

Spatial Proliferation of Epithelial Cells Is Regulated by E-Cadherin Force

Abhinav Mohan,¹ Kyle T. Schlue,¹ Alex F. Kniffin,¹ Carl R. Mayer,¹ Ashley A. Duke,¹ Vani Narayanan,¹ Paul T. Arsenovic,¹ Kranthidhar Bathula,¹ Brooke E. Danielsson,¹ Sandeep P. Dumbali,² Venkat Maruthamuthu,² and Daniel E. Conway^{1,*}

¹Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, Virginia and ²Department of Mechanical & Aerospace Engineering, Old Dominion University, Norfolk, Virginia

ABSTRACT Cell proliferation and contact inhibition play a major role in maintaining epithelial cell homeostasis. Prior experiments have shown that externally applied forces, such as stretch, result in increased proliferation in an E-cadherin force-dependent manner. In this study, the spatial regulation of cell proliferation in large epithelial colonies was examined. Surprisingly, cells at the center of the colony still had increased proliferation as compared to cells in confluent monolayers. E-cadherin forces were found to be elevated for both cells at the edge and center of these larger colonies when compared to confluent monolayers. To determine if high levels of E-cadherin force were necessary to induce proliferation at the center of the colony, a lower-force mutant of E-cadherin was developed. Cells with lower E-cadherin force had significantly reduced proliferation for cells at the center of the colony but minimal differences for cells at the edges of the colony. Similarly, increasing substrate stiffness was found to increase E-cadherin force and increase the proliferation rate across the colony. Taken together, these results show that forces through cell-cell junctions regulate proliferation across large groups of epithelial cells. In addition, an important finding of this study is that junction forces are dynamic and modulate cellular function even in the absence of externally applied loads.

INTRODUCTION

In normal-tissue homeostasis, epithelial cells are contact inhibited, only proliferating in response to cell death, tissue injury, and cell turnover (1). Homophilic ligation of cadherins has been shown to suppress cell proliferation (2), which may explain increased cell proliferation in subconfluent *in vitro* cultures and increased cell proliferation adjacent to wounding. However, *in vivo* epithelial cells undergo frequent division to replenish lost cells while simultaneously maintaining an intact epithelial layer with intact cell-cell junctions that are crucial for barrier function (3). Therefore, there must be additional factors that can stimulate proliferation in cells within an epithelial cell monolayer.

Endothelial and epithelial cells exhibit significant mechanical tension across cell-cell contacts (4,5). Using fluorescence resonance energy transfer (FRET)-based force biosensors, we and others have shown that cadherins experience mechanical tension (6,7). Junctional forces have been proposed to be an important signal in regulating collective

cell migration, contact inhibition, and proliferation (8,9). Furthermore, mechanical stretch, a well-established inducer of cell proliferation, is dependent on mechanically coupled E-cadherin (10). Taken together, these observations suggest a hypothesis that cellular-initiated changes in E-cadherin force could modulate cellular proliferation.

We observed that cells at the center of large epithelial cell colonies continue to proliferate at high rates. This observation indicates that these cells, which should otherwise be contact inhibited, are receiving a proliferative stimulus. To examine the relationship in which E-cadherin force is coupled to cell proliferation, we used MDCK cells with an existing E-cadherin FRET-based force biosensor (6) to measure endogenous E-cadherin forces. We observed significantly higher E-cadherin force for cells in the center of these larger colonies, as compared to completely confluent monolayers. By varying the force across E-cadherin, we show that E-cadherin force contributes to increased proliferation of cells at the center of the colony that would otherwise be contact inhibited. Our results show that cell-cell junction forces, even without externally applied force (e.g., stretch), can coordinate proliferation across large groups of cells.

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*Correspondence: dconway@vcu.edu

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MATERIALS AND METHODS

Cell culture

Madin-Darby Canine Kidney (MDCK) II cells, a gift from Rob Tombes (Virginia Commonwealth University, Biology), were used in all experiments. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher, Waltham, MA) with 10% fetal bovine serum (Thermo Fisher) and 1% penicillin/streptomycin (Thermo Fisher).

To generate stable cell lines expressing canine full-length E-cadherin tension sensor module (TSmod), dileucine endocytosis mutant, or tailless (E-cadherin cytoplasmic tail detection (Ecad Δ cyto)), cells were transfected with lipofectamine 2000 and selected using 500 μ g/mL G418 (Thermo Fisher). Colonies were selected based on similar fluorescent brightness that was suitable for FRET imaging. All three forms of E-cadherin were found to be expressed at similar or greater levels than endogenous E-cadherin (Fig. S3). Full-length canine E-cadherin TSmod and Ecad Δ cyto were gifts provided by Alex Dunn (6). The full-length dileucine endocytosis mutant was generated by cloning the K743R, L746V, and L747A portion of Ecad Δ cyto into the full-length E-cadherin TSmod using SpeI/XmaI. The mutation of the dileucine motif (L746 and L747) was previously shown to block E-cadherin endocytosis (11,12).

In all proliferation experiments, cells were grown on both glass dishes coated with 40 μ g/mL fibronectin and on the Culture-Insert 2 Well dishes (Ibidi, Planegg, Germany). In experiments with confluent cells, cells were grown on glass bottom dishes and the Culture-Insert 2 Well dishes (where the silicone insert was already removed) for a period of 2 days postconfluence. These cells had a density of \sim 2800 cells/mm². To develop uniform large epithelial colonies, cells were seeded in both wells of Culture-Insert 2 Well dishes. The silicone insert was removed, and cells were incubated another 24 h. Because cells readily closed the small 500- μ m gap between wells within 6 h (unpublished data), at post-24 h, the cells were in one large circular colony with a diameter of 10 mm. Cells at the edge of the colony had a density of 870 cells/mm², whereas cells at the center had a density of 2700 cells/mm². In select experiments, 15 μ M Gefitinib (Sigma) was used to inhibit epidermal growth factor receptor (EGFR) activity.

FRET imaging and analysis

Cells were seeded onto a 35 mm dish with a 1.5 NA (numerical aperture) 20-mm glass-bottom coverslip dishes (Cellvis, Sunnyvale, CA) that were pre-coated with 40 μ g/mL fibronectin (Alfa Aesar, Haverhill, MA). Living cells in glass-bottom dishes expressing one of the three force sensors were imaged using a 63 \times -magnification oil immersion objective on a Zeiss (Oberkochen, Germany) 710 laser-scanning microscope, using spectral unmixing with a 458 laser to collect both mTFP and mEYFP, as previously described (13).

Intensity images were further processed and analyzed using a custom Python code, as previously described (13). For each data set, the data were acquired for at least nine image frames per condition.

For polyacrylamide gel experiments, cells were grown on glass coverslips coated with the polyacrylamide gel. To image these cells, the coverslip was inverted into a glass-bottom coverslip dish.

Statistics

Statistical significance was measured using an unpaired, two-tailed Student's *t*-test for data containing two groups. For data involving more than two groups, the analysis of variance test was performed to obtain the statistical analysis for the data sets concerned. A further comparison of the groups was conducted using the Tukey (Honestly Significant Difference) test to obtain significant differences between multiple groups, if any. All statistical tests were conducted at a 5% significance level ($p < 0.05$). The R statistical software was used for statistical analyses. Data are shown as mean \pm standard error.

FRET

Upon measuring the E-cadherin force using the FRET based biosensor, the data were analyzed in Python to determine the average FRET ratio and then the standard error about the mean. Each data set had a minimum of seven images to deem the results statistically significant. Statistical significance was measured using the Student's *t*-test (unpaired and two-tailed for two groups) and analysis of variance for multiple groups and for differences between pairs, and the Tukey Honestly Significant Difference test was done. The significance value for the statistical tests was set at 5%. R and JMP Pro 12 were used as the software for conducting the statistical analysis.

Formation of polyacrylamide gels

Polyacrylamide gels of varying stiffness were made on glass coverslips by varying concentrations of acrylamide (Sigma, St. Louis, MO) and bisacrylamide (Sigma), as previously described (14). These gels were functionalized with 25 μ g/mL fibronectin using sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate. The concentrations of acrylamide and bisacrylamide used to give the stiffnesses used in this study were chosen based on prior work by Tse and Engler (14).

Immunofluorescence staining

Cells on glass-bottom dishes or polyacrylamide gels were fixed with 4% para-formaldehyde (Thermo Scientific, Rockford, IL) in phosphate-buffered saline with Ca²⁺ (Life Technologies, Eugene, OR). The primary antibody used was anti-Ki67 (anti-Rabbit; Abcam, Cambridge, MA, and anti-Rabbit; Thermo Fisher) with secondary AlexaFluor 647 chicken anti-Rabbit immunoglobulin G (IgG) (Life Technologies). Along with the secondary antibody for the non-E-cadherin FRET tension-sensor-expressing cells, LifeAct 488 phalloidin (Cytoskeleton, Denver, CO) was used to stain for actin and Hoechst 33342 (Life Technologies) to stain nuclei. Cells were mounted and imaged on the Zeiss 710 laser scanning confocal microscope.

Immunofluorescence staining of EdU

The Invitrogen Molecular Probes Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher) was used for staining the presence of 5-ethynyl-2'-deoxyuridine (EdU) incorporation into DNA. Cells were seeded in sparse and confluent colonies as previously described, with the exception that EdU was added a day postseeding sparse and 2 days postseeding confluent and left for an extra day to allow EdU incorporation if the cells went through the synthesis (S) phase of the cell cycle.

Biaxial stretch of subconfluent monolayer

Biaxial tension experiments were performed using the FlexCell FX-5000 Tension System (Flexcell International Corporation, Burlington, NC). Cells were seeded as subconfluent monolayers on the amino-functionalized BioFlex Culture 6-Well Plate (Flexcell International Corporation). The wells were coated with 40 μ g/mL fibronectin before the cells were seeded. The cells were seeded overnight, and experiments were performed the next day. A 15% biaxial strain was applied for a period of 1 h, and after 4 h, the cells were fixed and stained for Ki67. In select experiments, cells were treated with 10 μ g/mL of DECMA-1 E-cadherin-blocking antibody (EMD Millipore, Burlington, MA) or 10 μ g/mL of control rat IgG (Catalogue 31933; Thermo Fisher) at the time of seeding, and the antibody was maintained in the media for the duration of the experiment.

FRET analysis of E-cadherin force changes under stretch was measured using a custom-made cell-stretching device compatible with live cell

imaging, in which subconfluent colonies of cells were seeded on silicone (thickness of 0.005 inches; Specialty Manufacturing, Saginaw, MI). Cells expressing the E-cadherin tension sensor or dileucine endocytosis mutant sensor were tracked, and FRET images were acquired at 0% (static), 15, and 30% biaxial strain to allow paired comparisons of E-cadherin junctional FRET from the same cell.

Stitching image tiles

Stitching of the image tiles was manually done on Microsoft Word, making sure the image dimensions and positions were precise. The images were then saved as high-quality tagged image file format files and analyzed using FIJI ImageJ software.

Intensity plot analysis

Intensity plots were generated in FIJI ImageJ for the Ki67- and EdU-stitched image channels. The red line shown is the average intensity, which is found by averaging the data points of the intensities output by the ImageJ software.

Traction force imbalance measurements

The intercellular force in MDCK cell pairs (expressing either wild-type (WT) or dileucine mutant E-cadherin) was measured using traction force imbalance measurements. Here, Collagen I (BD Biosciences, Franklin Lakes, NJ) was first coated onto polyacrylamide gels (doped with fluorescent fiducial markers) of stiffness 8.4 kPa using the hydrazine hydrate (Sigma-Aldrich) method (prepared as in (15)). Then, MDCK cells were plated sparsely overnight such that they formed cell pairs. From images of the fiducial markers with and without the cells, the displacement field was calculated with the use of particle imaging velocimetry software in MATLAB (The MathWorks, Natick, MA; available at <http://www.oceanwave.jp/software/mpiv/>). Traction force reconstruction was performed using regularized Fourier-transform traction cytometry (16,17). The intercellular force was then calculated as the imbalance in the traction force under each cell in a cell pair as in (5). We observed $n = 11$ cell pairs for MDCK cells expressing WT E-cadherin and $n = 10$ cell pairs for MDCK cells expressing the dileucine mutant E-cadherin.

RESULTS

Cells at the center of large epithelial cell colonies continue to proliferate

To assess the spatial proliferation of confluent and subconfluent cells, MDCK epithelial cells were seeded as either large (8–10 mm in diameter) colonies or confluent monolayers (2 days postconfluence) (Fig. 1 A). Positive immunostaining for Ki67, a marker for cells in the gap 1, S, and gap 2 phases, was observed for a large number of cells at the edge of the colony and in the center (Fig. 1 B). To further examine cell proliferation, cells were exposed to EdU, a thymidine analog, which is incorporated into cellular DNA only during the S phase of the cell cycle. Similar to Ki67, EdU-positive cells were seen at both the edges and center of the colony (Fig. 1 C). To qualitatively assess the distribution of proliferation, we generated intensity plots of Ki67 and EdU staining across the entire colony (Fig. 1 D).

Cells in a confluent monolayer, although similar in cell size to cells at the center of an epithelial colony, had minimal positive Ki67 and EdU cells (Fig. 1 E). To quantify differences across edge, center, and confluent cells, images from each condition were scored for positive Ki67 or EdU and then normalized to the total cell count (based on Hoechst staining). Quantification of the fractions of Ki67- and EdU-positive cells revealed that there was a significantly higher fraction of proliferating cells at the edge of the colony as compared to the center; however, cells at the center still had elevated proliferation compared to cells in a confluent monolayer (Fig. 1 F).

To assess the role of serum in the media on spatial proliferation, these experiments were repeated under low serum conditions (1% FBS). In reduced serum media, there was a marked decrease in the proliferation of cells in the center (Fig. S1), showing that growth factors do augment proliferation of cells in the center of the colony, which is in agreement with prior work showing spatial differences in cell proliferation triggered by epidermal growth factor (18). In separate experiments with normal serum conditions (10% FBS), we observed that more frequent media replenishment of confluent cells did not alter cellular proliferation (Fig. S2), indicating that confluent cells were not inhibited by a lack of growth factors.

E-cadherin force is increased in subconfluent cells as compared to confluent monolayers

Stable MDCK cells expressing the E-cadherin force biosensor (6) were seeded as confluent monolayers or subconfluent colonies. A schematic of the sensor, in which FRET is inversely related to force, is shown in Fig. 2 A. FRET was measured at the edge of the colony (defined as three to four rows into the colony), and the center (30–40 rows into the subconfluent colony), as well as for an additional dish with a confluent monolayer (images for the confluent colony were taken across the colony) (Fig. 2 B). The edge of a sparse colony had the highest level of E-cadherin force (indicated by lower FRET), followed by cells in the center of the colony, and the lowest level of force was observed in the confluent colony (Fig. 2, C and D), correlating to the proliferative state of the cells (Fig. 1 F).

Previous work by Muhamed et al. (19) has shown that force on E-cadherin can activate integrins in an EGFR-dependent manner. To investigate the role of this pathway in the spatial proliferation of cells, we inhibited EGFR activity with 15 μ M of gefitinib. Gefitinib did not affect the number of Ki67-positive cells (Fig. S3 A), suggesting that this pathway is not necessary for proliferation of cells at the edge or center of the colony. Gefitinib did attenuate the number of EdU-positive cells at the edge and center of the colony (Fig. S3 B). We also did not observe a

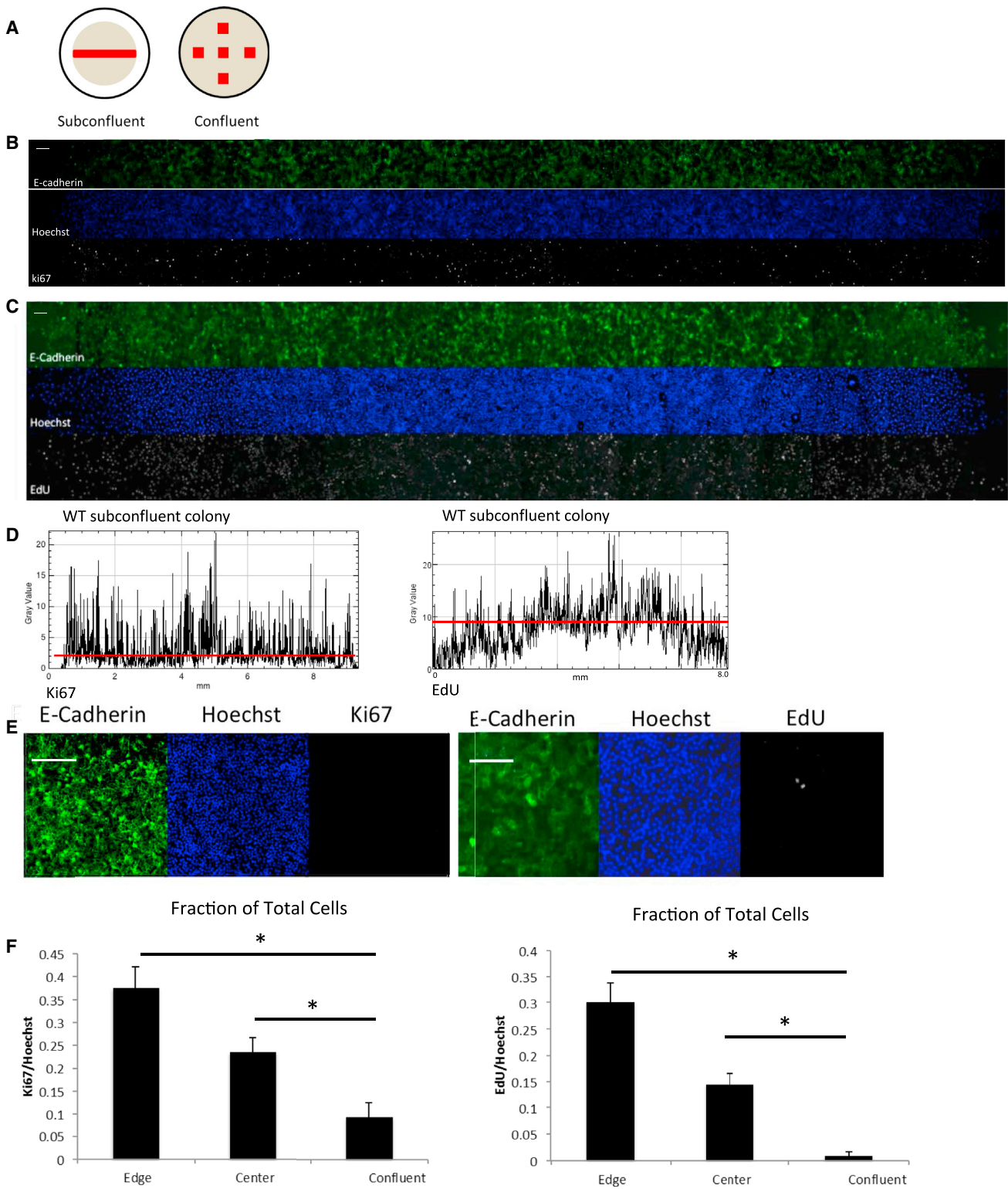


FIGURE 1 Spatial distribution of proliferating cells in a subconfluent colony. (A) A schematic of the distribution of cells (in *gray*) and the regions imaged (*red*) is shown. A single row of images was taken from the beginning to the end of the sparse colony, whereas in the confluent, images were taken randomly throughout the colony. The subconfluent colonies were ~ 10 mm in diameter. (B and C) Positive Ki67 and EdU cells, respectively, were observed at the center of the colony (scale bars, 150 μm). (D) Intensity plots of Ki67 and EdU intensity across the colony are shown. The red line indicates mean intensity. (E) Minimal Ki67- and EdU-positive cells were observed in confluent colonies. (F) The fraction of Ki67- and EdU-positive cells was quantified for edge and center cells in the colonies as well as cells in the confluent monolayers (a minimum of 10 frames per condition were quantified and three independent experiments showed the same trend; $*p < 0.05$). To see this figure in color, go online.

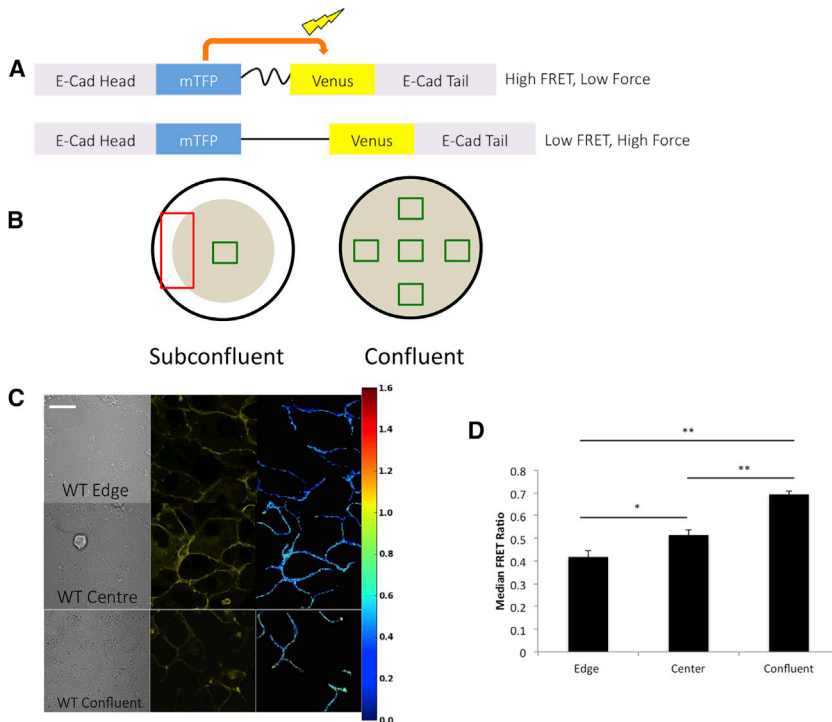


FIGURE 2 Spatial distribution of force in a subconfluent colony. (A) A schematic of the E-cadherin tension sensor, in which force is inversely proportional to FRET, is shown. (B) A diagram showing areas defined as the edge and center of the subconfluent colony is shown. The edge (*red*) was defined as 1–5 rows into the colony and the center (*green*) as 30–50+ rows into the colony. (C and D) FRET ratio images (a minimum seven frames per condition) of the E-cadherin sensor at the edge and center of colonies and in confluent monolayers are shown (scale bars, 20 μm). The edge has the highest force, whereas the confluent colony has the lowest force (three repeated experiments; * $p < 0.0063$, ** $p < 0.0001$). To see this figure in color, go online.

difference in the force of E-cadherin when EGFR was inhibited (Fig. S3 C).

Development of reduced-force E-cadherin mutants

To investigate if E-cadherin forces were important for the spatial regulation of proliferation observed in Fig. 1, we developed two lower-force mutants of E-cadherin (dileucine endocytosis mutant and tailless (also known as Ecad Δ cyto)) and compared them to WT E-cadherin (Fig. 3 A). Tailless, previously developed in (6), lacks the catenin-binding domain of E-cadherin required for cytoskeletal connectivity and therefore cannot experience mechanical loading. It also contains a mutation in the dileucine motif to prevent endocytosis and to stabilize the truncated protein at cell-cell junctions. The endocytosis mutant lacks the dileucine endocytotic motif, stabilizing E-cadherin at the junction, but retains the rest of the cytoplasmic tail, remaining connected to the cytoskeleton. These two E-cadherin mutations were expressed at high levels (greater than or equal to endogenous E-cadherin; Fig. S4). Previously, it was shown that E-cadherin lacking its cytoplasmic tail functions as a dominant negative (20). Similarly, the dileucine mutant has been shown to displace endogenous E-cadherin from cell-cell contacts (11), indicating that it can likewise function as a dominant negative. FRET measurements of the three sensors in confluent cells showed that the dileucine mutant E-cadherin is still subject to mechanical tension, because the

FRET from this sensor was lower than the zero-force tailless mutant (Fig. 3, B and C). No significant difference was observed between the dileucine mutant and WT E-cadherin in confluent cells (Fig. 3, B and C), indicating that in the confluent condition, there is no force difference between WT and dileucine mutant E-cadherin. Next, we examined the force differences with the dileucine mutant for cells at the edge and center of epithelial colonies. The dileucine mutant had increased FRET for subconfluent cells (both at the edge and center of the colony) as compared to WT E-cadherin (Fig. 3, D and E), indicating that the dileucine mutant experiences reduced tension in subconfluent cells, creating a condition in which E-cadherin exists in a reduced force state. The force-insensitive tailless E-cadherin-expressing cells had no differences in FRET between edge, center, and confluent cells (Fig. 3, D and E), further confirming the force sensitivity of the FRET sensor.

To assess the role of the overall junction forces between cells for these different E-cadherin constructs, traction force imbalance measurements (5) of WT, dileucine endocytosis mutant, and tailless E-cadherin-expressing cell pairs (subconfluent) were performed. Results from this technique showed that the overall intercellular force was not significantly different between any of the three conditions (Fig. 3 F). Thus, the force transmitted specifically through E-cadherin was the distinguishing feature of the WT and mutant E-cadherin constructs used here and not the total intercellular force, which can also be influenced by other cell-cell adhesion molecules including K-cadherin

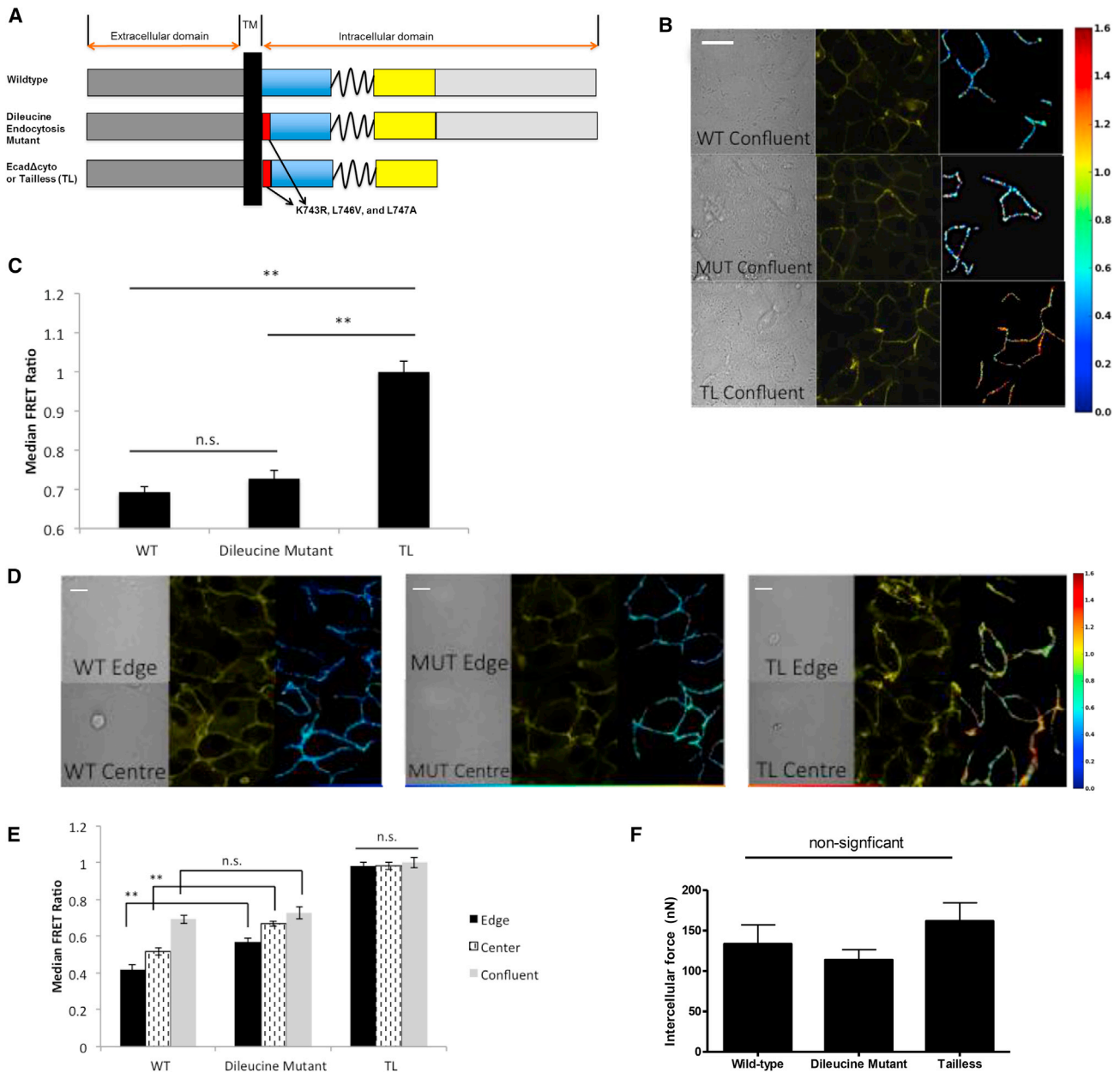


FIGURE 3 Lower-force mutants of E-cadherin. (A) A schematic of the wild-type (WT), dileucine endocytosis mutant, and tailless E-cadherin is shown. (B and C) E-cadherin FRET-force measurements were done in confluent colonies as a test of functionality of the mutants (scale bars, 20 μ m). The zero force control (tailless) showed the highest FRET ratio, corresponding to the lowest relative force of the three cell lines (a minimum of seven frames per condition; $**p < 0.0001$). (D and E) FRET-force measurement at the edge and center of a subconfluent colony in the mutant cell lines is shown (scale bars, 20 μ m; three repeated experiments; $**p < 0.01$). (F) Intercellular force measurement between the WT and the Dileucine mutant was not significantly different. n.s., nonsignificant; TM, transmembrane. To see this figure in color, go online.

(21), nectins (22), and desmosomal cadherins (23), among others.

E-cadherin force is necessary for the proliferation of center cells

Having developed E-cadherin mutants with reduced force, next we examined the role of E-cadherin force in spatial

proliferation. Cells were plated as single cells sparsely across a cell culture dish, and cell number was counted daily for 4 days. Both the dileucine endocytosis mutant and tailless mutant grew slower when compared to cells expressing the WT E-cadherin force sensor (Fig. 4 A). As a control, the WT E-cadherin sensor-expressing cells were compared to the parental MDCK cell line, and no significant difference was observed, meaning that

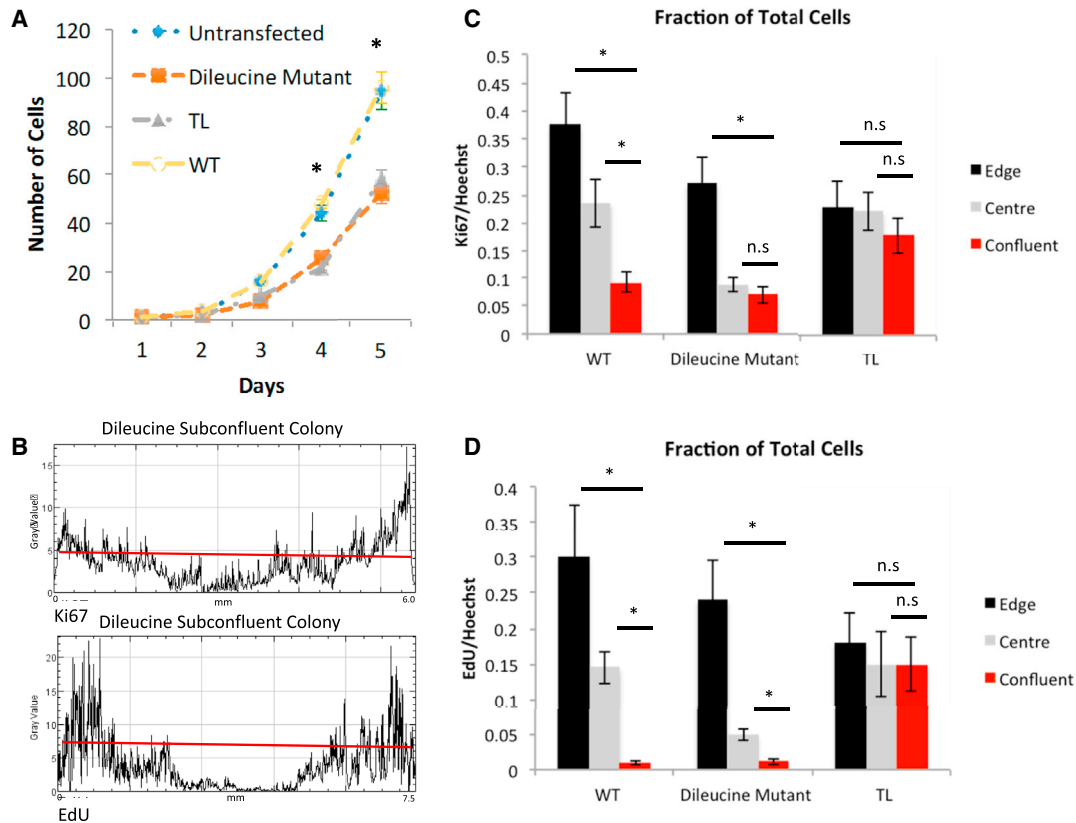


FIGURE 4 Proliferation is decreased when E-cadherin force is reduced. (A) E-cadherin force mutants show an impaired growth rate relative to the WT (a minimum of eight colonies per condition per day, and three independent experiments showed the same trend; * indicates WT and parental cells (MDCK) were significantly different from tailless and dileucine endocytosis mutant; $p < 0.05$). The figure shows the average number of cells per colony observed per day. (B) Intensity plots of Ki67 and EdU intensity across dileucine mutant subconfluent colonies are shown. The red line indicates mean intensity. (C) A fraction of Ki67-positive cells in a subconfluent colony at the edge and center and in a confluent colony is shown (a minimum of 10 frames per condition was quantified, and three independent experiments showed the same trend; $p < 0.05$). (D) A fraction of EdU-positive cells in a subconfluent colony at the edge and center and in a confluent colony is shown (a minimum of 10 frames per condition was quantified, and three independent experiments showed the same trend; $p < 0.05$). n.s., nonsignificant. To see this figure in color, go online.

overexpression of E-cadherin does not increase proliferation, but rather expression of dileucine mutant and tailless E-cadherin slows proliferation. Next, dileucine mutant cells in a large colony were stained for Ki67 and EdU. Intensity plots showed that dileucine-mutant-expressing cells had less proliferation for cells at the center of the colony (Fig. 4 B; Fig. S5) as compared to WT cells (Fig. 1 D). These images were then quantified for Ki67- and EdU-positive cells as a fraction of the total cells. We observed a significant decrease in the proliferating cells at the center of the colony in the dileucine-mutant-expressing cells (Fig. 4, C and D), which supports the hypothesis that higher E-cadherin force promotes cellular proliferation. Tailless-expressing cells exhibited high proliferation in the confluent condition (Fig. 4, C and D), which is consistent with prior results (10) in which the adherens junction has a role in the contact inhibition of proliferation, which is likely a separate biological process from E-cadherin force-induced proliferation. We did not observe any significant differences in proliferation between the edge, center,

and confluent conditions for the tailless-expressing cells (Fig. 4, C and D). Notably, the proliferation of tailless cells at the edge of the colony was less than the proliferation of WT and dileucine-mutant edge cells, which further supports the role of E-cadherin forces for inducing cellular proliferation.

Substrate stiffness affects E-cadherin force and proliferation

ECM stiffness has been known to be positively correlated to cell proliferation (24). We therefore sought to understand how substrate stiffness affects E-cadherin force. Subconfluent cells expressing WT, dileucine endocytosis mutant, and tailless E-cadherin were grown on polyacrylamide gels of varying stiffness (1, 8, and 20 kPa). The results for the WT indicated that E-cadherin force increased as substrate stiffness increased (Fig. 5, A and B), whereas for the dileucine mutant, there was only a slight increase in force between 1 and 8 kPa but no

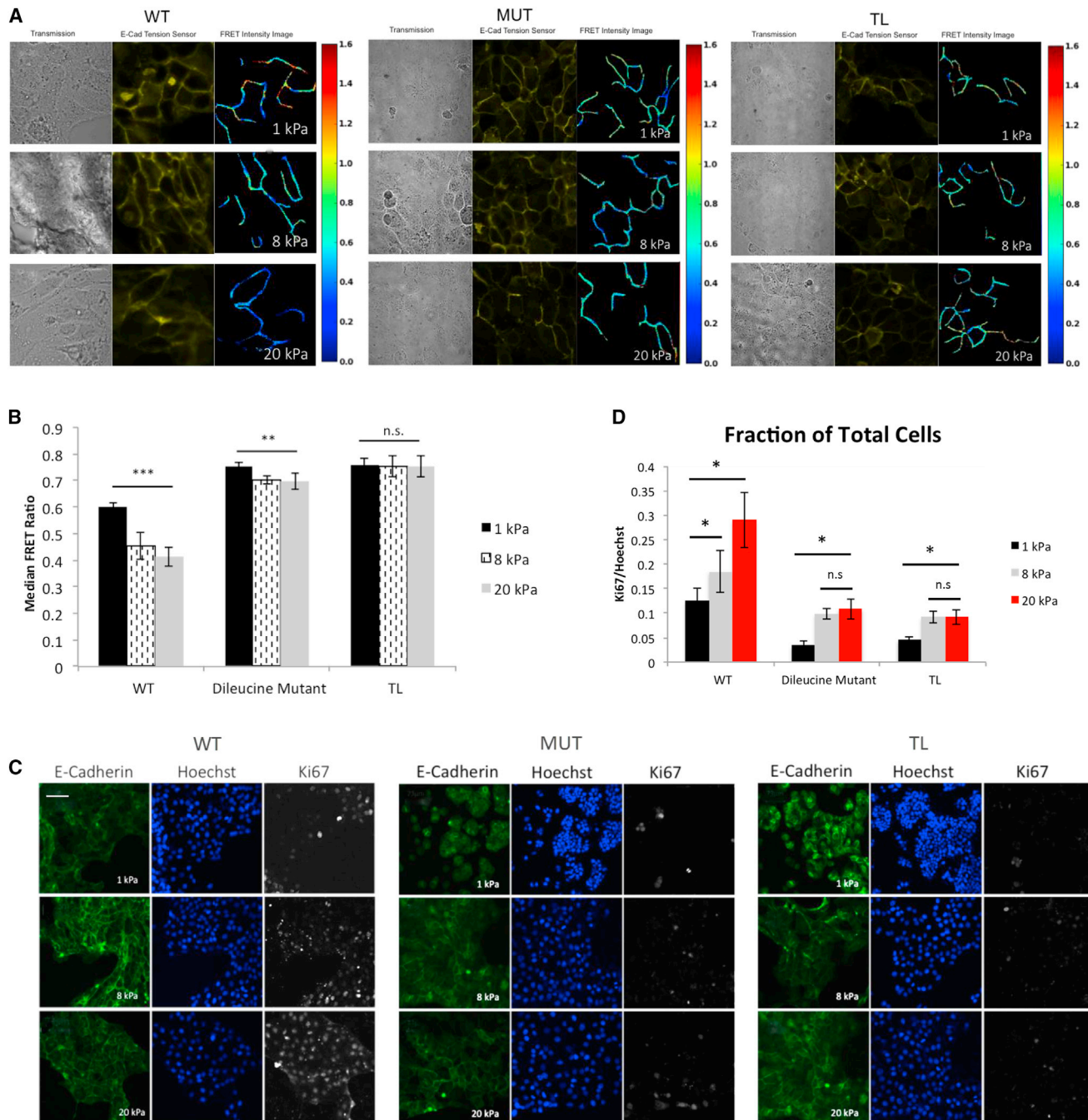


FIGURE 5 E-cadherin force has a positive correlation to substrate stiffness. (A) Transmission and FRET ratio images of the WT and mutants on polyacrylamide gels of varying stiffness are shown (scale bars, 20 μ m). (B) Quantification of the FRET ratios between the WT and mutants is shown (a minimum of six frames per condition; $p < 0.0001$). (C) Ki67 staining of the WT and mutants on the gels is shown (scale bars, 100 μ m). (D) Quantification of Ki67 staining is shown (a minimum of 10 frames per condition was quantified, and two independent experiments showed the same trend; $p < 0.05$). n.s., nonsignificant. To see this figure in color, go online.

difference between 8 and 20 kPa. There was no difference in force for the tailless mutant across the different gels. Next, we examined Ki67 expression and found that each group of cells had higher Ki67-positive cells when going from 1 to 8 kPa (Figs. 5, C and D). Because tailless E-cadherin-expressing cells experienced increased

proliferation, it suggests that an additional mechanism beyond E-cadherin force mediates stiffness-induced changes in proliferation. Only WT E-cadherin cells had further increased proliferation between 8 and 20 kPa (Fig. 5, C and D), which is correlated to increased E-cadherin force.

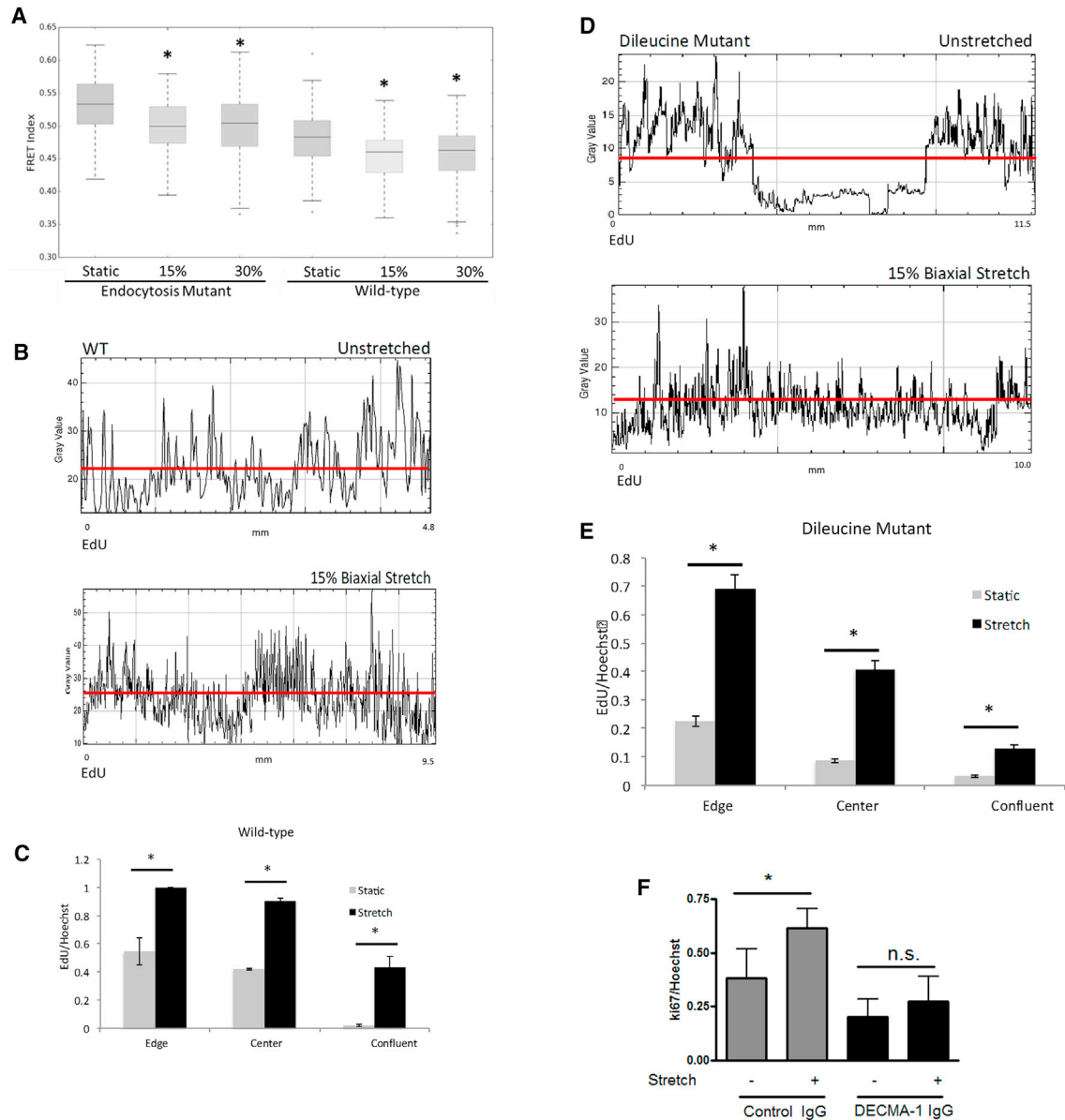


FIGURE 6 Stretch increases proliferating cells for both wild-type (WT) and dileucine mutant E-cadherin. (A) Cells expressing WT or dileucine endocytosis mutant E-cadherin force sensors were imaged at 0% (static), 15, and 30% biaxial stretch; $*p < 0.05$ between stretched and static condition. (B) Intensity plots of EdU across the WT colony before and after stretch are shown. The red line indicates mean intensity. (C) Quantification of EdU staining of unstretched and stretched WT cells. (D) Intensity plots of EdU across the dileucine endocytosis mutant colony before and after stretch are shown. The red line indicates mean intensity. (E) Quantification of EdU staining of unstretched and stretched dileucine mutant cells is shown. (F) Cells were seeded in the presence of DECMA-1 blocking antibody or control rat IgG, cultured under static conditions for 24 h, and then subjected to stretch. Ki67 staining was performed, and the fraction of Ki67 positive cells at the center of the colony was normalized to the total number of cells. To see this figure in color, go online.

Biaxial stretch rescues proliferation of center cells in dileucine endocytosis mutant colonies

To test if the principal defect of the dileucine mutant E-cadherin was reduced force (rather than endocytosis), we sought to develop an experimental approach to increase forces in these cells. Biaxial stretch was previously shown to increase E-cadherin force (6,10). First, we tested whether biaxial stretch would increase E-cadherin forces in the

dileucine mutant. We observed that a 15% biaxial stretch decreased FRET for both WT and dileucine mutant E-cadherin (Fig. 6 A), indicating that biaxial stretch is a suitable method to increase E-cadherin tension in the endocytosis-resistant dileucine mutant E-cadherin cells. In light of this observation, we used a 15% biaxial stretch as a method to test the hypothesis that increased tension across E-cadherin would rescue proliferation of mutant cells in the center of

the colony. Stretch increased proliferation in both edge and center cells in colonies of WT cells (Fig. 6 B), in agreement with prior work (10). Intensity plots showed that EdU staining increased evenly throughout the center of the colony (Fig. 6 C; Fig. S6 A). Stretch was also able to induce proliferation in both edge and center cells of dileucine-mutant-expressing cell colonies (Fig. 6 D). Notably, stretch eliminated the nonproliferative central region of the colony (Fig. 6 E; Fig. S6 C), returning the center cells to similar levels of proliferation as unstretched WT cells (Fig. 6 C). Tailless-expressing colonies were not responsive to stretch (Fig. S6, E–G), similar to a previous report showing that a cytoskeletal-connected E-cadherin is necessary for stretch-induced proliferation in confluent monolayers (10). To further test the role of E-cadherin forces in the context of force-induced proliferation, E-cadherin adhesions were inhibited with the E-cadherin-blocking antibody DECMA-1. Cells treated with E-cadherin-blocking antibody (DECMA-1) had reduced proliferation at the center of the colony that was not rescued with stretch (Fig. 6 F).

DISCUSSION

There is limited knowledge about how cell proliferation is spatially regulated across a large group of cells. In this study, we performed a careful analysis of how groups of cells proliferate, observing that cells in the center of the colony (completely surrounded on all sides with cell-cell contacts) had significantly higher levels of proliferation than confluent monolayers (Fig. 1 F) despite having similar cell densities. Although these cells in the center had a reduced proliferation rate compared to cells at the free edge of the colonies, the proliferation rate of the center cells did not appear to depend on the distance to the free edge, which is surprising given the large size of colonies studied (10 mm in diameter). At the center of the colony, cells have well-formed adherens junctions. Because ligation of E-cadherin inhibits growth-signaling pathways (2), there must be a mechanism by which cells at the center of the colony overcome contact inhibition.

Our work identifies cell-cell junction forces as a contributing signal for cell proliferation in these center cells that otherwise would be contact inhibited. We initially observed higher E-cadherin force in subconfluent cells as compared to confluent monolayers, with high E-cadherin forces also present at the center of the colony (Fig. 2 C). To confirm the contributory role of E-cadherin force to the proliferation of these cells, we identified a mutant of E-cadherin (defective in endocytosis) that resulted in a phenotype of lower E-cadherin force (Fig. 3, D and E) but no significant difference in the overall forces between subconfluent cells (Fig. 3 F). This allowed us to identify E-cadherin force, rather than global junction forces, as being the mediator of cell proliferation in epithelial colonies. Cells expressing the lower-force E-cadherin mutant had high proliferation

at the edges of the colony but impaired proliferation at the center of the colony (Fig. 4, B and D), suggesting that a protein (or proteins) in the E-cadherin adherens junction complex is the primary mechanosensor. We hypothesize that the lack of force by these center cells is what leads to the reduced proliferation rate of these cells (Fig. 4 A). However, it is also possible that the increased accumulation of E-cadherin at cell-cell contacts (as a result of reduced E-cadherin endocytosis) is the principal reason for reduced proliferation in these mutant cells. To further investigate the role of force in the dileucine endocytosis mutant cells, we subjected these mutant cells to biaxial stretch. Stretch was sufficient to increase E-cadherin force (Fig. 6 A) and rescue proliferation to the center cells (Fig. 6, D and E). Taken together, our data suggest that the major proliferation defect of the dileucine mutant of E-cadherin is force mediated.

Mechanical forces across cell-cell junctions have already been shown to regulate proliferation in the context of externally applied biaxial stretching forces (10). Our work extends upon this prior finding to show that this E-cadherin-dependent force-sensing proliferative mechanism occurs even when cells are not subject to externally applied force, but rather experiences changes in cell-generated force. We have also observed that cell proliferation of center cells is nonhomogeneous, frequently occurring in clusters or hot spots (Fig. 1, B and C). Similarly, we have observed nonhomogeneous distributions of E-cadherin force in both large colonies and confluent monolayers (D.E.C., unpublished data), which is in agreement with traction-force microscopy measurements that have shown similar hot spots of force (25). It will be interesting in future work to determine if and how these hot spots of force are correlated to hotspots of proliferation.

In addition to mechanical forces across cell-cell junctions, changes in focal adhesion forces can initiate cellular mechanical signaling events (26–28). Because forces at cell-cell junctions have been shown to be proportional to cell traction forces (4), it is possible that our efforts to perturb E-cadherin forces are also simultaneously affecting focal adhesion forces. This is an especially important consideration in the stretch experiments (Fig. 6), in which stretch also imparts mechanical force on focal adhesions. We sought to address this by using a blocking antibody to prevent the formation of E-cadherin adherens junctions. The blocking antibody inhibited stretch-induced proliferation of cells at the center of the colony (Fig. 6 F). This result, combined with similar work by Benham-Pyle et al. (10) and Hart et al. (29), suggests that the E-cadherin-containing adherens junction is necessary for stretch-induced proliferation; however, additional mechanosignaling events through focal adhesions may further contribute to stretch-induced proliferation. It is also possible that the E-cadherin dileucine and tailless mutants used in these studies could affect overall cellular contractility, thereby influencing the forces across focal adhesions. However, we observed that the expression

of E-cadherin dileucine and tailless mutants did not affect the overall cell-cell junction force (Fig. 3 F), arguing against the idea that changes in E-cadherin forces are inducing large-scale changes in cellular contractility.

Recent work on E-cadherin mechanotransduction has shown that increased forces on E-cadherin lead to integrin activation and changes in cell stiffness in a process that is dependent on EGFR activity (19). To investigate whether this pathway was required to mediate proliferation of cells at the center of the colony, we used an inhibitor of EGFR and assessed both proliferation and E-cadherin force. EGFR inhibition did not affect the distribution of Ki67-positive cells (Fig. S3 A) or E-cadherin force (Fig. S3 C), suggesting that this pathway is not involved in the induction of cell proliferation. However, the use of low-serum media (Fig. S1) and EGFR inhibition (Fig. S3 B) did reduce EdU incorporation, indicating that growth factors do affect the spatial proliferation across an epithelial colony. Interestingly, stretch experiments performed in which EGFR or focal adhesion kinase activity was inhibited resulted in significant monolayer detachment (D.E.C., unpublished data), suggesting that the E-cadherin/EGFR/focal adhesion pathway is important in cellular adaptation (stiffening and increased adhesion strength) in response to large changes in force.

Our finding of increased E-cadherin force at the edge of a colony (Fig. 2 C) is in agreement with a number of studies showing higher traction forces at the edges of colonies (15,30). Larger forces at the edges of colonies may be balanced by higher forces across cell-cell junctions, as has been previously measured for cell-cell pairs (4,5). Our current work using the E-cadherin force sensor also extends on these prior cell-cell pair studies by showing that increasing substrate stiffness also affects the force across cell-cell junctions in larger groups of cells (Fig. 5, A and B). Interestingly, we also show that increased proliferation at lower stiffnesses (from 1 to 8 kPa) is independent of E-cadherin force (Fig. 5, C and D). This increased proliferation may be the result of increases in cell spread area as well as stiffness-dependent activation of integrins (26). The major effect of substrate stiffness is to increase the proliferation of the center cells, as edge cells remain in a proliferative state even on 1-kPa hydrogels, which is in agreement with a prior report showing that substrate stiffness increased proliferation of center cells in response to epidermal growth factor (31). We also wish to note the methods of fibronectin attachment to glass (passive adsorption) was different from hydrogels (covalently cross-linked); therefore, our results between glass (Figs. 1, 2, 3, and 4) may not be directly comparable to hydrogels (Fig. 5).

A number of questions remain as to how these forces are generated deep into the colony. Given the large distance from the center to the edge of the colony, we presume that the center cells are not passively pulled by the edge cells, but that they must actively contribute to the force via internal mechanisms, such as increased myosin contractility.

Although E-cadherin forces have been shown to be myosin dependent (6), the detailed biological mechanisms for how E-cadherin force is regulated remain to be elucidated. A recent report has suggested that E-cadherin, similar to integrins, undergoes an activation process by which the cadherin is switched from a low to high adhesive state (32); it will be interesting to see the role force might play in switching the adhesive state of E-cadherin. It is also worth noting that we observed the highest E-cadherin forces at the edge of the colonies, which also had the largest cell sizes, suggesting that cell size could regulate cell-cell junction force (or vice versa). A prior study showed that the mitotic rate is correlated to cell size (33).

In conclusion, we have demonstrated that mechanical forces across E-cadherin vary as a function of cell confluence. E-cadherin tension is required for the proliferation of cells located at the center of larger colony; loss of E-cadherin force results in these cells becoming contact inhibited. Our results illustrate that cell-cell junction forces are dynamic and can modulate proliferation across a large colony of cells even in the absence of externally applied loads.

SUPPORTING MATERIAL

Six figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(18\)30917-2](http://www.biophysj.org/biophysj/supplemental/S0006-3495(18)30917-2).

AUTHOR CONTRIBUTIONS

A.M. and D.E.C. designed the research project. A.M., K.T.S., A.F.K., C.R.M., A.A.D., V.N., P.T.A., K.B., B.E.D., S.P.D., and V.M. performed experiments and analyzed data. A.M. and D.E.C. wrote the article.

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REFERENCES

1. Eisenhoffer, G. T., and J. Rosenblatt. 2013. Bringing balance by force: live cell extrusion controls epithelial cell numbers. *Trends Cell Biol.* 23:185–192.
2. Perrais, M., X. Chen, ..., B. M. Gumbiner. 2007. E-cadherin homophilic ligation inhibits cell growth and epidermal growth factor

- receptor signaling independently of other cell interactions. *Mol. Biol. Cell.* 18:2013–2025.
3. Garrod, D. 2010. Desmosomes in vivo. *Dermatol. Res. Pract.* 2010: 212439.
 4. Liu, Z., J. L. Tan, ..., C. S. Chen. 2010. Mechanical tugging force regulates the size of cell-cell junctions. *Proc. Natl. Acad. Sci. USA.* 107:9944–9949.
 5. Maruthamuthu, V., B. Sabass, ..., M. L. Gardel. 2011. Cell-ECM traction force modulates endogenous tension at cell-cell contacts. *Proc. Natl. Acad. Sci. USA.* 108:4708–4713.
 6. Borghi, N., M. Sorokina, ..., A. R. Dunn. 2012. E-cadherin is under constitutive actomyosin-generated tension that is increased at cell-cell contacts upon externally applied stretch. *Proc. Natl. Acad. Sci. USA.* 109:12568–12573.
 7. Conway, D. E., M. T. Breckenridge, ..., M. A. Schwartz. 2013. Fluid shear stress on endothelial cells modulates mechanical tension across VE-cadherin and PECAM-1. *Curr. Biol.* 23:1024–1030.
 8. Trepats, X., M. R. Wasserman, ..., J. J. Fredberg. 2009. Physical forces during collective cell migration. *Nat. Phys.* 5:426–430.
 9. Mertz, A. F., Y. Che, ..., V. Horsley. 2013. Cadherin-based intercellular adhesions organize epithelial cell-matrix traction forces. *Proc. Natl. Acad. Sci. USA.* 110:842–847.
 10. Benham-Pyle, B. W., B. L. Pruitt, and W. J. Nelson. 2015. Mechanical strain induces E-cadherin-dependent Yap1 and β -catenin activation to drive cell cycle entry. *Science.* 348:1024–1027.
 11. Miranda, K. C., S. R. Joseph, ..., J. L. Stow. 2003. Contextual binding of p120ctn to E-cadherin at the basolateral plasma membrane in polarized epithelia. *J. Biol. Chem.* 278:43480–43488.
 12. Hong, S., R. B. Troyanovsky, and S. M. Troyanovsky. 2010. Spontaneous assembly and active disassembly balance adherens junction homeostasis. *Proc. Natl. Acad. Sci. USA.* 107:3528–3533.
 13. Arsenovic, P. T., I. Ramachandran, ..., D. E. Conway. 2016. Nesprin-2G, a component of the nuclear LINC complex, is subject to myosin-dependent tension. *Biophys. J.* 110:34–43.
 14. Tse, J. R., and A. J. Engler. 2010. Preparation of hydrogel substrates with tunable mechanical properties. *Curr. Protoc. Cell Biol.* Chapter 10:Unit 10.16.
 15. Maruthamuthu, V., and M. L. Gardel. 2014. Protrusive activity guides changes in cell-cell tension during epithelial cell scattering. *Biophys. J.* 107:555–563.
 16. Butler, J. P., I. M. Tolić-Nørrelykke, ..., J. J. Fredberg. 2002. Traction fields, moments, and strain energy that cells exert on their surroundings. *Am. J. Physiol. Cell Physiol.* 282:C595–C605.
 17. Plotnikov, S. V., B. Sabass, ..., C. M. Waterman. 2014. High-resolution traction force microscopy. *Methods Cell Biol.* 123:367–394.
 18. Kim, J. H., K. Kushiro, ..., A. R. Asthagiri. 2009. Tunable interplay between epidermal growth factor and cell-cell contact governs the spatial dynamics of epithelial growth. *Proc. Natl. Acad. Sci. USA.* 106:11149–11153.
 19. Muhamed, I., J. Wu, ..., D. E. Leckband. 2016. E-cadherin-mediated force transduction signals regulate global cell mechanics. *J. Cell Sci.* 129:1843–1854.
 20. Levine, E., C. H. Lee, ..., B. M. Gumbiner. 1994. Selective disruption of E-cadherin function in early *Xenopus* embryos by a dominant negative mutant. *Development.* 120:901–909.
 21. Stewart, D. B., A. I. Barth, and W. J. Nelson. 2000. Differential regulation of endogenous cadherin expression in Madin-Darby canine kidney cells by cell-cell adhesion and activation of beta-catenin signaling. *J. Biol. Chem.* 275:20707–20716.
 22. Sato, T., N. Fujita, ..., Y. Takai. 2006. Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. *J. Biol. Chem.* 281:5288–5299.
 23. Lowndes, M., S. Rakshit, ..., W. J. Nelson. 2014. Different roles of cadherins in the assembly and structural integrity of the desmosome complex. *J. Cell Sci.* 127:2339–2350.
 24. Provenzano, P. P., and P. J. Keely. 2011. Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. *J. Cell Sci.* 124:1195–1205.
 25. Trepats, X., and J. J. Fredberg. 2011. Plithotaxis and emergent dynamics in collective cellular migration. *Trends Cell Biol.* 21:638–646.
 26. Vogel, V., and M. Sheetz. 2006. Local force and geometry sensing regulate cell functions. *Nat. Rev. Mol. Cell Biol.* 7:265–275.
 27. Saxena, M., S. Liu, ..., M. P. Sheetz. 2017. EGFR and HER2 activate rigidity sensing only on rigid matrices. *Nat. Mater.* 16:775–781.
 28. Choquet, D., D. P. Felsenfeld, and M. P. Sheetz. 1997. Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell.* 88:39–48.
 29. Hart, K. C., J. Tan, ..., M. Gloerich. 2017. E-cadherin and LGN align epithelial cell divisions with tissue tension independently of cell shape. *Proc. Natl. Acad. Sci. USA.* 114:E5845–E5853.
 30. du Roure, O., A. Saez, ..., B. Ladoux. 2005. Force mapping in epithelial cell migration. *Proc. Natl. Acad. Sci. USA.* 102:2390–2395.
 31. Kim, J. H., and A. R. Asthagiri. 2011. Matrix stiffening sensitizes epithelial cells to EGF and enables the loss of contact inhibition of proliferation. *J. Cell Sci.* 124:1280–1287.
 32. Petrova, Y. I., M. M. Spano, and B. M. Gumbiner. 2012. Conformational epitopes at cadherin calcium-binding sites and p120-catenin phosphorylation regulate cell adhesion. *Mol. Biol. Cell.* 23:2092–2108.
 33. Puliafito, A., L. Hufnagel, ..., B. I. Shraiman. 2012. Collective and single cell behavior in epithelial contact inhibition. *Proc. Natl. Acad. Sci. USA.* 109:739–744.

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Supplemental Information

**Spatial Proliferation of Epithelial Cells Is Regulated by E-Cadherin
Force**

Abhinav Mohan, Kyle T. Schlue, Alex F. Kniffin, Carl R. Mayer, Ashley A. Duke, Vani Narayanan, Paul T. Arsenovic, Kranthidhar Bathula, Brooke E. Danielsson, Sandeep P. Dumbali, Venkat Maruthamuthu, and Daniel E. Conway

Figure S1 | Untransfected (**A,B**) and MUT (**C,D**) cells grown as subconfluent colonies were subject to normal growth factor conditions and serum starvation to determine whether that impacted proliferation at the centre of the monolayer. Scale = 150um. (**E**) Quantification of the EdU positive cells at the edge and center of the subconfluent colonies (n = 10 images for edge and 10 images for the center).

Figure S2 | Media was changed every 24 hours over a period of 48 hours and not changed at all over a period of 48 hours to determine whether or not growth factors affected proliferation in the confluent state (Scale = 150um).

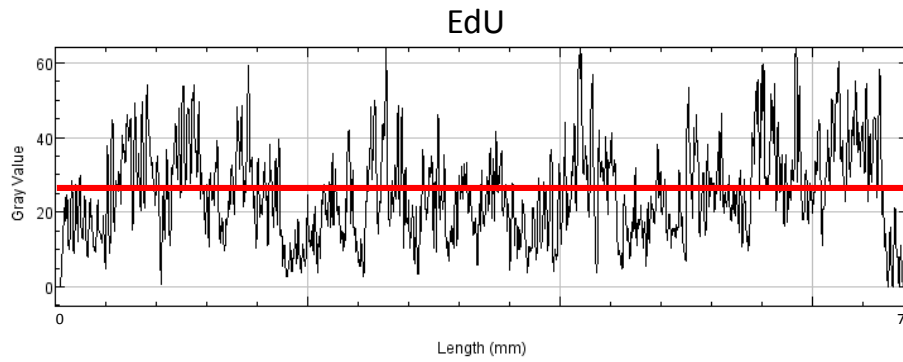
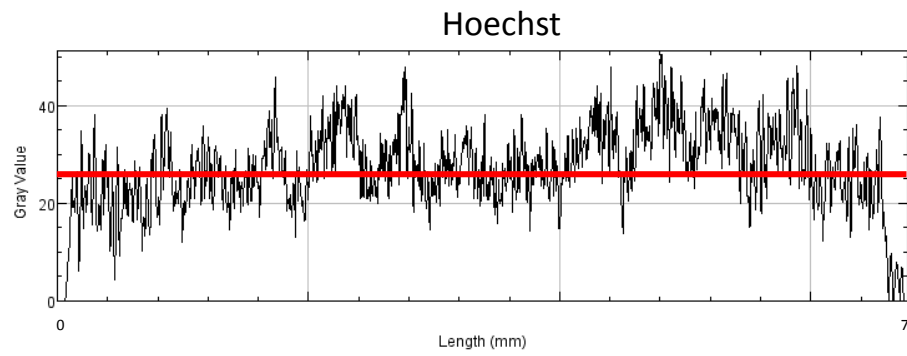
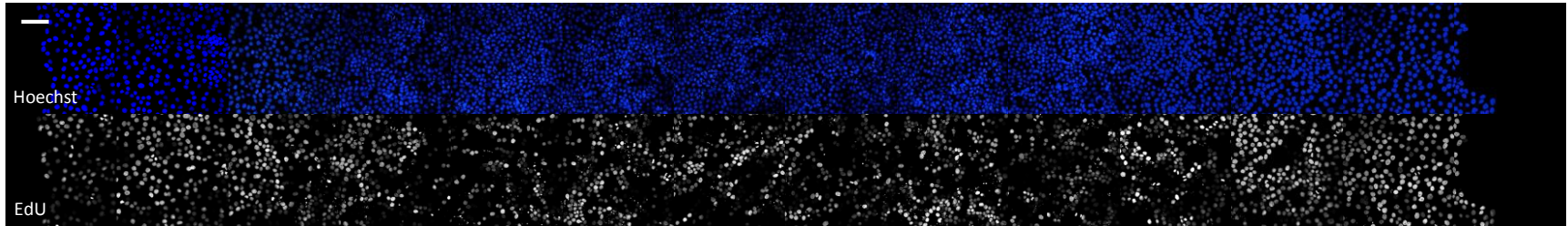
Figure S3 | Cells were untreated or treated with gefitinib (15 uM) for 24 hours to investigate the role of EGFR activity in proliferation as measured by ki67 staining (**A**) and EdU incorporation (**B**). (**C**) FRET measurements with the E-cadherin force sensor (wild-type) showed that there was no significant difference in E-cadherin force (at the center of the colony) with EGFR treatment.

Figure S4 | Blot of the endogenous E-Cadherin and TSmad constructs. Notable point is that the TSmad construct is significantly over expressed as compared to the endogenous E-Cadherin.

Figure S5 | Stitched image of Ki67 and EdU for the Dileucine Mutant corresponding to Figure 4 in the paper. Scale = 150um.

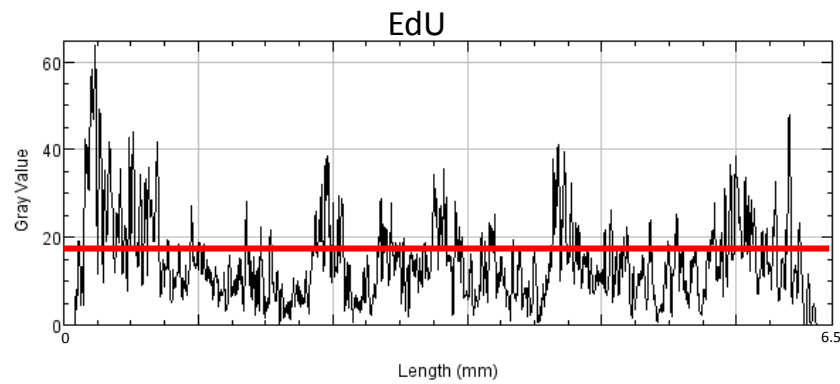
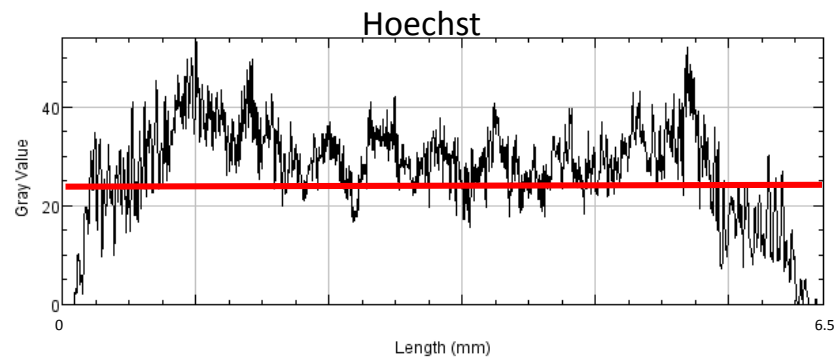
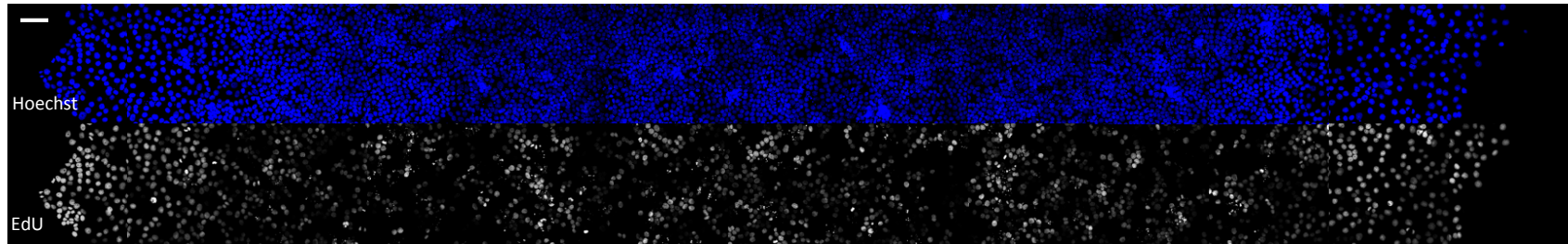
Figure S6 | EdU stained images from stretch experiments in Figure 6. **A**) Subconfluent monolayer of unstretched and stretched WT cells subjected to 15% biaxial stretch. **B**) Unstretched and stretched confluent WT cells. **C**) Subconfluent monolayer of unstretched and stretched Dileucine mutant cells subjected to 15% biaxial stretch. **D**) Unstretched and stretched confluent Dileucine mutant cells. **E**) Quantification of EdU staining on unstretched and stretched colonies expressing tailless E-cadherin. **F**) Subconfluent monolayer of unstretched and stretched Tailless cells subjected to 15% biaxial stretch. **G**) Unstretched and stretched confluent Tailless cells. (Scale = 150µm).

Supplemental Figure 1A



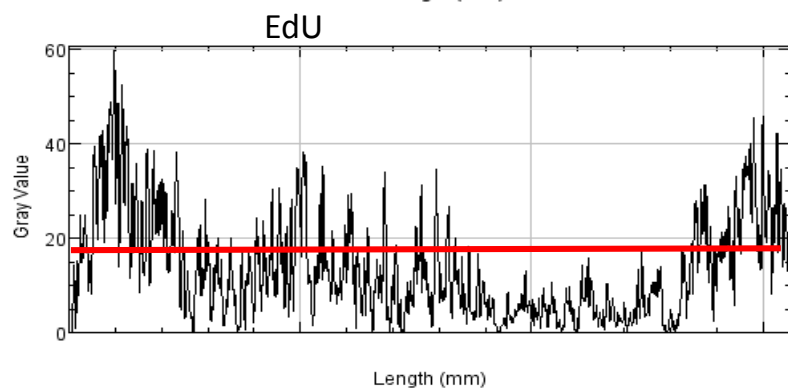
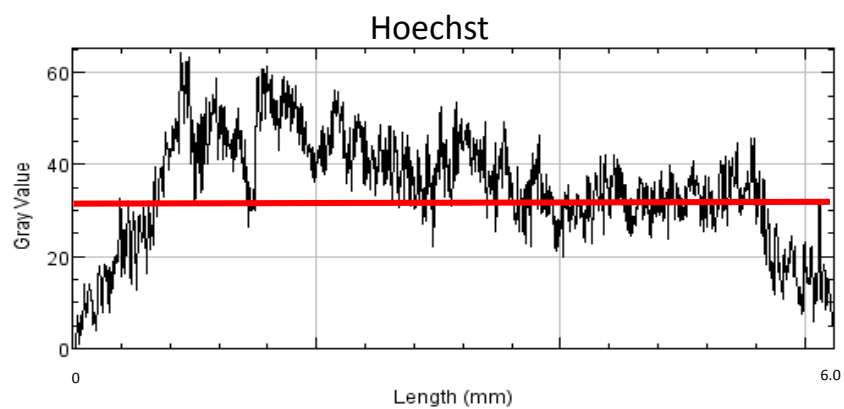
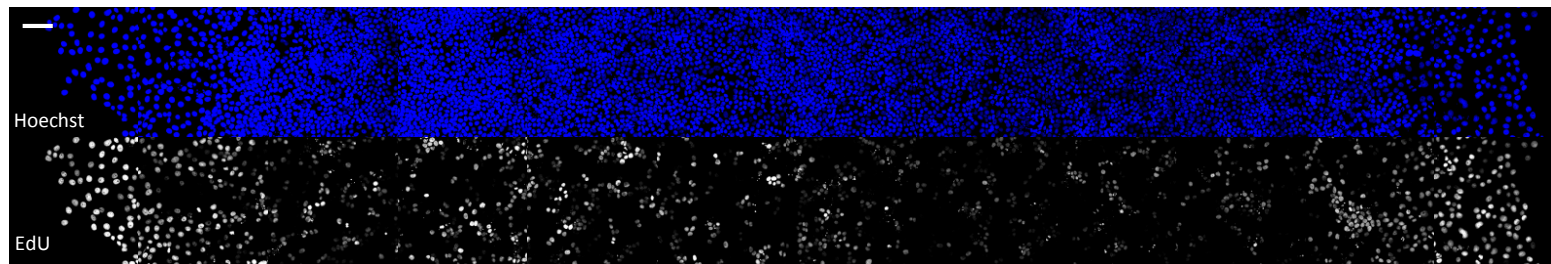
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Supplemental Figure 1B



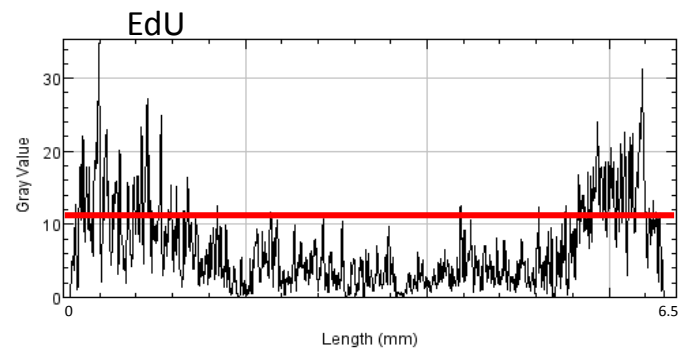
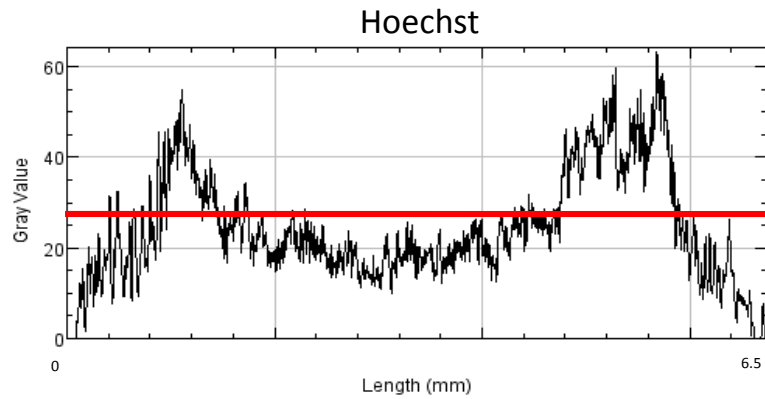
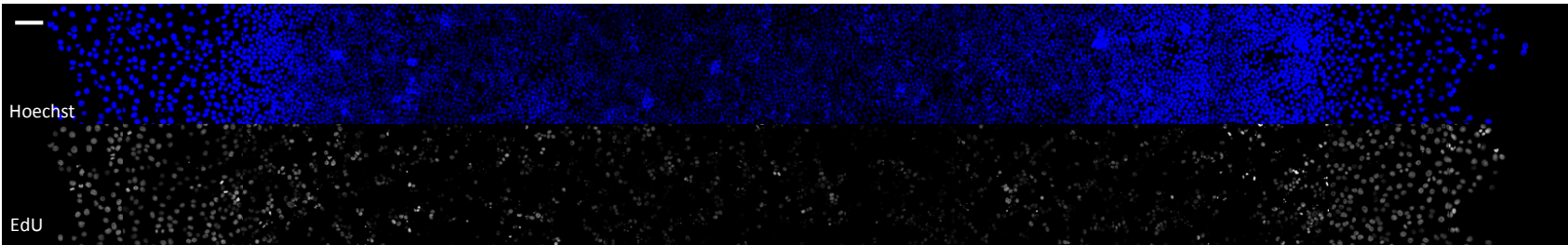
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Supplemental Figure 1C



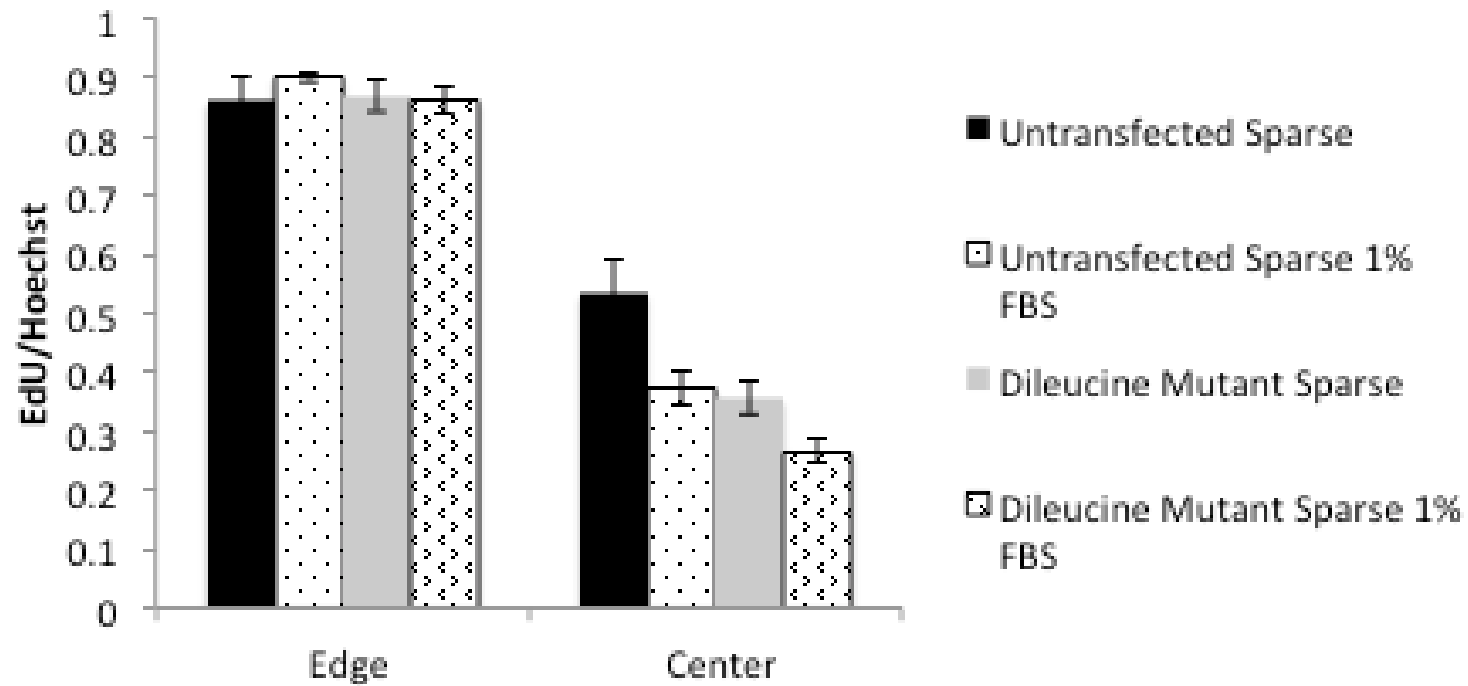
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Supplemental Figure 1D

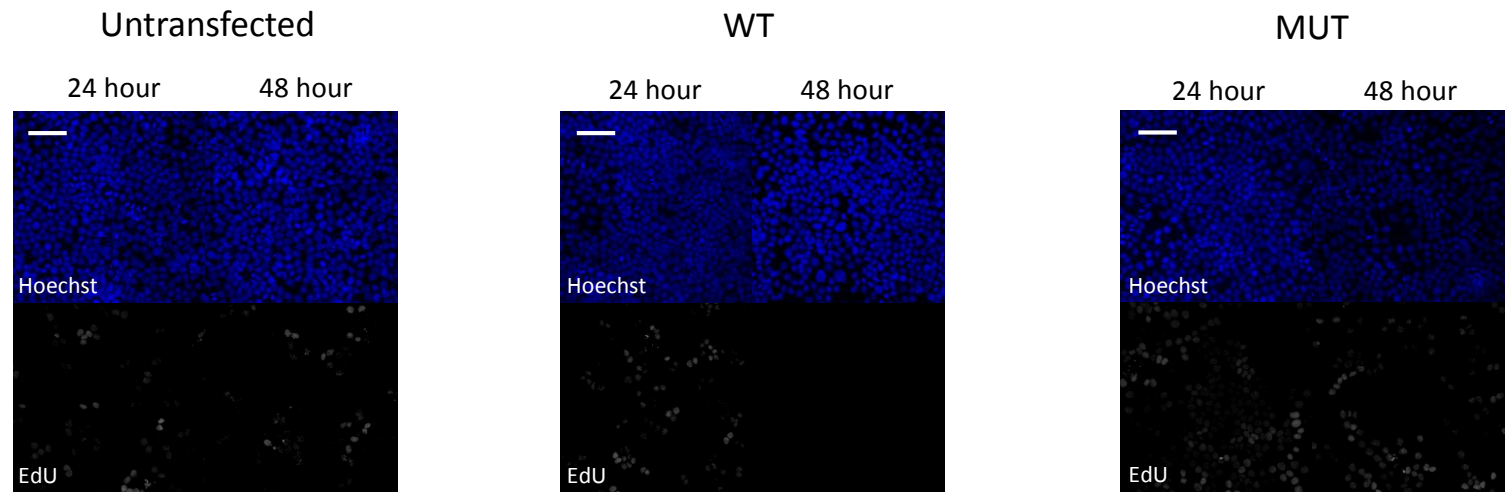
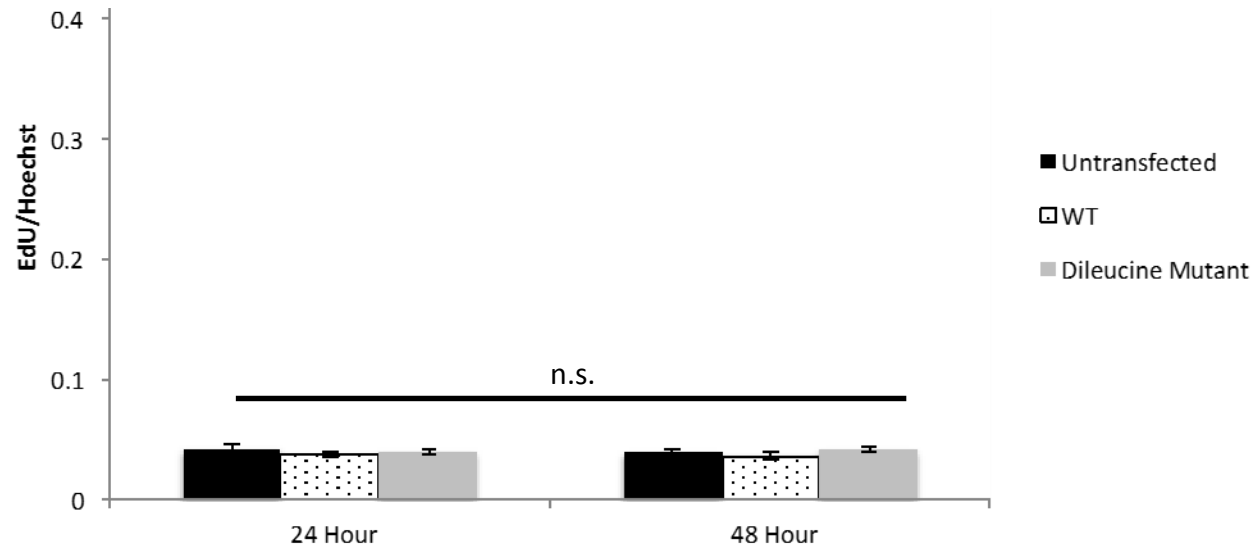


MUT sparse 1% FBS

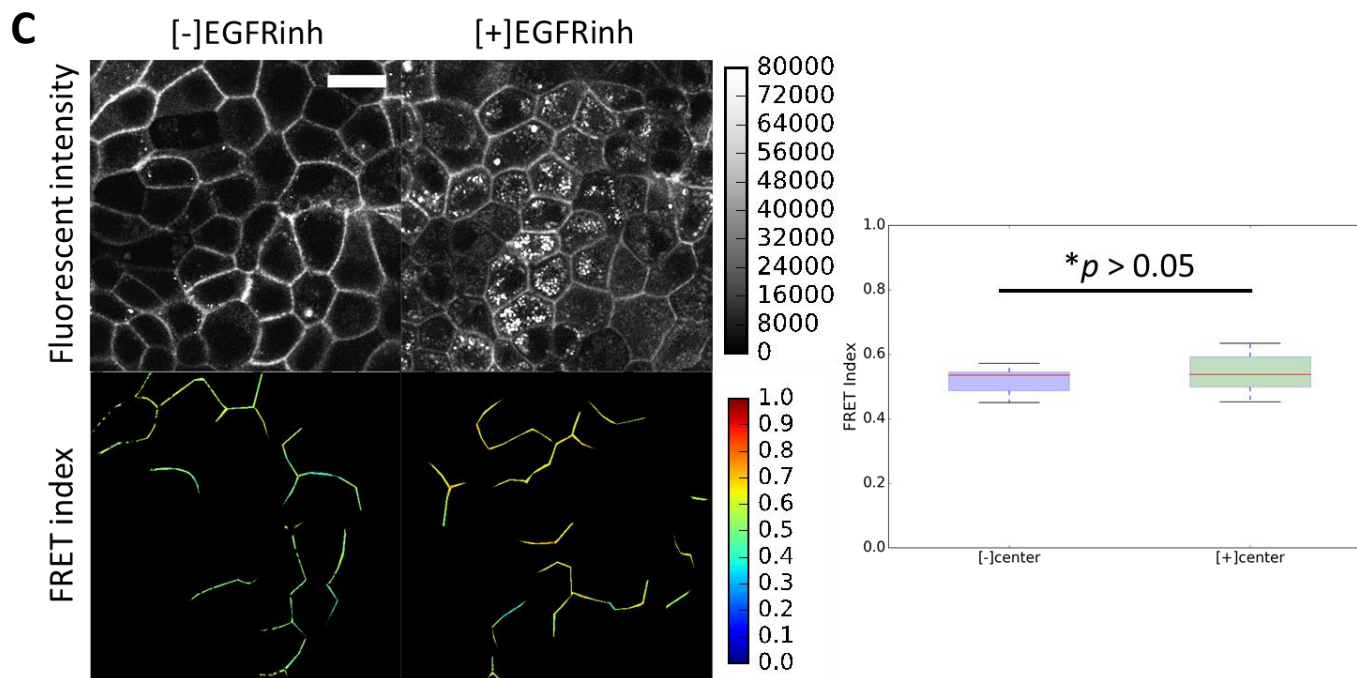
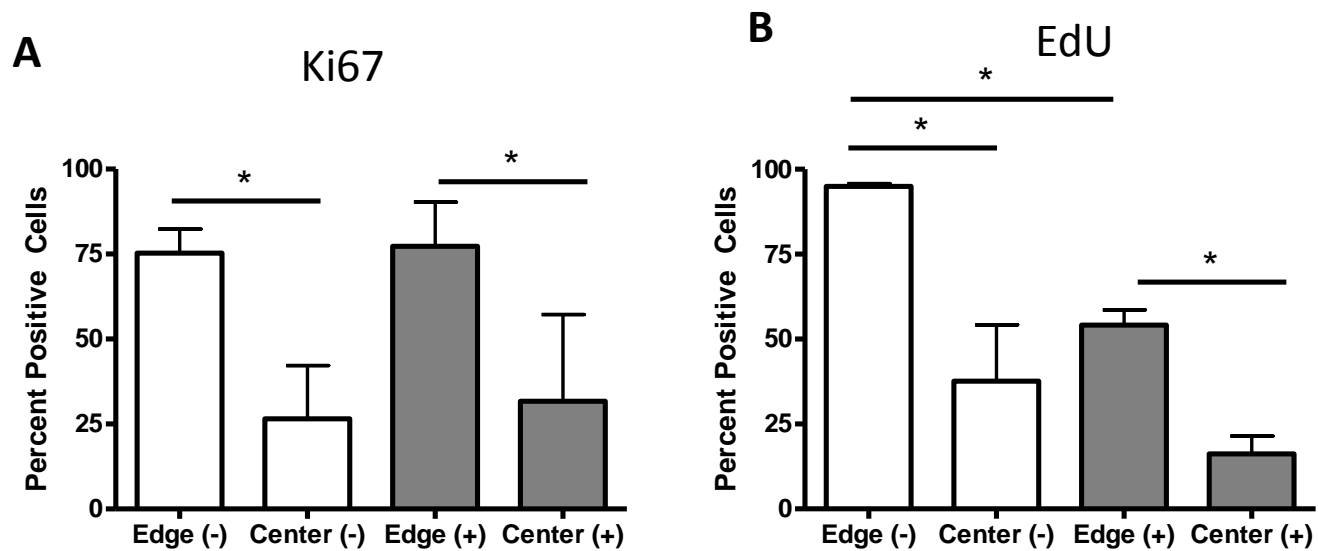
Supplemental
Figure 1E



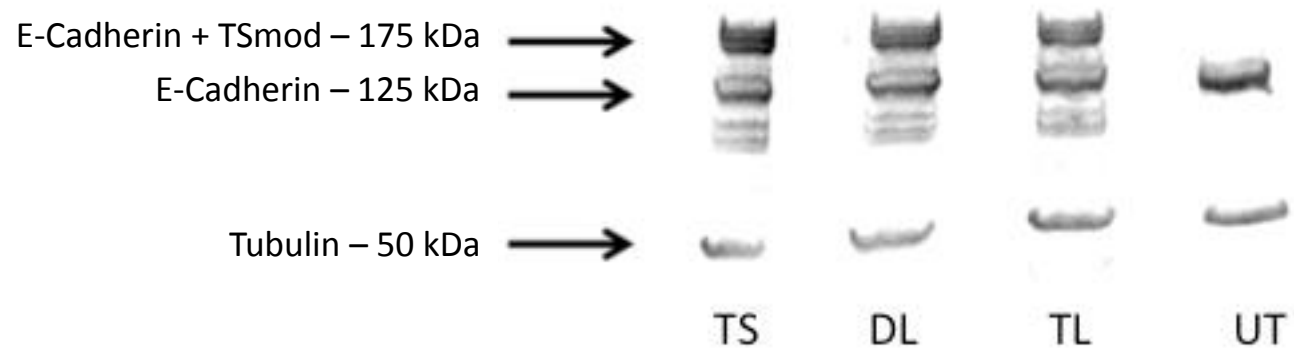
Supplemental Figure 2



Supplemental Figure 3



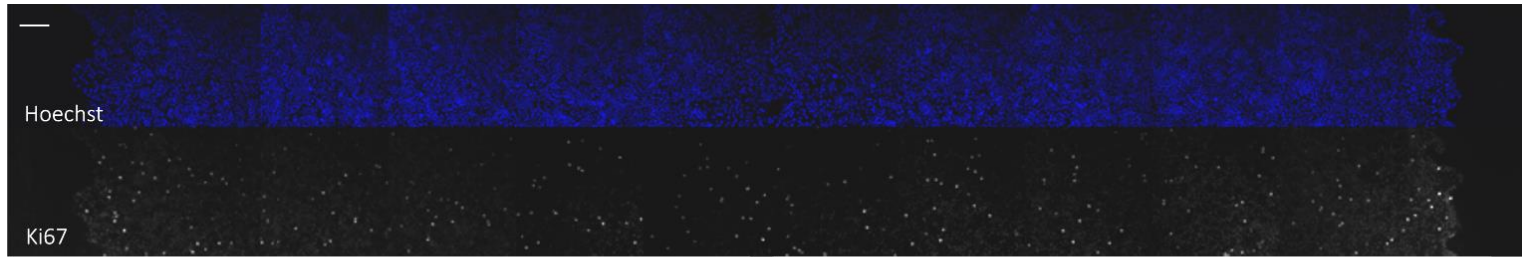
Supplemental Figure 4



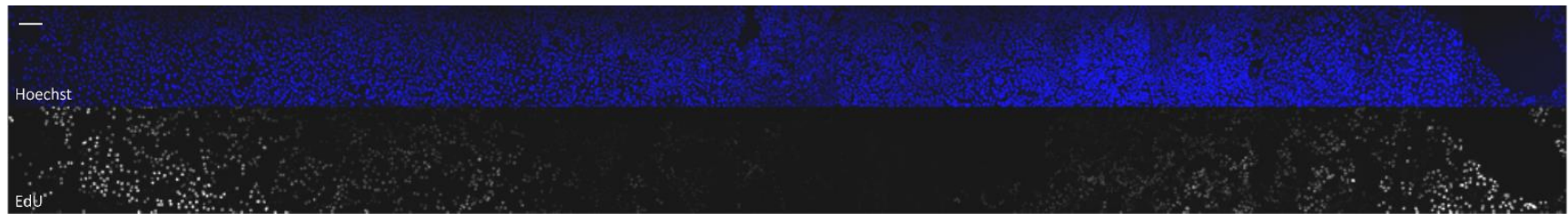
Supplemental Figure 5

Dileucine Mutant

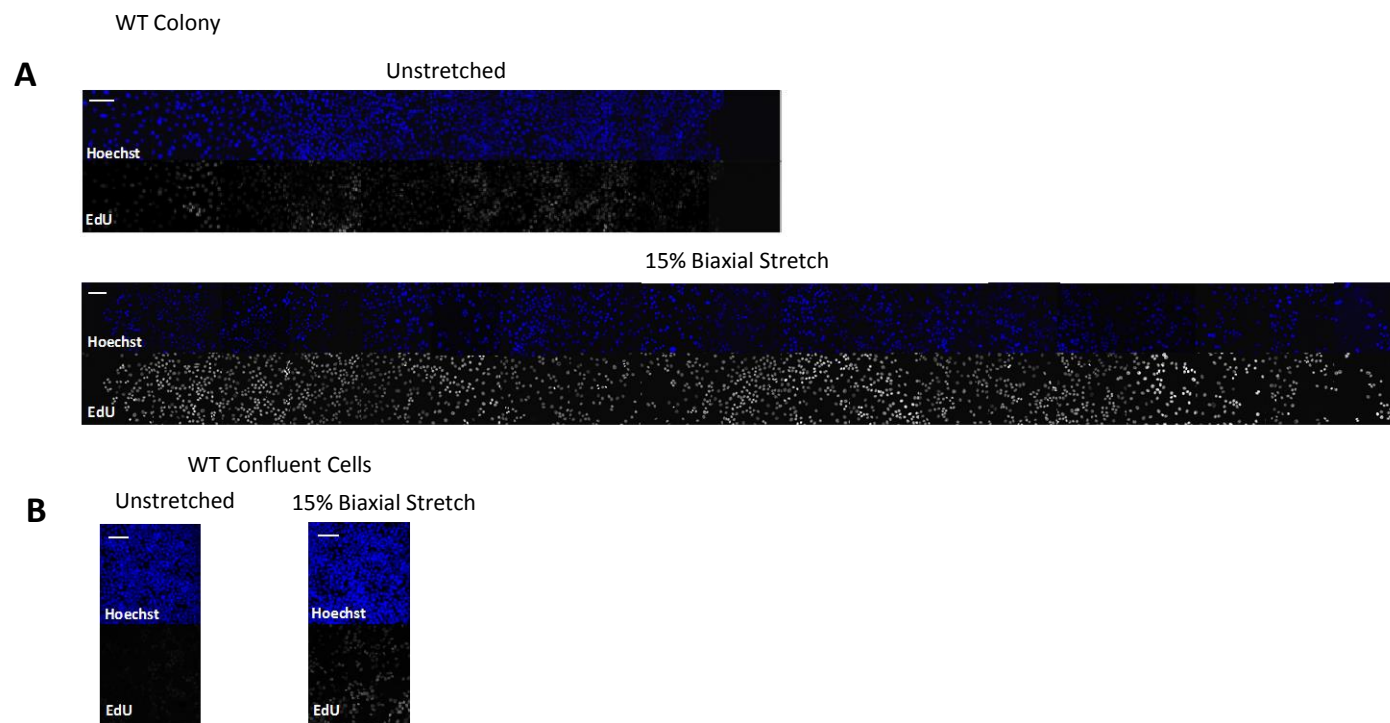
Ki67



EdU

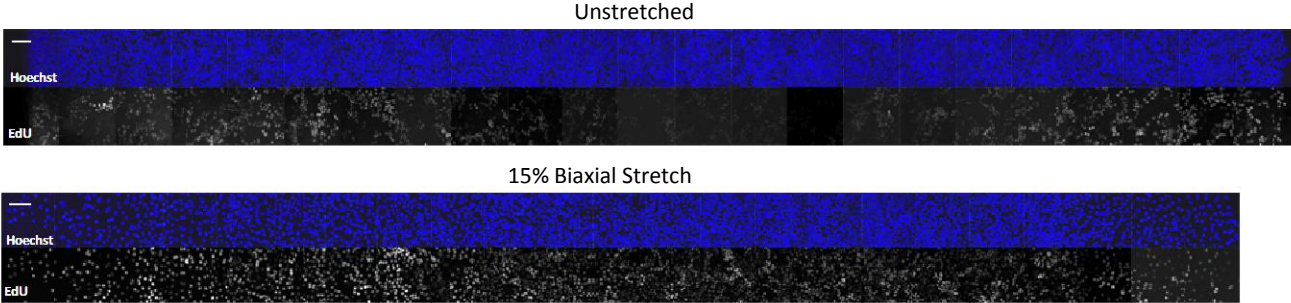


Supplemental
Figure 6

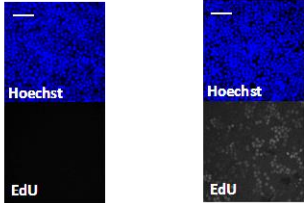


Supplemental
Figure 6, cont.

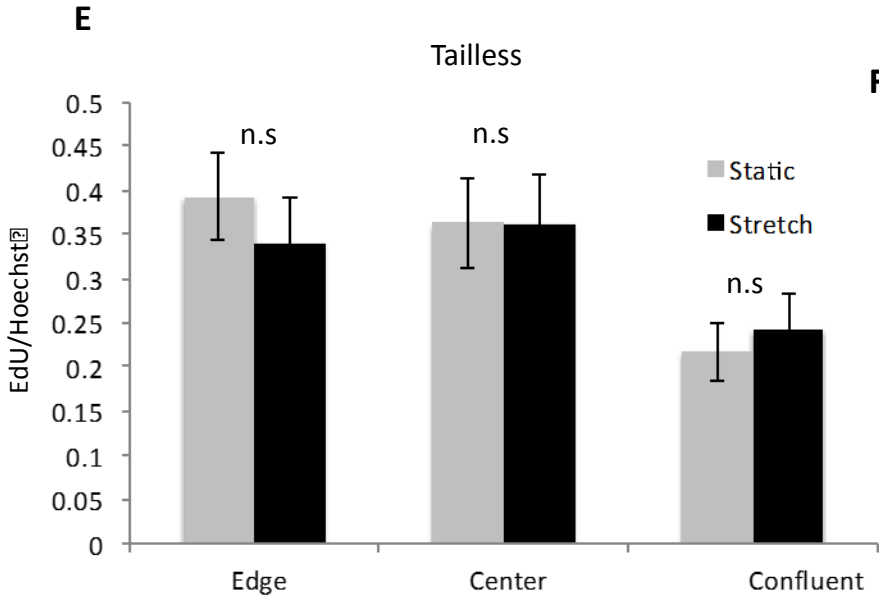
C Dileucine Mutant Colony



D Dileucine Mutant Confluent Cells
Unstretched 15% Biaxial Stretch



Supplemental Figure 6, cont



Tailless Colony

