were taken every 5 min for 1 h (13 images). Experiments were carried out in a chamber under controlled environment and maintaining 37 °C, 70% humidity and 5% CO_2. Images capturing the cells and the fluorescent dot array were analyzed using custom MATLAB scripts, as reported by Polio et al. [27]. The program determines the displacement vector (**u**) of the geometrical center of each dot and calculates the corresponding tangential traction force vector (**F**) as follows

$$\mathbf{F} = \frac{\pi E a \mathbf{u}}{2 + \nu - \nu^2}, \tag{1}$$

where $a = 1$ μm is the radius of the dot markers [29].

2.3. Contractile moment and tension.

The magnitude of the contractile moment (M) was used as a quantitative metric of the magnitude of the cell traction field [18,20,30]. Physically, $M > 0$ represents a strength by which the contracting cell "pinches" the substrate as it probes its rigidity; $M < 0$ is not physically feasible. The significance of M is that, for a plane state of stress in the cell (i.e., two-dimensional state of stress), M is equivalent to the mean normal stress (tension) within the cell times the cell volume [31]. Given that cells do not change their volume during the experiments, M is a direct indication of the cytoskeletal tension.

The contractile moment was calculated at each 5-min time interval (t) as follows

$$M(t) = \sum_{i=1}^{N} \mathbf{r}_i(t) \cdot \mathbf{F}_i(t), \tag{2}$$

where $\mathbf{r}_i$ denotes the position vector of the center of a micropatterned dot (i.e., a moment arm vector), $\mathbf{F}_i$ is the corresponding traction force vector, the dot denotes the scalar product between the vectors, and N is the number of FAs within a cell.

2.4. Data analysis.

For each image taken, measured traction forces were adjusted to satisfy mechanical equilibrium as described previously [31]. If this equilibration process yields forces of unusually high magnitudes (>15 nN), those cells were excluded from the analysis. Traction forces below 0.3 nN were also not considered since displacements corresponding to these forces are indistinguishable from background noise [21].

For each 5-min time interval, $M(t)$ was calculated according to Eq. 2. Cells where $M(t) < 0$ in more than 3 (out of 13) time intervals were discarded from further analysis. Otherwise, negative Ms were replaced by zero values. For each cell, we computed the time-average value ($\langle M \rangle$) of $M(t)$ and the corresponding standard deviation (SD_M) over

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the 1-h observation time. The coefficient of variation (CV_M) was subsequently obtained as the ratio of both parameters, $CV_M = SD_M/\langle M \rangle$ [20].

For each FA identified within a cell, we computed the time-average traction force ($\langle F \rangle$), the corresponding standard deviation (SD_F), and the corresponding coefficient of variation (CV_F) as $CV_F = SD_F/\langle F \rangle$. Values of $\langle F \rangle > 0.3$ nN obtained from all FAs were then sorted in an ascending manner; the difference between the highest and lowest values of $\langle F \rangle$ was calculated and divided by ten. Hence, ten bins of data were obtained for $\langle F \rangle$ and the corresponding CV_F. For each bin, we calculated the mean values of $\langle F \rangle$ and of CV_F and the corresponding standard errors [21].

2.5. Quantitative metrics of tensional homeostasis.

The coefficients of variation CV_M and CV_F indicate the extent of temporal variability of $M(t)$ and $F(t)$ relative to $\langle M \rangle$ and $\langle F \rangle$, respectively. Thus, we used CV_M and CV_F as quantitative metrics of tensional homeostasis at the whole cell level and at the FA level, respectively. As CV_M and CV_F approach zero values, it indicates that a cell and its FAs are close to the state of tensional homeostasis.

2.6. Statistical analysis.

For statistical analysis, median values ± median absolute deviation (MAD = the median of the absolute deviations from the data's median) were compared using the Mann-Whitney Rank Sum Test since data for $\langle M \rangle$ of individual cells did not exhibit a normal distribution. Normality of the distribution was evaluated through the Shapiro-Wilk test. Significance was established at $p < 0.05$ or $p < 0.1$, as indicated. The statistical analysis was carried out using SigmaPlot (version 13).

3. Results

3.1. Contractile moments of juxtamembrane, intracellular E-cadherin mutants, and mock cells exhibit greater temporal variability than wild-type cells and extracellular mutants.

To investigate the effects of E-cadherin dysfunction in cellular traction forces, we used cells transfected with wild-type E-cadherin or variants associated to cancer. We have selected variants affecting different protein domains to evaluate potential domain-specific functions.

Traction microscopy measurements of cells were carried out on Fn+Vt micropattern, which was described as an advantageous substrate for adhesion of E-cadherin mutant cells. For comparison of $M(t)$ between different cells, we normalized $M(t)$ with its time average $\langle M \rangle$. Time lapses of $M(t)/\langle M \rangle$ exhibited erratic temporal fluctuations over the 1-h observation time in all cell types (Figure 1). However, the dynamics of the WT (Figure 1A) and A634V cells (Figure 1B), was less fluctuating than that of the R749W (Figure 1C), V832M (Figure 1D), and Mock cells (Figure 1E).

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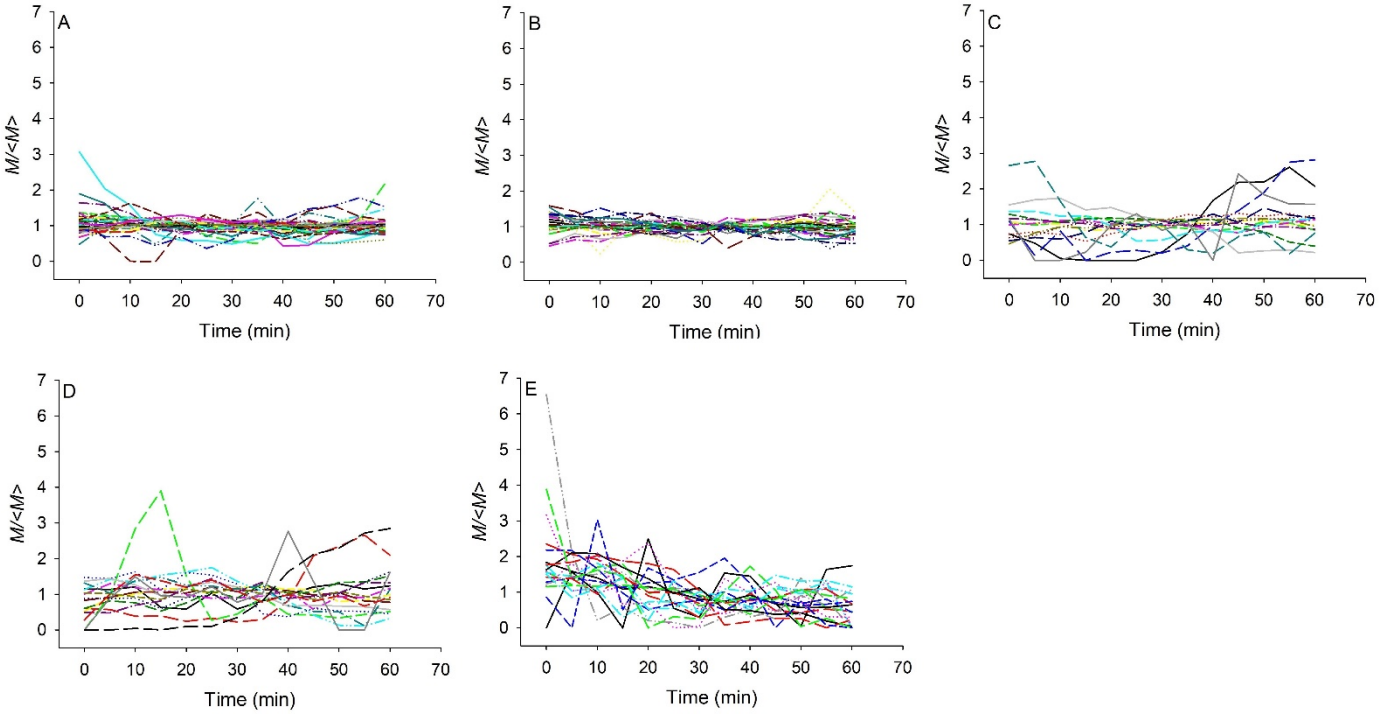

Figure 1. Time lapses of normalized contractile moments of different types of AGS cells cultured on the combination of Fibronectin and Vitronectin micropatterns over the course of 60 min experiments. The graphs of WT cells (A) and A634V cells (B) exhibit smaller temporal fluctuations than the graphs of R749W cells (C), V832M cells (D), and Mock cells (E). Contractile moment (M) was normalized by it time-averaged value ($\langle M \rangle$). Different colors and different lines correspond to different cells.

3.2. E-cadherin expression promotes cell tension.

The cells transfected with the WT E-cadherin or the different mutants had significantly greater median values of $\langle M \rangle$ than the Mock cells, which do not express E-cadherin ($p < 0.001$ for WT and A634V; $p = 0.005$ for R749W ; $p = 0.013$ for V832M; Figure. 2A). This suggests that the presence of E-cadherin promotes cell contractility, regardless its status. Median values of $\langle M \rangle$ of the mutants were not significantly different from the WT cells. Interestingly, the V832M cytoplasmic mutant presented an evident, although not significant ($p = 0.151$), decrease in $\langle M \rangle$ when compared with the WT cells, further suggesting its deleterious effect. There was, however, a significant difference between the V832M and A634V cells, where the median $\langle M \rangle$ of the former was significantly smaller than that of the latter ($p = 0.034$).

To evaluate whether the observed differences in $\langle M \rangle$ might be explained by differences in the cells' ability to establish FAs, we computed the median number of FAs in each cellular condition. The Mock cells exhibited a significantly lower median number of FAs ($p < 0.05$) than all the E-cadherin-transfected cells (Figure 2B), which was consistent with the significantly lower median value of $\langle M \rangle$ of the Mock cells in comparison with the all transfected cells (Figure 2B vs. 2A). The R749W cells exhibited significantly greater number of FAs in comparison with the WT-cells ($p = 0.011$), which was consistent with the difference in their respective median values of $\langle M \rangle$ albeit non-significant (Figure 2B vs. 2A). On the other hand, the A634V and V832M cells had nearly the same median number of FAs although their respective median values of $\langle M \rangle$ were significantly different (Figure 2B vs. 2A). Together, these results suggest that, aside the differences in the number of FAs, other factors may contribute to the differences in $\langle M \rangle$ observed across cell variants.

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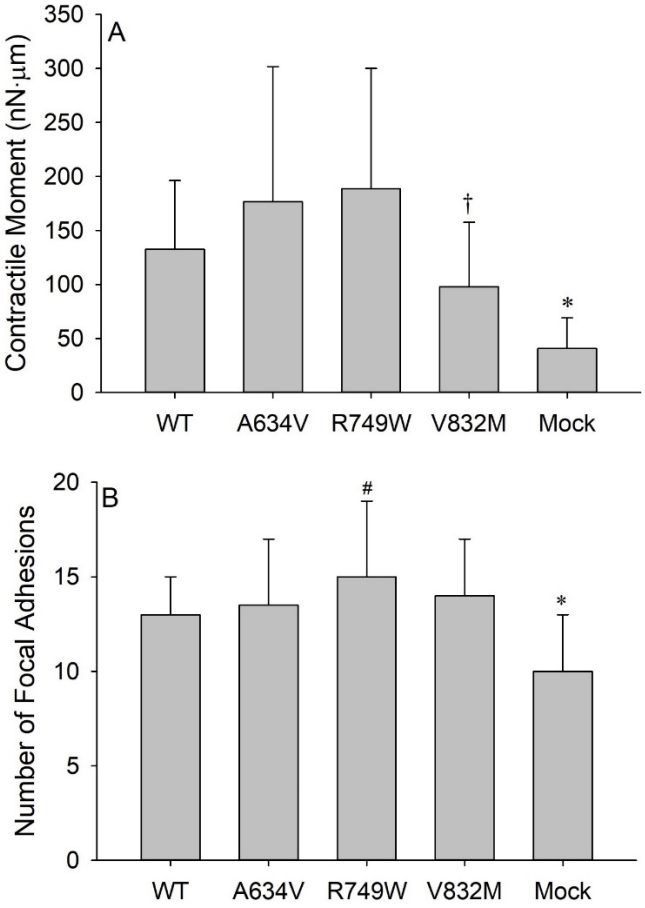

Figure 2. A) Median values of time-averaged contractile moments of WT, A634V, R749W, V832M, and Mock AGS cells cultured on the combination of Fibronectin and Vitronectin micropatterns. The Mock cells exhibit a significantly smaller contractile moment than the other cell types (*$p < 0.05$), whereas the V832M cells exhibit a significantly smaller contractile moment than the A634V cells ($^{u}p < 0.05$). B) Median values of the number of focal adhesions (FAs) of WT, A634V, V832M, R749W, and Mock AGS cells cultured on the combination of Fibronectin and Vitronectin micropatterns. The Mock cells exhibit a significantly smaller number of FAs than the other cell types (*$p < 0.05$), whereas the R749W cells exhibit a significantly greater number of FAs than the WT cells ($^{\#}p < 0.05$). Graphs are median ± MAD.

3.3. Juxtamembrane and intracellular E-cadherin mutants compromise tensional homeostasis.

We next investigated the temporal variability of the contractile moment in our cell lines using CV_M as metric of tensional homeostasis. Recall that the lower the value of CV_M, the closer the cell to the state of tensional homeostasis is (see Materials and Methods). We verified that the R749W and V832M mutants, as well as the Mock cells had significantly higher median values of CV_M when compared with the WT cells ($p = 0.011$, $p < 0.001$, and $p < 0.001$, respectively; Figure 3).

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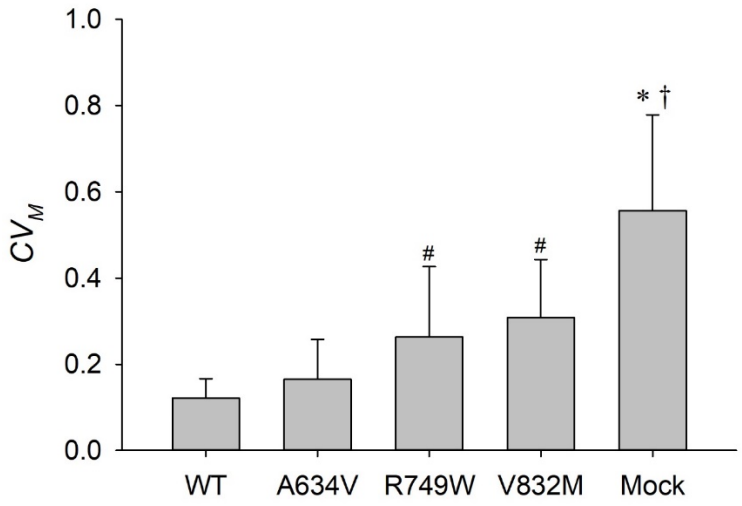

Figure 3. Median values of the coefficient of variation of the contractile moment (CV_M) of WT, A634V, V832M, R749W, and Mock AGS cells cultured on the combination of Fibronectin and Vitronectin micropatterns. The Mock cells exhibit a significantly greater CV_M than the WT, A634V, R749W ($^{*}p < 0.05$), and V832M ($^{\cup}p < 0.1$), whereas the R749W and V832M exhibit a significantly greater CV_M than the WT and A634V cells ($^{\#}p < 0.05$). Graphs are median ± MAD.

Together, these results indicate that E-cadherin forms compromising the juxtamembrane or the intracellular portions of the protein interfere with the cell's ability to maintain tensional homeostasis. The higher values of the median CV_M in the V832M and Mock cells may by partially explained by their lower values of the median $\langle M \rangle$, when compared with WT or the A634V cells (Figure 2C vs. 2A) since, by definition, CV_M and $\langle M \rangle$ are inversely related. However, $\langle M \rangle$ is not the sole determinant of CV_M and the standard deviation, SD_M, which is indicative of temporal variability of $M(t)$, is also an important factor (recall that $CV_M = SD_M/\langle M \rangle$). Indeed, we found that the WT cells had the lowest median values of SD_M, while the R749W and V832M cells had the highest values, which is consistent with the differences in CV_M between these cell types. A different interpretation of the above results follows from a consideration of temporal variability of traction forces at the FAs level.

In our previous study, we measured variability of individual traction forces applied to FAs in endothelial and vascular smooth muscle cells [21]. We found that their respective mean values of CV_F did not change significantly, until their corresponding mean values of $\langle F \rangle$ reached a threshold value beyond which CV_F precipitously decreased, indicative of FAs tensional homeostasis. In the present study, we did not find such a threshold of $\langle F \rangle$ in the AGS cell model. The mean values of CV_F of the E-cadherin-transfected cells and Mock cells generally deceased with increasing mean $\langle F \rangle$ (Figure 4). However, the maximum mean values of $\langle F \rangle$ in the R749W (5.8 nN), V832M (5.5 nN), and Mock cells (4.3 nN) were smaller than that observed in the WT (8.0 nN) and A634V cells (6.8 nN) and hence, the corresponding values of CV_F were higher in the R749W, V832M, and Mock cells than in the WT and A634V cells (Figure 4). This is in accordance with the observed lower values of CV_M in the WT and A634V cells, than in the R749W, V832M, and Mock cells (Figure 3).

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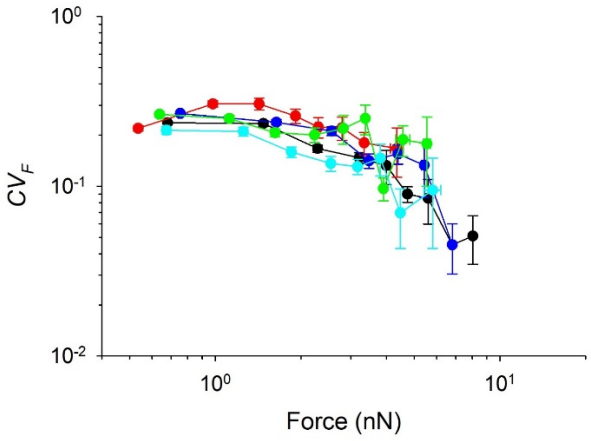

Figure 4. With increasing of the time-averaged focal adhesions traction forces their coefficient of variation (CV_F) decreases. Different colors correspond to different cell types WT cells (black), A634V cells (blue), R749 cells (cyan), V832M cells (green), and Mock cells (red) cultured on the combination of Fibronectin and Vitronectin micropatterns. Data are mean ± standard error.

3.4. Collagen VI enhances traction field magnitude and fluctuations.

To study the effects of the ECM on cell mechanical response, we compared tensional homeostasis of cells seeded on gels micropatterned with Fn+Vt or with Col VI, which were described as attractive or repulsive substrates, respectively [22]. Our results indicated that the differences observed for the WT, mutant, and Mock cell were consistent between the Fn+Vt combination or the Col VI micropattern. However, the measurements carried out on the Col VI patterns yielded higher values of the median $\langle M \rangle$ in all cell types relative to the corresponding data obtained from the Fn+Vt experiments and this difference was significant in the Mock cells ($p = 0.049$, Figure 5A). The higher values of $\langle M \rangle$ may reflect higher numbers of FAs in the cells cultured on the Col VI micropatterns, when compared with that formed on the Fn+Vt micropatterns (Figure 5B), although the differences were not significant.

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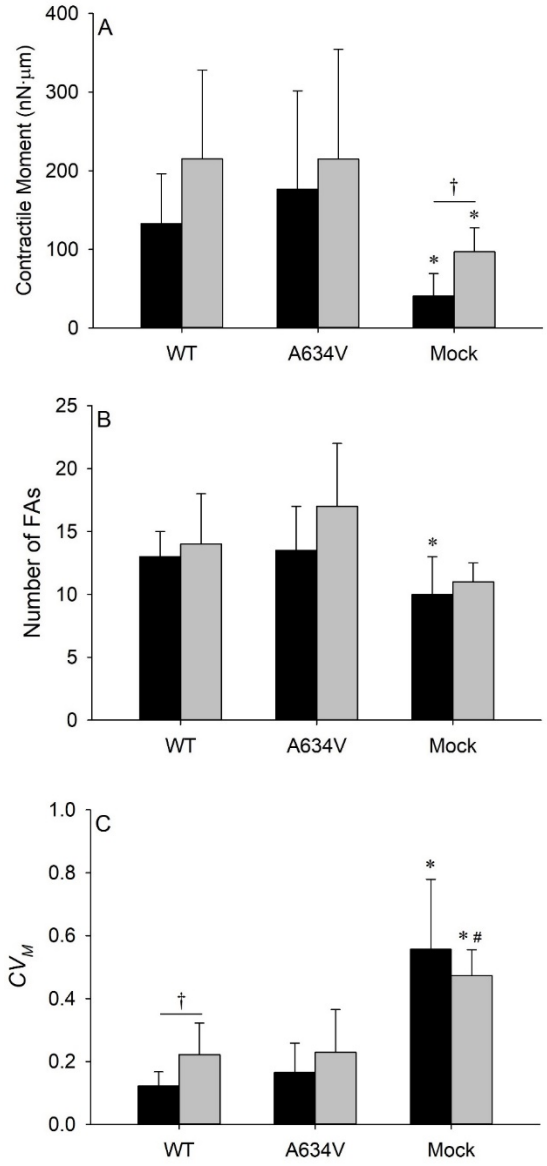

Figure 5. Comparison of the median values of the time-averaged contractile moments (A); of the corresponding median numbers of focal adhesions (FAs) (B); and of the corresponding median values of the coefficient of variation of the contractile moment (CV_M) (C) of the WT cells, A634V cells, and Mock cells cultured on the combination of Fibronectin and Vitronectin micropatterns (Fn+Vt, black bars) and on Collagen VI (Col VI, gray bars). Only the Mock cells exhibit significantly different values between the contractile moments of obtained on the Fn+Vt versus Col VI micropatterns ($^u p < 0.05$). No significant differences in the median number of focal adhesions between the two micropatterns is observed. Only the WT cells exhibit significantly different values of CV_M obtained on the Fn+Vt versus Col VI micropatterns ($^u p < 0.05$). The graph bars are median values ± MAD; *significantly different contractile moment and number of FAs ($p < 0.05$) than WT and A634V cells in the cases of Fn+VT and of Col VI micropatterns; *significantly different CV_M ($p < 0.05$) than WT cells and $^\#$significantly different ($p < 0.1$) than A634V cells in the case of Col VI micropatterns. Graphs are median ± MAD.

Median values of CV_M obtain from the measurements on the Col VI micropatterns were higher in the WT and A634V mutant cells than the corresponding values obtained from the measurements on the Fn+Vt micropatterns ($p = 0.009$ and $p = 0.236$, Figure 5C), indicating that this ECM component induced increased traction fluctuation and is less favorable for tensional homeostasis.

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Taken together, the results obtained on the different micropatterns suggest that the Fn+Vt combination decreases the variability of the traction field and supports a stable cell-ECM interplay, promoting tensional homeostasis.

4. Discussion

Tensional homeostasis of malignant cells has been studied almost exclusively in the cases of breast cancer cells, in the context of mechanoreciprocity between the cell's contractile forces and the stiffness of the extracellular matrix [cf. 4,5]. Those approaches describe tensional homeostasis as a static phenomenon. Here, we studied tensional homeostasis in AGS cells by focusing on the effect of E-cadherin and cancer-associated E-cadherin mutants on intracellular force transmission. Since cytoskeletal contractile forces vary over time, we approached tensional homeostasis as a dynamic process. Our strategy is consistent with the notion that homeostasis, in general, is continuously changing and oscillating around a set point. Moreover, the cellular environment is always ready to reset itself, but also to provide the reference point for a change if necessary for survival in an ever-changing environment [32]. Our major findings are as follows.

In AGS cells, E-cadherin expression enhanced the magnitude and reduced temporal variability of their contractile moment. The extent of these effects depended on whether E-cadherins were WT or cancer-associated mutants. The WT cells and the cells with the extracellular mutant variant exhibited high magnitude and low temporal fluctuations of the contractile moment, thus promoting tensional homeostasis. The cells with the juxtamembrane mutant variant exhibited nearly the same magnitude and a higher variability of the contractile moment in comparison with the WT cells. On the other hand, the cells with the intracellular mutant variant exhibited a lower magnitude and a higher variability of the contractile moment in comparison with the WT cells. The higher variability of the contractile moment of the juxtamembrane and intracellular mutants interfere with the cell's ability to maintain tensional homeostasis. In the absence of E-cadherins, the Mock cells had the lowest magnitude and the highest variability of the contractile moment than the all E-cadherin-transfected cells, corroborating the evidence that E-cadherin expression was essential for tensional homeostasis.

The Fn+Vt micropattern substrates yielded lower magnitudes and lower variability of the contractile moment than the Col VI substrates in the E-cadherin-transfected cells, whereas in the Mock cells the Fn+Vt substrates yielded lower magnitude and higher variability of the contractile moment than the Col VI substrates. These findings suggests that the combination of Fn and Vt was more favorable for homeostasis than Col VI alone.

We may speculate that the WT cells and the cells expressing the extracellular variant sustain an intact contractile actin cytoskeleton. Thus, the intracellular force transmission between E-cadherin and FAs across the cytoskeleton remains uninterrupted. Furthermore, this E-cadherin-FA crosstalk allows cells to develop a high level of cytoskeletal tension with relatively small temporal fluctuations, maintaining thereby tensional homeostasis [33]. It has been shown that Myosin VI plays an important role in coupling of the E-cadherin juxtamembrane domain to the actin cytoskeleton [34]. It is also possible that Myosin VI may bind to E-cadherin along its full intracellular tail [34]. Thus, E-cadherin mutations affecting the juxtamembrane and the intracellular domains may induce a fragile interaction between E-cadherins and Myosin VI, consequently hindering the intracellular force transmission among E-cadherin and FAs, and thus preventing the development of a high cytoskeletal tension with low fluctuations. Under such conditions, cells may attempt to build up stable tension by establishing a high number of FAs and by depositing ECM proteins on the substrate, creating a favorable condition for tensional homeostasis. Accordingly, our results demonstrated that cells expressing the juxtamembrane or the intracellular E-cadherin variants exhibited an increased number of FAs, when compared to than detected in the WT cells or in the cells carrying the extracellular mutation. Importantly, it was previously reported that E-cadherin dysfunctional cells are able to produce and secret ECM components such as Laminin to survive and invade [35]. In

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contrast to cells expressing E-cadherin, the Mock cells could not develop high and stable cytoskeletal tension and thus they were the furthest from the state of tensional homeostasis.

It is noteworthy that our previous study of tensional homeostasis of AGS cells showed that cell clustering was much less effective for achieving homeostasis than in endothelial cells [20]. We found that temporal fluctuations of the contractile moment in clusters were insignificantly smaller than in single cells in both the WT and the Mock cells cultured on the Fn+Vt micropatterned gels. Together, these findings suggest that the E-cadherin-mediated intercellular force transmission, which was present in the WT clusters and absent from the Mock clusters, may have a minor impact on tensional homeostasis of AGS cells. Corroborating these data, our present work showed that the loss of cell-cell adhesion and thereby of intercellular force transmission caused by extracellular mutations of E-cadherin might not affect tensional homeostasis of AGS cells.

Regarding the effect of ECM composition in our system, we verified that different matrix proteins did not result in qualitatively different behaviors of the cells tested. Based on the distinct adhesion affinities of the WT cells and the cells with the extracellular mutations towards the combination of Fn and Vt versus Col VI alone [22], we anticipated that the extracellular mutations cells would exhibit a higher value of $\langle M \rangle$ on the Fn+Vt micropatterns than that observed on the Col VI micropatterns, whereas the WT cells would exhibit the opposite behavior. Surprisingly, our results showed that both the WT cells and the extracellular mutants exhibited higher values of $\langle M \rangle$ and of CV_M on the Col VI micropatterns than on the Fn+Vt micropatterns, which is possibly related to a higher number of integrins that bind Col VI than they do for the Fn+Vt combination. In this context, it is relevant that high traction forces are supported by $\alpha_5\beta_1$ integrins, whereas less stable $\alpha_v\beta_3$ integrins provide reinforcement of integrin-cytoskeleton linkages [36]. In the Mock cells, the difference observed between the median $\langle M \rangle$ on the Col VI and on the Fn+Vt micropatterns is further exacerbated. Taking into account that Mock cells display complete absence of E-cadherin, it appears that the presence of this protein (even if not functional) and the activation of its downstream signaling award cells an increased ability to adapt to distinct ECM compositions, as reflected in higher values of $\langle M \rangle$ and higher number of focal adhesions in cells transfected with WT or mutant forms of E-cadherin.

Ultimately, we would like to point out that in our previous publication, we were able to show a clear dependence of the magnitude of the traction field and the adhesion affinities of cells expressing WT and mutant E-cadherin cells towards the Fn+Vt combination and Col VI [22]. In that study, we used a different metric of the magnitude of the traction field – namely the sum of magnitudes of traction forces, which is different from the magnitude contractile moment that we have used in the present work. In fact, for the purpose of tensional homeostasis evaluation, we believe that it is more appropriate to use the magnitude of the contractile moment since it is directly associated to the mean cytoskeletal tension. Furthermore, the contractile moment accounts for the vectorial nature of traction forces and for the size of the cell, whereas the sum of the magnitudes of traction forces does not.

5. Conclusion

A breakdown of tensional homeostasis is the hallmark of epithelial cancers. Here, we showed that cancer-associated mutations of E-cadherin located at the juxtamembrane or at the intracellular region of the protein might lead to loss of tensional homeostasis in AGS cells, in contrast to extracellular mutants. The behavior of the cells expressing an extracellular mutation was indeed similar to that of the WT cells in the sense that it was closer to the state of tensional homeostasis than the cells carrying juxtamembrane or intracellular mutations. Overall, our data suggest that juxtamembrane and the intracellular domains of E-cadherin are critical for tensional homeostasis by establishing an E-cadherin-cytoskeletal linkage, which sustains cellular tension. This work provides the first

evidence that specific E-cadherin mutations are detrimental for tensional homeostasis, contributing to the disease progression.

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References

1. Chien, S. Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell. *Am. J. Physiol. - Heart Circ. Physiol.* **2007**, 292, H1209–H1224.
2. Humphrey, J. D. Vascular adaptation and mechanical homeostasis at tissue, cellular, and sub-cellular levels. *Cell Biochem. Biophys.* **2008**, 50, 53–78.
3. Stamenović, D.; Smith, M. L. Tensional homeostasis at different length scales. *Soft Matter*, **2020**, 16, 6946-6963.
4. Paszek, M. J.; Zahir, N., Johnson, K. R.; Lakins, J. N.; Rozenberg, G. I; Gefen, A.; Reinhart-King, C. A.; Margulies, S. S.; Dembo, M.; Boettiger, D.; Hammer, D. A.; Weaver, V. M. Tensional homeostasis and the malignant phenotype. *Cancer Cell* **2005**, 8, 241–254.
5. Butcher, D. T.; Alliston, T.; Weaver, V. M. A tense situation: forcing tumour progression. *Nat. Rev. Cancers* **2009**, 9: 108-122.
6. Mayer, B.; Johnson, J. P.; Leitl, F.; Jauch, K. W.; Heiss, M. M.; Schildberg, F. W.; Birchmeier, W.; Funke, I. E-cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res*. **1993**, 53, 1690-1695.
7. Figueiredo, J.; Soderberg, O.; Simoes-Correia, J.; Grannas, K.; Suriano, G.; Seruca, R.; The importance of E-cadherin binding partners to evaluate the pathogenicity of E-cadherin missense mutations associated to HDGC. *Eur. J. Hum. Genet*. **2013**, 21, 301-309.
8. Rübsam, M.; Mertz, A. F.; Kubo, A.; Marg, S.; Jüngst, C.; Goranci-Buzhala, G.; Schauss, A. C.; Horsley, V.; Dufresne, E. R.; Moser, M.; Ziegler, W.; Amagai, M.; Wickström, S. A.; Niessen, C. M. E-cadherin integrates mechanotransduction and EGFR signaling to control junctional tissue polarization and tight junction positioning. *Nat. Commun.* **2017,** 8, 1250.
9. DuFort, C.C.; Paszek, M. J.; Weaver, V. M. Balancing forces: architectural control of mechanotransduction. *Nat. Rev. Mol. Cell Biol.* **2011**, 12, 308-319.
10. Lecuit, T.; Yap, A. S. E-cadherin junctions as active mechanical integrators in tissue dynamics. *Nat. Cell Biol.* **2015**. 17, 533-539.
11. Paredes, J.; Figueiredo, J.; Albelgaria, A.; Oliviera, P.; Carvalho, J.; Ribeiro, A. S.; Caldeira, J.; Costa, A. M.; Simões-Correia, J.; Oliveira, M. J.; Pinheiro, H.; Pinho, S. S.; Mateus, R.; Reis, C. A.; Leite, M.; Fernandes, M. S.; Schmitt, F.; Carneiro, F.; Figueiredo, C.; Oliveira, C.; Seruca, R. Epithelial E- and P-cadherins: role and clinical significance in cancer. *Biochim. Biophys. Acta* **2012** 1826, 297-311.
12. Winkler, J.; Abisoye-Ogunniyan, A.; Metcalf, K. J.; Werb, Z. Concepts of extracellular matrix remodeling in tumour progression and metastasis. *Nat. Commun.* **2020**, 11, 5120.
13. Henke, E.; Nandigama, R.; Ergün, S. Extracellular matrix in the tumor environment and its impact on cancer therapy. *Front. Mol. Biosci.* **2020**, 6, 160.
14. Wozniak, M.A.; Desai, R.; Solski, P.A.; Der, C.J.; Keely, P.J. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J. Cell Biol.* **2003**, 163, 583–595.
15. Provenzano, P. P.; Keely, P. J. Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. J. Cell Sci. **2011**, 124, 1195-1205.
16. Moreira, A. M.; Pereira, J.; Melo, S.; Fernandes, M. S.; Carneiro, P.; Seruca, R., Figueiredo, J. The extracellular matrix: an accomplice in gastric cancer development and progression. *Cells* **2020**, 9, 394.
17. Webster, K.D.; Ng, W. P.; Fletcher, D. A.. Tensional homeostasis in single fibroblasts. *Biophys. J.* **2014**, 107, 146–155.
18. Canović, E.P.; Zollinger, A. J.; Tam, S. N.; Smith, M. L.; Stamenović, D. Tensional homeostasis in endothelial cells is a multicellular phenomenon. *Am. J. Physiol. Cell Physiol.* **2016**, 311, C528-535.
19. Weng, S.; Shao, Y.; Chen, W.; Fu, J. Mechanosensitive subcellular rheostasis drives emergent single-cell mechanical homeostasis. *Nat. Mater.* **2016**, 15, 961-967.

20. Zollinger, A. J.; Xu, H.; Figueiredo, J.; Paredes, J.; Seruca, R.; Stamenović, D. Smith, M. L. Dependence of tensional homeostasis on cell type and on cell-cell interactions. *Cell. Mol. Bioeng.* **2018**, 11: 175-184.
21. Xu, H.; Donegan, S.; Dreher, J. M.; Stark, A. J. Canović, E. P. Stamenović, D.; Smith, M. L. Focal adhesion displacement magnitude is a unifying feature of tensional homeostasis. *Acta Biomater.* **2020**, 113, 372-379.
22. Figueiredo, J.; Ferreira, R. M.; Xu, H.; Gonçalves, M.; Barros-Carvalho, A.; Cravo, J.; Maia, A. F.; Carneiro, P.; Figueiredo, C.; Smith, M. L.; Stamenović, D.; Morais-de-Sá, E; Seruca, R. Integrin β1 orchestrates the abnormal cell-matrix attachment and invasive behaviour of E-cadherin dysfunctional cells. *Gastric Cancer* **2022**, 25, 124-137.
23. Suriano, G.; Oliveira, C.; Ferreira, P.; Machado, J. C.; Brodin, M. C.; De Wever, O.; Bruyneel, E. A.; Moguilevsky, N.; Grehan, N.; Porter, T. R. Richards, F. M.; Hruban, R. H.; Roviello, F.; Huntsman, D.; Mareel, M.; Carneiro, F.; Caldas, C.; Seruca, R. Identification of CDH1 germline missense mutations associated with functional inactivation of the E-cadherin protein in young gastric cancer probands. *Hum. Mol. Genet.* **2003**, 12, 575–582.
24. Kaurah, P.; MacMillan, A.; Boyd, N.; Senz, J.; De Luca, A.; Chun N.; Suriano, G.; Zaor, S.; Van Manen, L.; Gilpin, C.; Nikkel, S.; Connolly-Wilson, M.; Weissman, S.; Rubinstein, W. S.; Sebold, C.; Greenstein, R. Stroop, J.; Yim, D.; Panzini, B.; McKinnon, W.; Greenbaltt, M.; Wirtzfeld, D.; Fontaine, D.Coit, D.; Yoonm, S.; Chung, D.; Lauwers, G.; Pizzuti, A.; Vaccaro, C.; Redal, M. A.; Oliveira, C.; Tischkowitz, M.; Olschwang, S.; Gallinger, S.; Lynch, H.; Green, J.; Ford, J.; Pharoah, P.; Fernandez, B.; Huntsman, D. Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer. *JAMA* **2007**, 297, 2360-2372.
25. More, H.; Humar, B.; Weber, W. Ward, R.; Christian, A.; Lintott, C.; Graziano, F.; Ruzzo, A.-M.; Acosta, E.; Boman, B.; Harlan, M.; Ferreira, P.; Seruca, R.; Suriano, G.; Guilford, P. Identification of seven novel germline mutations in the human E-cadherin (CDH1) gene. *Hum. Mutat.* **2007**, 28, 203.
26. Simões-Correia, J.; Figueiredo, J.; Oliveira, C.; van Hengel, J.; Seruca, R.; van Roy, F.; Suriano, G. Endoplasmic reticulum quality control: a new mechanism of E-cadherin regulation and its implication in cancer. *Hum. Mol. Genet.* **2008**, 17, 3566-3576.
27. Polio, S.R.; Rothenberg, K. E.; Stamenović, D.; Smith, M. L. A micropatterning and image processing approach to simplify measurement of cellular traction forces. *Acta Biomater.* **2012**, 8, 82–88.
28. Polio, S.R.; Parameswaran, H.; Canović, E.P.; Gaut, C. M.; Aksyonova, D.; Stamenović, D.; Smith, M. L. Topographical control of multiple cell adhesion molecules for traction force microscopy. *Integr. Biol.* **2014**, 6, 357-365.
29. Maloney, J. M.; Walton, E. B.; Bruce, C. M.; Van Vliet, K. J.. Influence of finite thickness and stiffness on cellular adhesion-induced deformation of compliant substrata. *Phys. Rev. E*, **2008**, 78, 041923.
30. Butler, J. P.; Tolić-Nørrelykke, I. M.; Fabry, B.; Fredberg, J. J. Traction fields, moments, and strain energy that cells exert on their surroundings. *Am. J. Physiol. Cell Physiol.* **2002**, 282, C595-C605.
31. Canović, E. P.; Seidl, D. T.; Polio, S. R.; Oberai, A. A.; Barbone, P. E.; Stamenović, D.; Smith, M. L. Biomechanical imaging of cell stiffness and prestress with subcellular resolution. *Biomech. Model. Mechanobiol.* **2014**, 13, 665-678.
32. Torday J. S. Homeostasis as the mechanism of evolution. *Biology* **2015**, 4, 573-590.
33. Canel, M.; Serrels, A.; Frame, M. C.; Brunton, V. G. E-cadherin-integrin crosstalk in cancer invasion and metastasis. *J. Cell Sci.* **2013**, 126, 393-401.
34. Mangold, S.; Norwood, S. J.; Yasp, A. S.; Collins, B. M. The juxtamembrane domain of the E-cadherin cytoplasmic tail contributes to its interaction with myosin VI. *Bioarchitecture* **2012**, 2, 185-188.
35. Caldeira, J.; Figueiredo, J; Brás-Pereira, C.; Carneiro, P.; Moreira, A. M.; Pinto, M. T.; Relvas, J. B.; Carneiro, F.; Barbosa, M.; Casares, F.; Janody, F.; Seruca, R. E-cadherin-defective gastric cancer cells depend on laminin to survive and invade. *Hum. Mol. Genet.* **2015**, 24, 5891-5900.
36. Roca-Cusachs, P.; Gauthier, N. C.; del Rio, A.; Sheetz, M. P. Clustering of $\alpha_5\beta_1$integrins determines adhesion strength whereas $\alpha_v\beta_3$ and talin enable mechanotransduction. *Proc. Natl. Acad. Sci. USA* **2009**, 106, 16245-16250.