A morphogenic cascade emerges from the co-evolution of spheroid fluidization and fracturing of multicellular barriers

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Abstract

Cell collectives migrate and undergo morphological transitions during development and diseases. How cells collectively invade a tissue barrier and its immediate consequence remains elusive. An outstanding challenge is to achieve a comprehensive understanding of the invasion cascade by integrating and applying principles from material science, biophysics, and cell biology. Using a three-dimensional model system of cancer spheroids invading the mesothelium, we show that collective cell invasion emerges from an interplay between spheroid fluidization and fracturing of the multicellular barrier. We found that mesothelial cell-cell contacts undergo tensile fracturing due to spheroid invasion. At the subcellular level, force transmission via intercellular integrin complexes between spheroid and mesothelial cells induces mesothelial cell apical constriction that leads to fracturing. Concurrently, fluidization and directed spheroid cells motility expand the mesothelial fracture. In response, the deformed mesothelium retards spheroid spreading, leading to spheroid cells crowding with contact inhibition of locomotion and proliferation. Our results demonstrate that collective invasion is

a morphogenic cascade, revealing that the multicellular barrier undergoing invasion-induced fracture can subsequently force the invaded cells into a dormant state of reduced proliferative potential. Thus, our conclusion may help explain why cancer cells that have invaded a foreign microenvironment tend to enter dormancy.

Introduction

Collective migration and heterotypic interactions between multicellular assemblies drive morphogenic transformation in tissue development and cancer progression [1-3]. For example, a critical step for Drosophila oogenesis entails border cell clusters interacting with their surrounding nurse cells, which promotes the migration of the border cell clusters towards the oocyte[4, 5]. In cancer, tumour spheroids penetrate tissues through tight interstitial spaces, ultimately colonizing distal peritoneal organs[5, 6]. Here, we study the 3D heterotypic culture model mimicking ovarian tumour invasion of the mesothelium during peritoneal metastatic implantation[7-10]. The mesothelium is a monolayer of simple squamous epithelium that lines the body's internal organs and serous cavities, such as the lungs and omentum[11]. As such, organs within the peritoneal cavity are covered with mesothelium[12-14]. Previous electron micrograph studies of patients' ovarian tumours attached to the peritoneum revealed that mesothelial cells were absent beneath the invading tumour[6, 13-15], implying that clearing of mesothelium by cancer spheroids is an early step of ovarian metastatic colonization. In 3D cultures, it was suggested that integrin and Talin-dependent activation of non-muscle myosin-II was required for spheroids to displace the mesothelial cells apart during invasion[7, 8]. An outstanding challenge is to elucidate spheroid invasion of a tissue barrier by integrating and applying concepts from material science, biophysics, and molecular cell biology. To that end, we present data that redefines collective invasion of the mesothelium as a morphogenic cascade that emerges from the co-evolution of spheroid fluidization and fracturing of multicellular barrier.

Fracture initiation of the mesothelium is akin to crazing during spheroid invasion

The human ovarian adenocarcinoma OVCA433 epithelial cells were cultured to form spheroids in suspension, bearing a grape cluster-like morphology (Fig. 1a)[16]. The wild-type OVCA433 cells exhibited an epithelial morphology when grown as a monolayer. The OVCA433 spheroids were introduced onto the ZT mesothelia[9], which express E-cadherin tdTomato (Fig. 1a).

As spheroids invaded the mesothelium, a phenomenon akin to thermoplastic polymer crazing was observed at the mesothelial cadherin junctions (Fig. 1a' and Video 1). Crazing is a unique feature when thermoplastic polymer materials fracture[17], characterised by a network of fine cracks within the material. It occurs when excessive tensile stress or "stretching" is applied to a polymer, producing gaps termed microvoids, which are between fibrillar bridges that are thought to help sustain some of the stress. (Fig. 1a'' and Video 1).

At the mesothelium, we observed the fibrillar bridges of intercellular adhesion molecule, E-cadherin, and the formation of microvoids similar to crazing prior to the complete mesothelial initial fracture (Fig 1a'' and Video 1). Mesothelial fracture initiation was also characterised by the elongation of E-cadherin puncta and we did not observe any dramatic reduction of E-cadherin at the intact areas of the mesothelium during crazing (Fig. 1a''', Video 1). These craze-like features are not specific to mesothelium cultured on glass, as we observe fibrillar bridges at sites of spheroid invasion when mesothelium is grown on matrigel (Video 2).

Analogous to crazing in thermoplastics, the fibrillar bridges that formed during mesothelium fracture initiation might have resisted fracture propagation (Figure 1a, Video 1 & 2). Thus, we investigated this possibility by specifically weakening cell-cell contacts. RhoA-Myosin-II mediated contractility supports E-cadherin cell-cell adhesion strength[18-21]. Hence, we reduced E-cadherin adhesiveness of the mesothelium by expressing a dominant negative RhoA mutant T19N fused to E-cadherin (Figure 1b and Supplementary Figure 1a). Strikingly, the craze-like structures formed during spheroid invasion were reduced in the mesothelium expressing E-cad-RhoA-T19N mutant (Fig. 1c,d). Although the rate of spheroid-induced mesothelial fracture remains unchanged in mesothelium expressing the E-cad-RhoA-T19N mutant (Supplementary Fig. 1b), more extensive mesothelial clearance by spheroid invasion was observed (Fig. 1e). Thus, these E-cadherin linked fibrillar bridges may resist mesothelium clearance by the invading spheroid.

Tensile fracture of the mesothelium by spheroid engagement

To directly analyse the fracturing process in the mesothelium, we tagged its apical surface with fluorescent microbeads[22] and imaged it undergoing spheroid invasion. 500nm beads were coated with WGA for binding to the cell surface[22].

A material's fracture response can be classified as brittle or more ductile according to the extent of its deformation during fracture. A ductile fracture is seen in readily deformable materials and is characterised by extensive plastic deformation before the material finally breaks apart[23]. By contrast, a brittle fracture exhibits little or no plastic deformation before its fracture[23]. Velocity magnitude quantification from particle image velocimetry (PIV) of the WGA-coated beads on the mesothelium apical surface revealed significant deformation of the mesothelium (Fig. 1f). This suggests that the tearing of the mesothelium is akin to ductile fracture. Additionally, we did not detect any behaviour that reflects ductile-to-brittle transition[22] as the sizes of deformation zones associated with fracture initiation and propagation are similar (Fig. 1g,h,i). This suggested that significant energy was absorbed by the deformed mesothelium throughout spheroid invasion and mesothelial clearance.

Generally, there are three principal modes by which fracture occurs, namely tensile fracture (mode I), shear fracture (mode II) and out-of-plane tearing (mode III). Since crazes typically form at the plane normal to tensile stress[24], spheroid invasion should likely induce mesothelial tensile fracture.

Indeed, PIV of the beads on the mesothelium apical surface after spheroid invasion revealed that there was a constriction zone next to the region where the crack was initiated (Fig. 1f). The small crack opens up a hole in the middle of the mesothelium after being pulled from both sides, an indication of a tensile fracture. A larger displacement of the beads at one side of the tensile fracture reveals anisotropy in the tensile fracture.

Given that cells did not evidently slide past each other upon crazing, it is unlikely that in-plane shear is an important driver of fracture initiation (Fig. 1a). To quantify the deformation nature of the mesothelium, we examined both the normal strain rate and shear strain rate of the mesothelium undergoing fracture (Fig. 1f). Here the normal strain rate represents the strength of velocity gradient in the direction of the local velocity and the shear strain rate represents the strength of velocity gradient perpendicular to the local velocity[25]. Physically, a high shear rate near the fracturing site means the fracture is largely dominated by in-plane shear. On the other hand, a small shear rate with high expansion velocity corresponds to tensile fracture (Methods).

Despite the higher flowing velocity of cells near the fracture compared to other areas away from the fracture site, such difference was not observed in the shear rate distribution (Fig. 1f).

Thus, fracture initiation and propagation in mesothelium are tensile-mode dominated. At the later stage of fracture propagation, strong heterogeneity in the distribution of shear rate was found near the fracture (Fig. 1f, 1k, Video 3), indicating the portion of in-plane shearing has increased during the propagation of mesothelial fracture.

Finally, using lattice light sheet microscopy to achieve a clear cross-section view, we did not observe any out-of-plane tearing (mode III) of the mesothelium caused by the spheroid (Fig. 1l). Protrusions penetrating the mesothelium E-cadherin junctions preceding mesothelial fracture were only observed in ~6% of the invading spheroids (Supplementary Fig. 1c). While in the majority of the spheroids, their protrusions were formed after mesothelial fracture. Indeed, lattice light sheet imaging revealed that mesothelial cells were forced apart from the apical side first, and such separation then propagated to the basal side, leading to a complete fracture of the mesothelium. Thus, tensile fracture is the principal mode initiating spheroid-induced mesothelial fracture.

Spheroid-induced mesothelial cell apical constriction initiates tensile fracture of the mesothelium

We then investigated the cellular mechanism underlying tension-induced mesothelial fracture by spheroids. We observed apical constriction starting from a mesothelial cell beneath the spheroid as the mesothelium begins to fracture (Fig. 2a). By contrast, such aggressive apical constriction is not detected in the other areas of the intact mesothelium away from any fracture sites as shown by a more constant cell apical area (Fig. 2b). It is conceivable that apical constriction of a cell could stretch its junctions with neighbouring cells and eventually result in the aforementioned tensile fracture (Fig. 1f). Apical constriction is a cell shape deformation mechanism promoting tissue remodelling during development[26-29] that is triggered by increased activation of myosin-II at the apical cortex[20, 28, 30, 31]. Structured illumination super-resolution imaging of myosin-regulatory-light-chain (MRLC)[32] revealed a myosin-II network extending across the apical region of the mesothelial cells (Fig. 2c, c'). In quantitative live-imaging, MRLC intensifies at the junctional and apical cortex region of a mesothelial cell beneath the interacting spheroid (Fig. 2a,d and Video 4) such that intensification of MRLC precedes junction constriction and fracture (Fig. 2d). Thus, supporting the notion that constriction of apical mesothelial myosin-II cortex exerts an anisotropic pull on E-cadherin junctions, initiating mesothelial fracture.

Besides the apical constriction mechanism, we use traction force microscopy (TFM) to analyse the contribution of in-plane traction-mediated migration of mesothelial cells during fracture[22, 33]. First, we find that the spheroid's leader cell tends to contract the PDMS substrate in the absence of mesothelium (Fig. 2e,f). Next, we introduced a spheroid on the mesothelium grown on PDMS but failed to detect an enhancement in the mesothelial cell traction force on the PDMS substrate (Fig. 2g,h) or any change in radial traction stress of mesothelial cells upon fracture initiation (Fig. 2g, Supplementary Fig. 2a). Thus, we did not observe traction-mediated lateral migration of mesothelial cells away from the invading spheroid during fracture initiation[7].

Intercellular and apical integrins trigger mesothelial fracture and collective cell invasion

We hypothesize that heterotypic interactions trigger mesothelial apical constriction. It was shown that mesothelial clearance is initiated by ovarian tumour attachment to the mesothelium via integrin alpha-5[7, 8], but the organization of intercellular integrin-matrix complexes in a 3D microenvironment remains unclear. We imaged Paxillin - a key component of the integrin matrix adhesion at the spheroid-mesothelium contact region to visualise the intercellular integrin-matrix complexes. Strikingly, when they attached to the mesothelium, spheroids expressing Paxillin-GFP exhibited strands of Paxillin at the spheroid-mesothelium interface (Fig. 3a, Supplementary Fig. 3a), which grew from smaller initial spots (Fig. 3b). The mesothelium tends to fracture before a spheroid's leader cell fully spread on a mesothelial cell (Supplementary Fig. 3b). These strands of Paxillin morphologically resemble the integrin adhesion complexes seen when cells spread on glass[34]. We observed that mesothelial fracture occurred after partial spreading of a spheroid's leader cell on the mesothelium in ~70% of invasion cases (Supplementary Fig. 3b).

As E-cadherin interactions between the heterotypic epithelial assemblies[3, 4, 30, 35, 36] were undetected with co-localization analysis between E-cad GFP spheroid and Ecad tdT mesothelium (Fig 3c,d), we continued to analyse the functional importance of integrin instead. Knocking down α5 integrin and using a thrombospondin-1 (TSP-1) peptide[37] to specifically block fibronectin-based integrin adhesion of cells successfully blocked mesothelial fracture by the spheroid (Fig. 3e-g, Supplementary Fig. 3c,d), validating the role of α5 integrin in the spheroid-mesothelium attachment.

Adhesion receptors such as integrins could be present at the apical surface of mesothelial cells, mediating their attachment to the spheroid. This is because in experiments where spheroid invaded mesothelial cell doublets on round fibronectin micropattern (Fig. 3h). Despite the confinement of matrix adhesive area, the mesothelial cells detached from the adhesive pattern and remained with the spheroid at the non-adhesive region of the substrate (Fig. 3h), whereas in areas without spheroid the mesothelial cells remained attached on the adhesive round micropattern (Supplementary Fig. 3e). Additionally, this observation was consistent with our TFM data, together suggesting that mesothelial cell's outward migration away from the spheroid by basal traction is dispensable for mesothelial fracture.

Apical integrin adhesion complexes have been reported for the blastoderm attachment to the vitelline envelope during insect gastrulation[38], albeit without direct visualization of these integrins. Here, strands of Paxillin were localized at the apical side of mesothelial cells and exhibited co-localization with immunostained fibronectin (Fig. 3i). Even in the absence of spheroid attachment to the apical side of the mesothelium, α5 integrin and other binding partners of the integrin complex, including Paxillin, kank2, tensin1, fibronectin and vinculin, co-localized at sites of apically localized integrin-based adhesions (Fig. 3j). Both mesothelial α5 integrin and mesothelial fibronectin knockdown respectively was sufficient to block spheroid-induced fracture (Fig. 3k & l, Supplementary Fig. 3f-i and Video 5). Of note, knockdown of α5 integrin primarily depleted apical integrins with a subtle reduction of basal integrins (Supplementary Fig. 3h). Thus, the mesothelium's apical integrin adhesion complex is required for spheroid invasion.

Our evidence supports the notion that mesothelial apical constriction from intercellular integrin engagement initiates the mesothelium tensile fracture. To directly test this, we blocked spheroid-induced mesothelial apical constriction by linking a dominant-negative RhoA mutant to α5 integrin (Fig. 3m and Supplementary Fig. 3j-l). This led to the significant reduction of spheroid-induced mesothelial fractures (Fig. 3n & o and Video 5). Thus, intercellular integrin engagement induces mesothelial fracture in part by apical myosin-II activation. As E-cadherin is the more well-studied mechanotransducer between epithelial cell-cell contacts instead[30], here we present an unexpected role for integrins at cell-cell contacts.

Fluidization and directed collective motility of contact-inhibited invading spheroids

Successful spheroid invasion and spreading on the submesothelial substrate can promote spheroid survival by preventing detachment and anoikis of spheroids[39] (Fig. 4a). We found that the spheroid spreading was significantly retarded when it occurred along a fractured mesothelium (Fig. 4b,c, Video 6). Without the mesothelium, the spheroid spreads freely (Video 7). Since the mesothelium fracture exhibits ductile behaviours(Fig. 1f-i), the fracturing mesothelium may absorb some of the spreading force exerted by the spheroid to retard fracture propagation. Thus, the mesothelium retards but does not promote spreading of the spheroids. This is in agreement with evidence that mesothelial E-cadherin fibrillar bridges contribute to spheroid spreading resistance (Fig.1e).

To assess the spheroid cell dynamics influencing mesothelial fracture propagation and their motility patterns, we quantitatively compared the cellular motility of (1) invading spheroids with (2) non-invading spheroids as a result of ITGA5 knockdown in the mesothelium and (3) spheroids spreading in the absence of the mesothelium-where we termed it as freely-spreading spheroid. We obtained tracking data of the spheroid's cell nuclei by deep-learning segmentation[40] and IMARIS tracking (Fig. 4d,e, Supplementary Fig. 4a,b). We first compared (1) invading spheroids with (2) non-invading spheroids- by knocking down ITGA5 in the mesothelium to identify the motility patterns induced by intercellular integrin adhesion engagement. There was an overall bias in spheroid cell movement towards the mesothelium base, even before mesothelial fracture (Fig. 4f,g, Video 8). However, the spheroids that did not invade due to mesothelium ITGA5 knockdown exhibit a random cellular motion instead (Fig. 4f,g). Thus, intercellular α5 integrin engagement induces collective motility of spheroid cells towards the mesothelium prior to fracture.

We then assess whether directed cell motility is involved in spheroid invasion by quantifying the persistency of cell velocity (see Methods). The results suggest that cells in non-invading spheroids move as random walk, while those in invading spheroids are moving in persistent directions. Particularly, the persistency is the highest in free-spreading spheroid. Overall, the spheroid invasion promoted directed cell motility, which could be further enhanced by removing the mesothelium barriers (Fig. 4h).

The observation that non-invading spheroid exhibit random cell motility whereas spheroid invasion involves directed cell motility may suggests the induction of fluidization for invasion[41]. Fluidization is the transition of cell collectives from a jammed to fluid state,

orchestrating collective migration[42, 43]. A conventional measure of fluidity is the dependence of mean-square displacement (MSD) of cells with time, where a linear relationship corresponds to a Brownian diffusion. To exclude the effect of a global drift of the spheroids, we adopted pair-wise MSD for our system (see Methods). We found that at long time scale about 50mins, MSD grows with lag time super-linearly for invading and free-spreading spheroids and sub-linearly in non-invading spheroid (Fig.4j). This suggests that transition from non-invasion to invasion could be regarded as fluidisation from sub-diffusive to super-diffusive regimes of the cellular dynamics in spheroids at the scale of 50mins, and apical mesothelial α5 integrin is required for such spheroid fluidization. Furthermore, we observed an increase in the slope of MSD growth from the top to bottom in invasive spheroids, suggesting that the fluidization is primarily due to the persistent spreading of cells outward in the base layer (Video 6).

During spheroid invasion of the mesothelium, cells flow like fluid at long timescales. At short timescales, cells usually remain less-motile and solid-like, with the motion of each cell constrained by cell crowding from its neighbours[44, 45]. We analysed the spheroid cell motility at short lag time to identify potential crowding effect on the invading spheroid. Indeed, they are less fluidic at a 10-minutes lag time (MSD slope < 1). At the same time, in free-spreading and non-invasive spheroids, cells move in a manner closer to Brownian diffusion (Fig. 4j). We also quantified the cell velocity at 10-minutes lag time and analysed the ratio of standard deviations of velocity magnitudes in XY directions over the magnitude along Z direction. This ratio is deemed as the measure of in-plane confinement of cell motions. We found a significant lower ratio (*i.e.* stronger motion constraint) in invading spheroids than in the free-spreading ones (Fig. 4k), implying that cells in spheroids invading the mesothelium could experience crowding from its neighbours[44, 45].

Indeed, the spheroid exhibited increasing cell density over time as it spread to propagate the mesothelial fracture (Fig. 4l). By contrast, the cellular density of a non-invading and freely-spreading spheroid remained constant (Fig. 4l). Additionally, the spheroids experiencing crowding have reduced proliferative potential at the lower region of the spheroids, a hallmark of contact inhibition, as the invaded spheroid base shows a decrease in nuclear Ki67 (Fig. 4m, n) and lower nuclear-to-cytoplasmic ratio of Yap (Fig. 4o and Supplementary Fig. 4c).

Cancer cells often enter dormancy characterised by low proliferative potential as they metastasize to a new secondary site[46]. Consistent with this idea, cancer spheroids experience

crowding as they propagate the mesothelial fracture. This resulted in spheroid cells with a lower proliferative potential (Fig. 4m,n,o). Thus, it is tempting to speculate that crowding-mediated contact inhibition may sometimes contribute to cancer cell dormancy. Indeed, this has been assumed in previous theoretical modelling of cancer studies[47]. Cancer cell dormancy typically invokes critical clinical implications, as these cells often evade treatment and contribute to cancer relapse[46]. This is because non-dividing cancer cells tend to evade cancer therapies, which mostly kill rapidly dividing cells; subsequently, cancer cells that evaded treatment contribute to relapse years after treatment[46].

In summary, we find that spheroid invasion of the mesothelium emerges from the dynamic interplay between spheroid fluidization and fracture processes. This emergent phenomenon encompasses several key properties, including the mesothelium's intercellular adhesion strength, its fracturing response, and spheroid cells' directed motility.

The mesothelium is facilitates spheroid invasion by presenting fibronectin fibrils on its apical integrin. Conversely, during mesothelial clearance, it assumes an inhibitory role, actively resisting spheroid spreading. Previously, spheroids spreading freely on glass was studied as active wetting; it occurs when the spheroid cell-substrate interactions and traction forces outcompete the E-cadherin induced intercellular stresses during spreading[48, 49]. In our current study, mesothelial cell neighbours join the competition with the intercellular and spheroid-substrate interactions, limiting spheroid spreading and thus may contribute to cellular crowding of the spheroid.

As envisioned by D'Arcy Thompson a century ago[50], the shaping of tissues shares physical principles with the sculpting of non-living materials, where transitions between solid and fluid states mould them into functional forms. The interplay between fluidization and fracture[51] likely yields a richer variety of supracellular structures, giving rise to diverse tissue physical properties and functionalities. In this study, we have identified fluidization accompanied with crowding as one such outcome. In conclusion, we propose that the co-evolution of fluidization and fracture processes may represent a general mechanism for the collective movement of cells into a foreign cellular microenvironment.

Figures

Figure 1. Spheroid-induced mesothelial tensile fracture.

(a) Mesothelial E-cadherin contact exhibits fracture initiation akin to “crazing” upon spheroid invasion. Representative time-lapse images of the mesothelium under a spheroid (colour-coded in hot orange). a’-a’’’ with detailed views. a’’’ shows a cadherin fibrillar bridge being extended as crazing proceeds. Red arrows point towards a fibrillar extension, and green arrows point towards a microvoid.

(b) Schematic of E-cad-GFP-RhoA-T19N fusion protein.

(c,d) Reduction of E-cad ‘crazing’ during mesothelial fracture when E-cad based contractility is reduced. Representative images (c) and quantification (d) of the percentage of mesothelium E-cad fibrillar bridges when either RhoA-T19N mutant is fused to E-cad or in controls.

(e) Increased size of mesothelial clearance when E-cadherin adhesion is compromised. Quantitative comparison of the mesothelial clearance size when either RhoA-T19N mutant is fused to mesothelial E-cad or in controls.

(f) Tension-induced fracture. PIV analysis of fluorescent microbeads on top of the mesothelium expressing Ecad-tdTomato. The heatmaps of shear strain rate (s^{-1}) or normal strain rate (s^{-1}) or velocity(µm/s) from the PIV analysis. Right magnified view of the velocity PIV heatmap shows outward vectors away from the fracture initiation site (grey dotted circle).

(g,h) Velocity magnitude line scan of the area beside fracture initiation(g) or fracture(h).

(i) Comparison of deformation zone size between fracture initiation and fracture. Data representative of two independent experiments.

(j) Diagram of three different modes of fracture which the mesothelium may undergo. The modes are Mode I: Opening (stress orthogonal to the local plane of the crack surface), Mode II: In-plane-shear (stress parallel to the crack surface but orthogonal to the crack front), and Mode III: Out-of-plane shear (stress parallel to the crack surface and the crack front). The arrows indicate the direction of stress, and asterisks indicate the point of crack.

(k) Tension-induced fracture is the dominant initiating mode of fracture. Comparison between the fold changes of the shear strain rate and normal strain rate with velocity magnitude analysis during fracture initiation and fracture.

(l) Mode III: Out-of-plane shear fracture is not detected when the spheroid begins to invade the mesothelium. XZ views of time-lapse lattice light-sheet microscopy images of E-cad GFP spheroid invading E-cad tdT mesothelium. Grey arrows indicate the opening of mesothelial cell-cell contacts.

All data are means ± SEM from at least n=2 independent experiments, except for (d) which are from n=8 independent experiments, ns, not significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0:0001$; analysed with unpaired t-test. Scale bars, 5µm.

Figure 2. Initiation of fracture from apical constriction of the mesothelial cell.

(a,b,c,d) Apical constriction precedes fracture initiation of the mesothelium. (a) Time-lapse images of the spheroid (brightfield, top right) invading mesothelium expressing E-cad tdT and MRLC-GFP. Intensification of MRLC filaments is observed as apical constriction of the mesothelial cell led to subsequent fracture initiation of the mesothelium. (b) Variance quantification of mesothelial apical cell area beneath spheroid during fracture compared to areas not undergoing fracture. (c) Network of myosin-II filaments spans the apical region of the mesothelium. Structured illumination microscopy of mesothelium expressing MRLC-GFP, with detailed view in (c') (d) Myosin-II filaments intensification precedes apical constriction. Representative temporal changes in myosin-II filaments intensity at the apical cortex and size of mesothelial cell apical area.

(e,f) Spheroid contracts basal substrate inwards. Time-lapse images (e) and quantification(f) of radial traction stress of E-cad GFP expressing spheroid attaching onto the PDMS substrate. Heat maps in (e,g) shows the direction and magnitude of radial traction stress. The colour scale bar indicates the magnitude of the outward (red) and inward (blue) radial traction stress from the mesothelial fracture site.

(g,h) Traction force of mesothelial cells did not exhibit obvious changes upon fracture. Time-lapse images (g) and quantification of fracture site average cellular traction stress (h) of EcadtdT expressing mesothelium when invaded by spheroid expressing E-cad GFP. Discontinuous line in (g) indicates the area of fracture.

All data are means ± SEM from n=7 cells from at least 3 independent experiments, except for (b) which is from 4 independent experiments, analysed with Mann Whitney U test. Scale bars, 5μm.

Figure 3. Intercellular integrin adhesion and fibronectin matrix complexes connect the spheroid to the mesothelium.

(a) Strands of integrin adhesion clusters at the spheroid-mesothelium interface from spheroid expressing Paxillin GFP with indicated XZ representative views (bottom).

(b) The spheroid leader cell's Paxillin GFP clusters growth at the spheroid-mesothelium interface. The cell that is first attached to the mesothelium is defined as the invasion leader cell. Time-lapse imaging of Paxillin GFP at the spheroid-mesothelium interface.

(c) Cadherin at the interface between spheroid (top left, brightfield) expressing E-cad GFP and mesothelium expressing E-cad tdTomato does not co-localize, with detailed views of c' in c'' and c'''.

(d) Representative fluorescence line-scan analysis of spheroid E-cad GFP and mesothelium E-cad tdTomato in c''' reveals a lack of homophilic E-cadherin interactions.

(e) Thrombospondin-1 peptide treatment prevents spheroid-induced mesothelial fracture. Rate of spheroid-induced fracture in control compared to thrombospondin-1 peptide treated cultures.

(f,g) Spheroid integrin knockdown blocks fracture. Representative images (f) and quantification of the spheroid (g) invading the mesothelium in control compared to ITGA5 knockdown in the spheroid.

(h) Time-lapse images of spheroid displacing mesothelial doublets from round adhesive fibronectin patterns into non-adhesive zones.

(i) Co-localization of fibronectin with apical mesothelial integrin clusters. Immunofluorescence images of immunostained fibronectin and mesothelium expressing Paxillin-GFP with detailed views (bottom).

(j) Mesothelial apical integrin adhesion complex without spheroid engagement. Immunofluorescence images of mesothelium apical region immunostained for Paxillin, ITGA5, FN1, Kank-2 and Vinculin.

(k,l) Mesothelial FN1 and ITGA5 are required for spheroid-induced mesothelial fracture. Rate of spheroid-induced fracture in control compared to either mesothelium FN1 knockdown or ITGA5 knockdown.

(m) Design of ITGA5-GFP fused to RhoA dominant negative T19N mutant for expression in the mesothelium.

(n,o) Spheroid-induced fracture requires activation of contractility at sites of mesothelial ITGA5 adhesion. Representative images(n) and quantification (o) of spheroid-induced mesothelial fracture rate in control mesothelium expressing ITGA5 knockdown with reconstitution of ITGA5-GFP compared to the reconstitution of ITGA5-GFP fused to RhoA dominant negative T19N mutant in the mesothelium.

All data are means ± SEM from at least with ~50 spheroid from n=3 independent experiments, $*p < 0.05$, $**p < 0.01$; analysed with unpaired t-test. Scale bars, 5μm.

Figure 4. Fluidization and directed collective motility of contact-inhibited invading spheroids.

(a) Model for spheroid-induced mesothelium tensile fracture

(b,c) Mesothelium retards spheroid spreading. (b) Representative images and (c) quantification of the size of mesothelial clearance by spheroid compared to the degree of spheroid spreading without mesothelium.

(d) Representative images of an OVCA433 spheroid labelled with H2B-mNeon segmented by 3D StarDist (left) and a 3D rendering of the segmented nuclei.

(e) Representative 2D projection of a 3D cell trajectories in spheroid invading the mesothelium over time: (left) in XY axis, projected from the top, (middle) map in XZ axis projected from the side and (right) map in Radial Z (RZ) axis, a radial projection of spheroid assuming the rotational symmetry around the axis at the in-plane centre of the spheroid.

(f) Spheroid cells exhibit motility bias towards the mesothelium prior to mesothelial fracture. Representative XZ and RZ side views velocity maps of a spheroid 6 and 3 minutes prior to control mesothelium fracture compared to spheroid that will not induce fracture due to ITGA5 knockdown in mesothelium.

(g) Mesothelium ITGA5 is required for spheroid cells motility bias towards the mesothelium. The ratio of spheroid cells Z velocity mean over standard deviation of XY velocities distribution of spheroid cells on control mesothelium compared to spheroid cells on ITGA5 knockdown mesothelium.

(h) Persistency of directed spheroid cell migration (see Methods) is the highest in freely spreading spheroid then spheroid invading the mesothelium and lowest in spheroid without invasion due to ITGA5 knockdown in mesothelium. Quantification of spheroid cells velocity auto-correlation persistency in freely spreading spheroid and spheroid with or without invasion.

(i,j) Pair-wise mean square displacement (MSD) (i) (see Methods) of representative samples for three conditions, namely freely spreading spheroid and spheroid with or without invasion. (j) Local slope of MSD value obtained from the pair-wise mean square displacement graph for six selected regions from top to base of spheroids (see Methods) for three different conditions.

(k) Spheroid spreading in the absence of mesothelium may experience less crowding. The ratio of the standard deviation of in-plane displacement (v_{xy}) over that of vertical displacement (v_z), with a time interval *dt* ~10 minutes, in freely spreading spheroid is higher than spheroid with or without invasion.

(l) Cellular crowding in invaded spheroid. Normalised mean distance of nearest neighbour (see Methods) decreases over time in spheroid that invades the mesothelium compared to freely spreading spheroid and spheroid that did not invade.

(m,n,o) A lower proliferation potential in spheroid cells at the base of the invaded spheroid. XZ views (m), nuclear Ki67 quantification (n, spheroid lower regions) and Yap

nuclear/cytoplasmic ratio quantification (o, spheroid lower regions) of invaded spheroid and freely spreading spheroid labelled with Ki67 or Yap and DAPI, after ~ 5 hours of attachment.

All data are means ± SEM from at least n=4 independent experiments, except for (n) which are means ± SD from n=133 spheroid cells from 2 independent experiments, *p < 0.05, **p < 0.01, ****p < 0:0001; analysed with Mann-Whitney U test. Scale bars, 5μm.

Supplementary Figure 1. Spheroid cells protrusion were rarely observed during the initial stages of invasion.

(a) Representative image of mesothelium expressing E-cad GFP wild-type and Ecad GFP fused with RhoA-T19N, immunostained for phospho-myosin light chain(red).
(b) Spheroid's rate of invading either mesothelium expressing wild-type E-cadherin or E-cadherin fused with RhoA-T19N.
(c) Quantification of spheroid protrusions before and after fracture.

All data are means ± SEM from n=8 independent experiments, ns, not significant; analysed with unpaired t-test. Scale bars, 5μm.

Supplementary Figure 2. Mesothelial fracture induced by spheroid was not accompanied by changes in radial traction stress.

Fracture site radial traction stress quantification of EcadtdT expressing mesothelium when invaded by spheroid expressing E-cad GFP.

Data are means ± SEM from n=2 independent experiments, ns, not significant; analysed with unpaired t-test.

Supplementary Figure 3.

(a) Additional representative image of XZ views of a spheroid cell's Paxillin-GFP clusters on a mesothelium expressing E-cadherin tdTomato.
(b) Spreading of a spheroid leader cell on a mesothelial cell is sufficient for mesothelial fracture. Quantification of the number of spheroid cells spreading on the mesothelium prior to spheroid induced mesothelial fracture.
(c) Blocking of integrin to fibronectin interactions with TSP-1 inhibits spheroid invasion. Representative image of spheroid invading mesothelium in control compared to when TSP-1 is introduced.

(d) Immunoblot of α5 integrin knockdown in OVCA433 spheroids with α–Tubulin as loading control.

(e) Mesothelial cells remained attached to the adhesive fibronectin round pattern in the absence of invading spheroids. Time-lapse imaging of mesothelial cells expressing E-cadherin tdTomato and cytoplasmic GFP on fibronectin round patterns.

(f) Immunoblot of fibronectin knockdown in mesothelium with α–Tubulin as loading control.

(g) Knockdown of mesothelium fibronectin inhibits spheroid invasion. Representative image of spheroid invading mesothelium in control compared to when fibronectin-1 is knocked down in the mesothelium.

(h) Knockdown of α5 integrin is most prominent at the apical region of mesothelium. Representative images of mesothelium with α5 integrin knockdown at the apical and basal region, immunostained with α5 integrin and Paxillin.

(i) Knockdown of mesothelial α5 integrin inhibits spheroid invasion. Representative image of spheroid invading mesothelium in control compared to when α5 integrin is knocked down in the mesothelium.

(j,k) Immunoblot of GFP, RhoA and GAPDH loading control of mesothelial cells expressing ITGA5-GFP and ITGA5-GFP fused with RhoA-T19N.

(l) ITGA5-GFP fused with RhoA-T19N reduces apical phosphoMLC. Mesothelial cells expressing ITGA5-GFP and ITGA5-GFP fused with RhoA-T19N immunostained for phosphoMLC.

Supplementary Figure 4. Spheroid cells tracks and Yap localization in spheroids.

(a, b) Representative XY top view, XZ and RZ side views of spheroid cells tracks that did not invade the mesothelium (a) and freely spreading spheroid (b) over time.

(c) Yap is localized in the cytoplasm of the spheroid that invaded the mesothelium. Representative XZ view of invaded spheroid and freely spreading spheroid immunostained with Yap and DAPI, after ~ 5 hours of attachment.

<u>Supplementary Videos</u>

Video 1. Formation of fibrillar bridges and microvoids at mesothelial E-cadherin (hot orange) cell-cell contacts during spheroid invasion.

Video 2. Formation of fibrillar bridges (Ecad tdTomato, red) and microvoids at mesothelium (GFP, green) grown on matrigel during spheroid invasion.

Video 3. Opposite sliding of mesothelial cell-cell contacts (Ecad tdTomato, red) during fracture propagation by spheroid invasion.

Video 4. Apical constriction of mesothelial cell expressing myosin regulatory light chain (MRLC-GFP, green) and E-cadherin tdTomato (red) during spheroid invasion.

Video 5. Representative multipanel videos of spheroid invasion of the mesothelium in the three following experimental conditions. 1) Fibronectin-1 and 2) α5 integrin siRNA mediated knockdown in mesothelium compared to controls. 3) Mesothelium expressing α5 integrin GFP fused with RhoA-T19N compared to control mesothelium expressing wild-type α5 integrin GFP. Arrows indicate four ends of the mesothelial fracture propagation.

Video 6. Representative multipanel video of mesothelial (E-cad tdT, red) clearance by spheroid (E-cad GFP, green/brightfield, grey)

Video 7. Representative multipanel video of freely spreading Ecad-GFP(green) spheroid (brightfield, grey).

Video 8. Representative multipanel video of spheroid expressing H2B-mNeon invading Ecad-tdT expressing mesothelium and the corresponding XZ and RZ velocity maps on the right.

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Author Contributions

S.K.W and B.C.L conceived the project. SKW and C.Z.H performed all experiments except for traction force microscopy and immunoblotting, which were shared with J.X and C.B.H. F.Q.S and Yuan Lin conducted fracture analysis. T.H and Yuting Lou conducted spheroid fluidization and crowding analysis ; C.B.H, I.Y and M.S contributed to traction force analysis and 3D StarDist A.I based nucleus segmentation. S.V and A.S.Y provided input on cadherin and myosin experiments. S.K.W wrote the manuscript. B.C.L, C.B.H, Yuan Lin and Lou Yuting, A.S.Y edited the manuscript. J.C contributed to imaging setup. All analysis were performed with S.K.W assisted by C.Z.H. All authors feedback on the manuscript.

Methods

Cell culture OVCA433 ovarian cancer cell lines and the ZT mesothelial cells were cultured in a 1:1 ratio of medium MCDB 105 (Cell Applications Inc.) and Medium 199 (GIBCO) supplemented with 10% FBS (GIBCO). The OVCA433 cell lines were obtained from Michael Sheetz's laboratory. ZT mesothelial cells were obtained from a benign pleural effusion, a gift from Ince Tan laboratory. These cells were immortalized by ectopic expression of SV40 T antigen and overexpression of human telomerase (fused to GFP), as previously described [9]. Non-GFP expressing ZT cells were obtained by sorting out non-fluorescence ZT cells and they were subsequently immortalized by transfecting them with SV40 T-antigen expressing plasmid (Addgene).

Heterotypic spheroid-monolayer coculture IWAKI 35mm dishes (12mm glass diameter) were coated with 1:100 Human Fibronectin plasma (Sigma-Aldrich) in PBS and incubated at

4°C overnight. Subsequently, ZT mesothelial cells were seeded and incubated at 37°C to form a confluent monolayer.

Spheroids of OVCA433 were prepared by dissociating cells with trypsin-free Cell Dissociation Buffer, PBS (Gibco) and incubated in suspension in 2ml of 1:1 ratio of medium MCDB 105 (Cell Applications Inc.) and Medium 199 (Gibco) with 10% FBS (Gibco) for 30 mins twice at 37°C to remove trace amounts of cell dissociation buffer and promote spheroid formation. Spheroids of approximately 50µm size were imaged and analysed. Spheroids were transferred to a dish containing a confluent monolayer and incubated for 15 min to allow spheroids to settle before live imaging. Imaging was performed using Nikon W1 Spinning Disk confocal microscope with the integrated Perfect Focus System on a 60x water-immersion magnification objective. Brightfield, GFP, and RFP images were captured every 3 min for 12 h. After live imaging, spheroids that invaded the monolayer were quantified by viewing data obtained using MetaMorph Microscopy Automation and Image Analysis Software. To quantify the mesothelial clearance area, the non-fluorescent area, created by the invading spheroid, in the GFP mesothelial monolayer images was measured at 24 h and divided by the initial area of the cancer spheroid 2D size at time 0. All measurements were taken using ImageJ software.

Plasmids, siRNA and shRNA For the expression of fluorescence transgenes we subcloned shRNA-resistant transgene fused with the fluorescence tag into the multiple cloning site of either lentiviral pLL3.7 or pLL5.0 vectors by recombination. To prevent overexpression artefacts, the shRNA targeting the corresponding endogenous proteins are cloned into the lentiviral pLL vectors between the XhoI and HpaI restriction sites. The fluorescent transgenes are designed to be shRNA resistant by using a non-human transgene or codon change with site-directed mutagenesis. The lentiviral constructs generated are 1. pLL3.7 human Paxillin shRNA knockdown reconstituted with gallus gallus Paxillin GFP. pLL3.7 Paxillin shRNA/gallus gallus Paxillin-GFP, 2. pLL5.0 human ITGA5 shRNA knock down reconstituted with shRNA resistant human ITGA5-GFP -- pLL5.0 human ITGA5 shRNA/shRNA resistant human ITGA5-GFP. Additionally, a linker (5′ - GGGTCTACATCTGGATCTGGGAAGCCGGGTTCTGGTGAGGGTTCT-3′) is fused between fluorescence transgenes and the RhoA dominant negative mutants for the following constructs. 3. pLL5.0 human ITGA5 shRNA knock down reconstituted with siRNA resistant human ITGA5-GFP fused to a linker and RhoA-T19N pLL5.0 human ITGA5 shRNA/shRNA resistant human ITGA5-GFP-RhoA-T19N, 4. pLL5.0 human E-cadherin shRNA knock down

reconstituted with mE-cadherin-GFP fused to a linker and RhoA-T19N pLL5.0 E-cadherin shRNA/mEcad–GFP-RhoA-T19N.

The following constructs were published previously. 1. pLL5.0 Myosin Regulatory Light Chain-GFP, 2. pLL5.0 human E-cadherin shRNA knockdown reconstituted with mE-cadherin-tdTomato and 3. pLL5.0 E-cadherin shRNA/mEcad–GFP used was as previously described[18], 4. Lentiviral H2B-mNeon is a gift from Rong Li's lab.

Fibronectin 1 and Integrin alpha-5 were silenced by siRNA directed against human ITGA5 5'-GGACCAGGATGGCTACAATGA-3' and Dharmacon Smartpool siRNA directed against human FN1(5'-GCAGCACAACUUCGAAUUA-3' ; 5'-GAAAUAGAUGCAACGAUCA-3'; 5'-GAGGAAAUCUGCACAACCA-3'; 5'-CCUAAAGACUCCAUGAUCU-3') respectively.

Protein coupling of microbeads PolyLink Protein Coupling Kit (Polysciences, Inc) was used to prepare 50µl of fluorescence beads solution. 200µg of Wheat Germ Agglutinin was added to the activated beads suspension and incubated for 2 hr at room temperature. Subsequently, the beads suspension was sonicated for 15 min and immediately added to a dish containing a confluent monolayer and centrifuged until no floating beads could be observed. Prepared OVCA433 spheroids were transferred to the dish and incubated for 15 min to allow spheroids to settle before live imaging.

Multi-cellular spheroids of OVCA433 were prepared by dissociating cells with trypsin-free Cell Dissociation Buffer, PBS (Gibco) and incubated in suspension in 2ml of 1:1 ratio of medium MCDB 105 (Cell Applications Inc.) and Medium 199 (Gibco) with 10% FBS (Gibco) for 30 mins twice at 37°C to remove trace amounts of cell dissociation buffer and promote spheroid formation. Spheroids were transferred to a dish containing a confluent monolayer and incubated for 15 min to allow spheroids to settle prior to live imaging. Imaging was performed using Nikon W1 Spinning Disk confocal microscope with the integrated Perfect Focus System on the 60x water-immersion magnification objective. Brightfield, GFP, and RFP images were captured every 3 min for 12 h. Spheroids that invaded the monolayer were quantified with MetaMorph.

Computational fracture analysis of microbeads Analysis of microbeads at regions of mesothelial fracture was performed as previously described with minor modifications and additional quantifications[22]. Particle image velocimetry (PIV) analysis (PIVlab package[52] in MATLAB) was used to quantify the flow fields in the top-viewed cell layer microbeads time-lapse datasets. The PIV analysis was carried out for specific duration segments of these datasets where the field of view was fixed. The time interval of each slice is 30 seconds. We then carry out the PIV analysis by dividing the images into 64×64 pixel interrogation windows with 50% overlap on the first pass, 32×32 pixel interrogation windows with 50% overlap for the second pass, and 16×16 pixel interrogation windows with 50% overlap for the third pass. Next, we select velocity threshold limits for post-processing the resulting velocity vector fields, smooth the data and use bicubic interpolation for missing vectors. After calibrating the results, velocity magnitude ($v = \sqrt{v_x^2 + v_y^2}$), normal strain rate [$\dot{\varepsilon} = \partial_x v_x - \partial_y v_y$][25] and shear strain rate [$\dot{\gamma} = \partial_x v_y + \partial_y v_x$] were calculated, where x and y are the directions of vectors while v_x and v_y represent the corresponding mesothelium velocity components[22].

Traction force microscopy TFM substrate was prepared as previously described [53] with minor modifications. Briefly, PDMS with an average stiffness of 15 kPa was prepared by dispensing 40 µL of 1:1 CyA and CyB mixture on the 14 mm well of the 35 mm glass bottom dish (Cellvis), spin-coating the dish at 1000 rpm for 1 min and letting it cure at 80°C for 2 h. PDMS surface was then silanised with 5% APTES in absolute ethanol, functionalised with filtered and sonicated 0.2% (v/v) 0.2 µm dark red fluorescent carboxylate-modified microspheres (FluoSpheres). Beads were inactivated by 0.1M Tri's treatment for 10 min. The substrate was coated with 100 µg/mL of poly-D-lysine for 1 hour, followed by the adding 50 µg/ml (1:100) of human plasma fibronectin (Sigma-Aldrich) for overnight incubation at 4°C.

Subsequently, ZT mesothelial cells were seeded and incubated at 37°C to form a confluent monolayer. Multi-cellular spheroids of OVCA433 were prepared and transferred to the confluent monolayer and incubated for 15 min to allow spheroids to settle prior to live imaging. Imaging was performed using Nikon W1 Spinning Disk confocal microscope with the integrated Perfect Focus System on the x60 water magnification objectives. After 12 h, 10% SDS was added to remove cells and the substrate was left alone to recover to the resting state for an hour prior to acquiring reference images.

TFM Analysis The fluorescent bead images were maximum projected for each timepoint and subsequently contrast-enhanced and registered to the maximum projected reference images using MultiStackReg. PIVlab[54] add-on for MATLAB was used to calculate the displacement field from the registered bead images using three passes of cross-correlation with the interrogation windows as followed: 128(64), 64(32), and 32(16). Outlier vectors with abnormally large values were removed post-processing. FTTC, an ImageJ plugin for a Fourier transform-based, semi-infinite half space method was used to reconstruct force field from the displacement field[55]. Customised codes were written to generate the force map or superimpose the traction force over the cell image. For downstream analysis requiring quantification for each cell, a code was customised to import ImageJ ROIs into MATLAB to calculate the parameters for each ROI. Radial traction stress is analysed as previously described, with the middle of initially spreading cells or fracture site being the point of reference[56]. Statistical analysis was performed using one-way ANOVA in GraphPad Prism.

Immunofluorescence and microscopy Cells were fixed at 4 ◦ C with either −20 ˚C methanol on ice for 5 min or with 4% paraformaldehyde in cytoskeleton stabilization buffer (10 mM PIPES at pH 6.8, 100 mM KCl, 300 mM sucrose, 2 mM EGTA and 2 mM MgCl2) on ice for 20 min and subsequently permeabilized with 0.2% Triton-X in PBS for 5 min at 4 ˚C.

For structured illumination microscopy, spinning-disc confocal microscopy (Yokogawa) coupled with the Live SR module (GATACA Systems), Nikon Eclipse Ti-E inverted microscope with Perfect Focus System, controlled by MetaMorph software (Molecular device) supplemented with a 100x oil 1.45 NA CFI Plan Apo Lambda oil immersion objective and sCMOS camera (Prime 95B, Photometrics)

For lattice light sheet microscopy, Zeiss Lattice Lightsheet 7 equipped with a 10x 0.4 NA illumination objective at 30° angle to cover glass and a 48x 1.0 NA detection objective at 60° angle to cover glass. Pre-defined Sinc3 beams with 30μm length x 1000nm thickness were used for image acquisition. Images were captured with Pco.edge 4.2 CLHS sCMOS camera and processed with Lattice Lightsheet 7 Processing Module.

Antibodies and blocking peptide Primary antibodies in this study were: mouse monoclonal antibody against human Fibronectin (1:100; BD Transduction Laboratories; catalogue number

610077); mouse monoclonal antibody against human CD49e/Integrin α5 Chain (1:1000 for western blotting; BD Pharmingen; catalogue number 555614) ; mouse monoclonal antibody against human Integrin alpha-5 (1:100 for immunofluorescence; EMD Millipore; catalogue number MABT201, clone number SNAKA51); rabbit monoclonal antibody [Y113] against mouse and human Paxillin alpha, beta, and gamma isoforms (1:200 for immunofluorescence; Abcam; catalogue number ab32084); mouse monoclonal antibody against GAPDH (1:1000 for western blotting; Santa Cruz Biotechnology; catalogue number sc-32233); rabbit polyclonal antibody against non-muscle myosin heavy chain IIA (1:200 for immunofluorescence; Sigma-Aldrich; catalogue number M8064)

Generation of micropatterned substrates Micropatterned substrate was fabricated by stencil patterning. Polydimethylsiloxane (PDMS) (Sylgard 184 kit, Dow Corning) was applied onto a photoresist mould, containing circular micropatterns. A mixture of elastomer to crosslinker in a 10:1 ratio (w/w) was used, followed by curing at 80°C for 2 hours. The resulting crosslinked PDMS layer was carefully detached and stamps were manually crafted. Inverting the PDMS stamp, it was positioned on an uncoated 35mm μ-dish (ibidi GmbH) with hydrophobic properties. Next, Norland Optical Adhesive 73 (NOA-73, Norland Inc.) was applied along one edge of the stamp. Through capillary action, NOA-73 flowed into the gaps between the PDMS stamp and the dish, effectively sealing the stamp on all sides. Curing the NOA-73 stencil took place under ultraviolet light for 15 seconds. Following removal of the PDMS stamp, the stencil and dish were subjected to incubation with fluorescence fibrinogen mixed fibronectin at 50 μg/ml concentration in PBS. This incubation occurred overnight at 4°C following a brief degassing at 10 mbar. After the incubation, the fibronectin solution was removed, and the stencil was detached. The dish's printed bottom was treated with 0.2% Pluronic acid-H2O for 30 minutes as a passivation step. Finally, the passivated dishes underwent three washes with PBS prior to cell seeding.

Image Processing and Analysis The images presented were processed with ImageJ (http://rsb.info.nih.gov/ij/). For quantification of apical fluorescent density and line-scan analysis, the mean intensity of the selected apical region was measured from the microscopy images. Line-scan analysis was performed as described previously[57].

To quantify crazing, time-lapse image stacks of mesothelial monolayer were analysed using Metamorph with Z-projection. Mesothelial monolayers were first visually verified to be intact prior to tearing. Timeframes showing the initial cell-cell contacts breakdown were then determined after playing through each movie and visually assessed for multiple fibrillar bridges and microvoids indicative of crazing.

ImageJ macros for image processing and analysis generated previously[58] were used to quantify the nuclear to cytoplasmic ratios of YAP fluorescence intensity and Ki-67 mean nuclear intensity in cells. For the quantification of Ki-67 mean nuclear intensity, a nuclei mask was generated from the Z-projection of DAPI for nuclear intensity quantification.

For the quantification of nuclear and cytoplasmic mean fluorescence intensity of YAP, a nuclei mask from Z-projected image stacks of DAPI, and 1 μm of the surrounding region around the nucleus was selected and designated as the cytoplasmic region. To ensure the separation of overlapping nuclei, a watershed function was applied on the nuclei mask. Background values were quantified in regions without cells and subtracted from the respective quantified intensities.

Spheroid nucleus segmentation with StarDist 3D deep learning model and cell tracking by IMARIS The spheroids' image stacks underwent manual labelling by utilising the Labkit plugin in ImageJ. These labels were subsequently employed as the ground truth masks for the StarDist 3D segmentation. A subset of the labelled stacks, selected at random, was allocated for both training (N_train=19) and testing (N_test=2) datasets. To augment the data, various techniques, including random flips, rotations, and intensity modifications, were applied during training to effectively expand the pool of training examples. The initial model parameters were set using the pre-trained StarDist model weights[59].

Throughout the training process, the last five Conv3D layers of the model were reinitialized and trained over 100 epochs. A learning rate of 10^{-4} was employed, along with a patch size of 8x96x96. All other training parameters were kept consistent as previously[59]. Following this, the best-performing model, determined by the lowest test dataset loss, underwent further fine-tuning for an additional 10 epochs. During this fine-tuning phase, a reduced learning rate of

10^{-6} was employed. All training and fine-tuning procedures were executed on a shared Jupyter notebook server equipped with an NVIDIA RTX 6000 GPU.

After model training, the trained model was deployed for predictions on new spheroid images. This inference step was carried out using a Google Colab notebook with GPU acceleration. After executing the prediction code, the resultant labels were compared with the original image stack to assess segmentation accuracy. Following this evaluation, the autoregressive motion algorithm was applied to enable automated tracking of the segmented H2B mNeon nuclei time-lapse images in IMARIS, resulting in the generation of nuclei tracks. These tracks were then subjected to manual refinement across all frames to capture the movement of spheroid cells accurately.

Pair-wise mean square displacement (MSD) analysis of cell nucleus Given a time t, the distance of two nuclei i and j is denoted as $\Delta x_{i,j}(t)$. The displacement of this distance over an time interval dt is $\Delta x_{i,j}(t, dt) = \Delta x_{i,j}(t+dt) - \Delta x_{i,j}(t)$. Then, the pair-wise MSD $\langle \Delta x^2 \rangle (dt)$ is the squared distance averaged over all time points and all nuclei pairs. MSD increases with dt with a power $\langle \Delta x^2 \rangle \propto dt^{\sigma}$, where $\sigma(dt) = \partial \ln \langle \Delta x^2 \rangle / \partial \ln dt$, is referred to as local slope in the main text. This local slope is 1 across all lag time dt, if the particles move by random diffusion.

Cell migration persistency analysis Let $\boldsymbol{x_i}$ be the position of cell nuclei i in the XYZ (Cartesian) coordinate space, where the origin of the space $\boldsymbol{x}_0 = \langle \boldsymbol{x} \rangle$ is the averaged positions of all cells in spheroids. Then, the velocity of nucleus i is calculated as the distance of the nucleus between two consecutive frames: $\boldsymbol{v}_i(t) = \boldsymbol{x_i}(t+dt) - \boldsymbol{x_i}(t)$, where dt = 3 minutes, the time resolution of tracking. The velocity direction is $\hat{v}_i(t) = v_i(t)/|v_i(t)|$, from which the auto-correlation of velocity directions $C(dt) = \langle \hat{v}_i(t) \hat{v}_i(t+dt) \rangle$ could be obtained. The decay of C with the lag time dt follows an exponential form as $C \propto \exp(-dt/\tau)$, where τ is the characteristic timescales of persistency in the cell velocity direction.

Z-stacks of spheroid Let S be the total number of stacks to be sliced and each stack is enumerated as integer s ranging from 1 (top) to S (base). We first calculate the cumulative distribution M of the cell nuclei in the spheroid along Z direction, then M=0 corresponds to the top surface and M =1 at the bottom surface. Next, we determine the upper bound z_s and lower bound z'_s of a stack s in such a way that $M(z_s) = (s-1)/S$ and $M(z'_s) = \min(1, (s-1)/S + h)$, where

h is a predefined effective thickness ($0 < h < 1$). Note that a fixed h imposes a similar number of cells within each stack and excludes the bias from the population number in the statistics. Finally, we select the cells with its vertical position z within the range of $[z_s, z'_s]$ for a stack s. In the main text, total number of stacks S is set as 6 and the effective thickness h is 0.3.

Nearest neighbour analysis Nearest neighbour distance of nuclei is the distance averaged over 5 nearest neighbour distances for each nucleus and then averaged over all nuclei in the selected region. Finally, the distance is normalised by the nearest neighbour distance of the whole spheroid at the initial time point.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary files. The raw data generated in this study are available from the corresponding authors upon reasonable request.

Code availability The scripts generated in this study are available from https://github.com/selwinwu/morphogenesis.git

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Figure 1

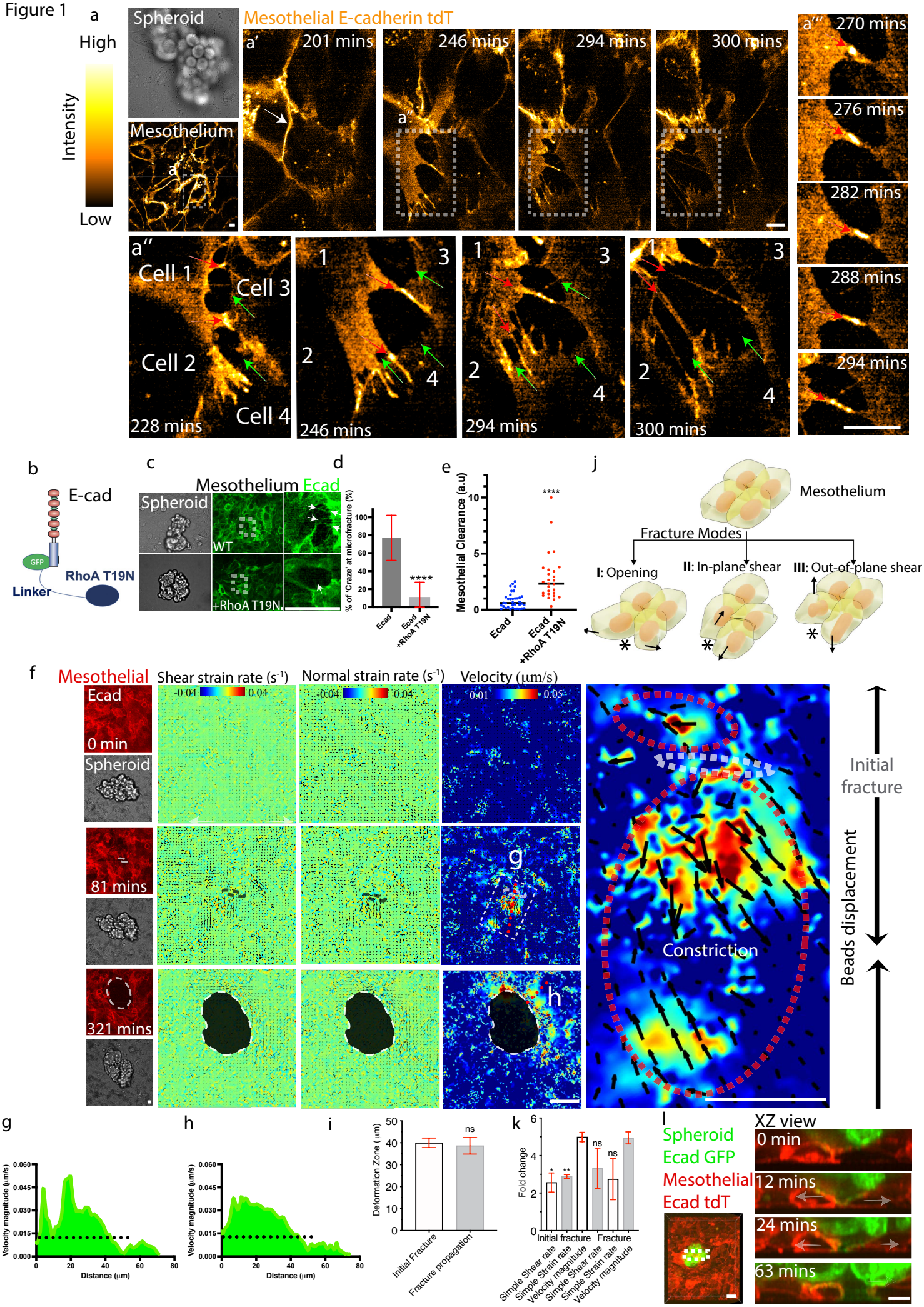
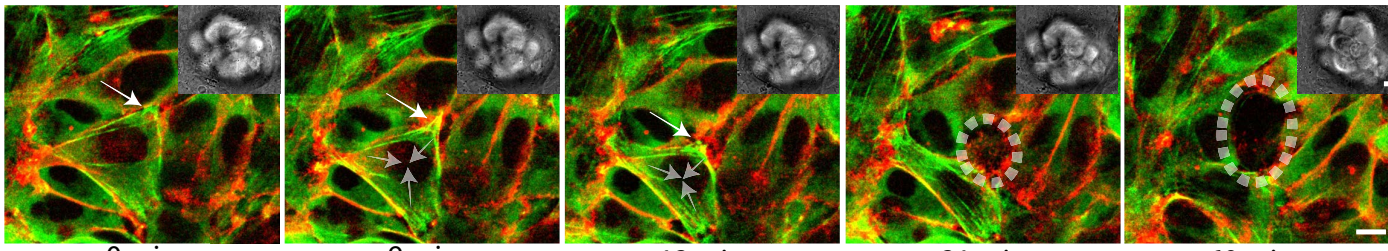

a
High
Intensity
Low
Spheroid
Mesothelium
Mesothelial E-cadherin tdT
a'
201 mins
246 mins
294 mins
300 mins
a''
a'''
270 mins
276 mins
282 mins
288 mins
294 mins
Cell 1
Cell 2
Cell 3
Cell 4
228 mins
246 mins
294 mins
300 mins
b
E-cad
GFP
RhoA T19N
Linker
c
Mesothelium Ecad
Spheroid
WT
+RhoA T19N
d
% of 'Craze' at microfracture (%)
Ecad
Ecad +RhoA T19N
****
e
Mesothelial Clearance (a.u)
Ecad
Ecad +RhoA T19N
****
j
Mesothelium
Fracture Modes
I: Opening
II: In-plane shear
III: Out-of-plane shear
f
Mesothelial
Shear strain rate (s-1)
Normal strain rate (s-1)
Velocity (μm/s)
Ecad
0 min
Spheroid
81 mins
321 mins
g
h
Constriction
Initial fracture
Beads displacement
g
Velocity magnitude (μm/s)
Distance (μm)
h
Velocity magnitude (μm/s)
Distance (μm)
i
Deformation Zone (μm)
ns
Initial Fracture
Fracture propagation
k
Fold change
Initial fracture
Fracture
Simple Shear rate
Simple Strain rate
Velocity magnitude
l
Spheroid
Ecad GFP
Mesothelial
Ecad tdT
XZ view
0 min
12 mins
24 mins
63 mins

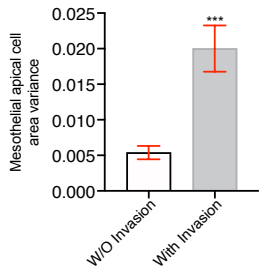
Figure 2

a Mesothelial E-cad tdT MRLC GFP

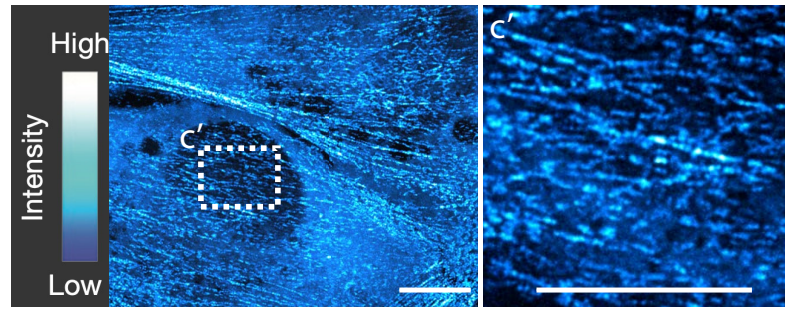


0 min 9 mins 12 mins 21 mins 60 mins

b



c Mesothelial medioapical MRLC-GFP



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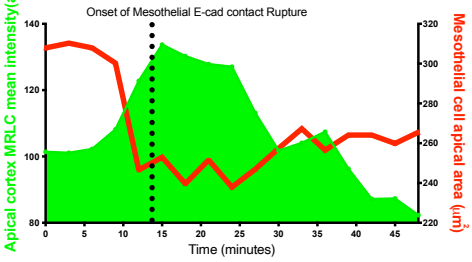

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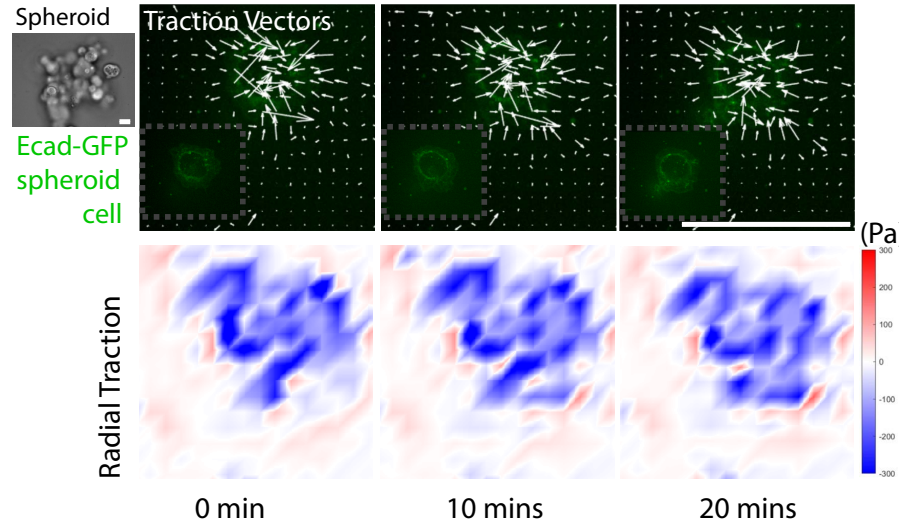

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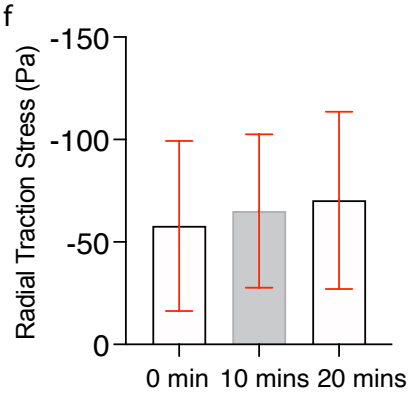
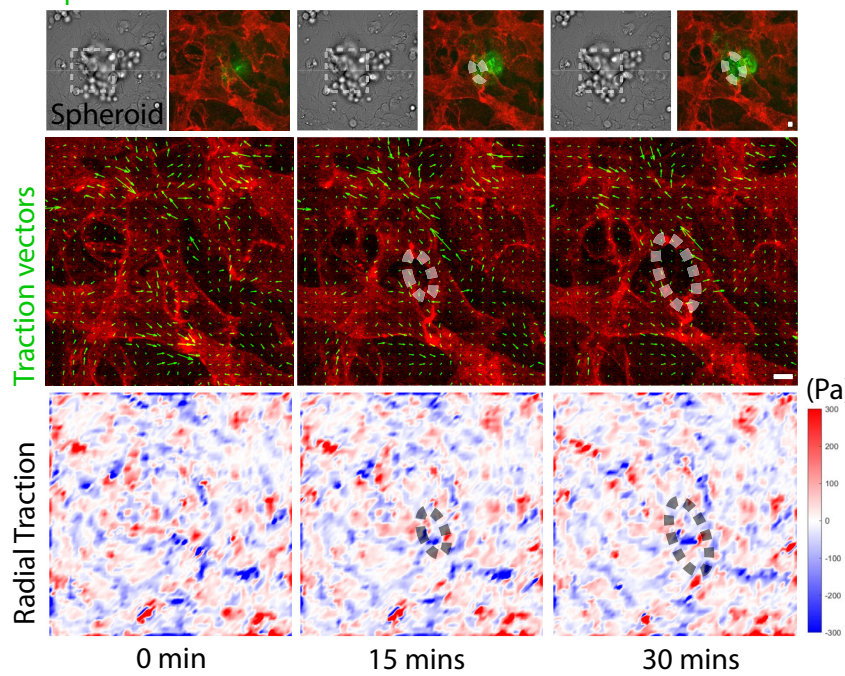


g Spheroid E-cad GFP Basal Mesothelial E-cad tdT



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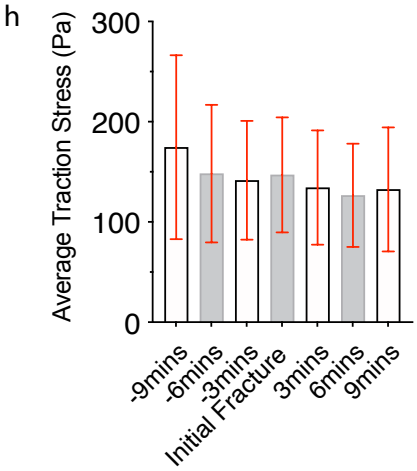


Figure 3

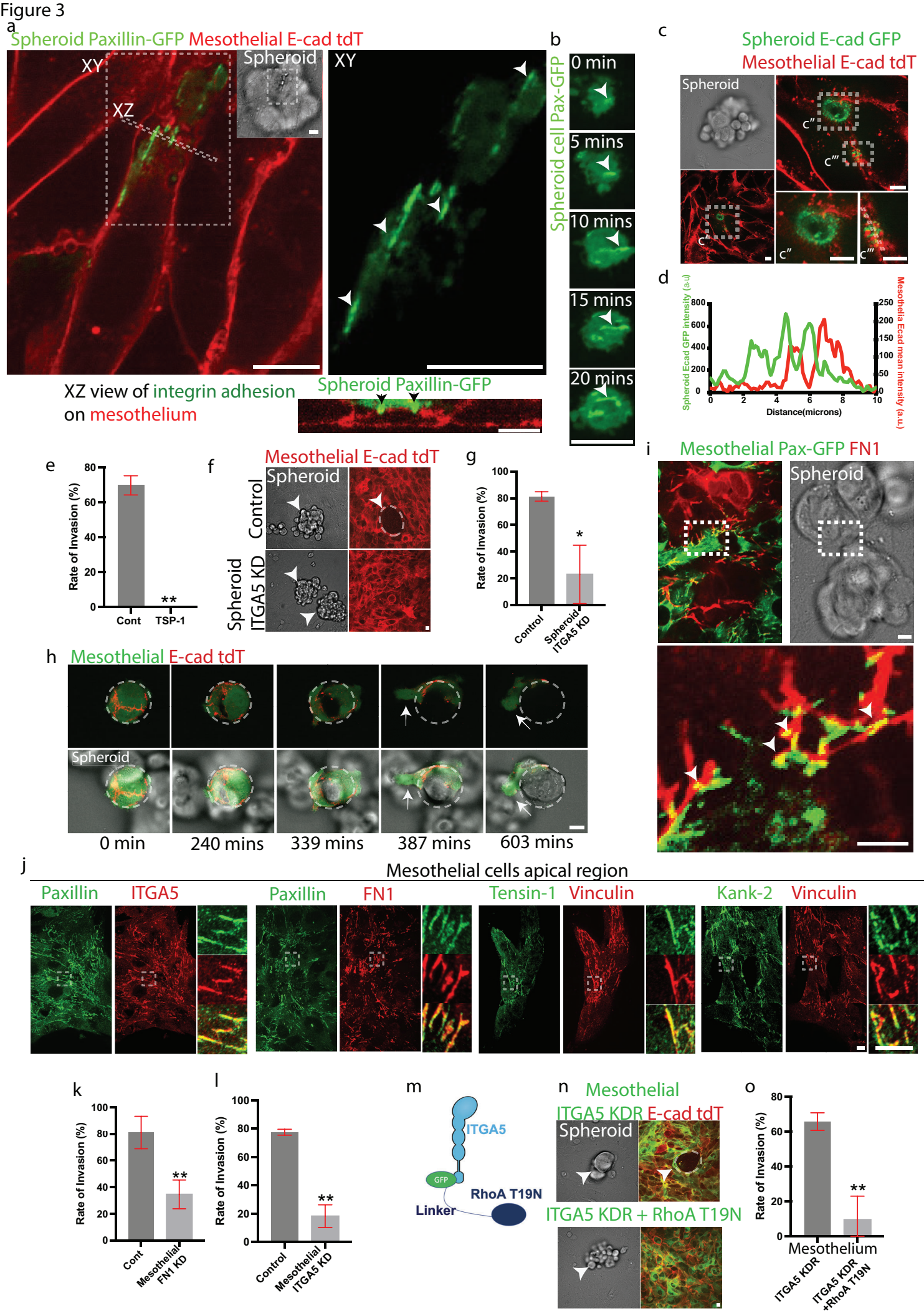
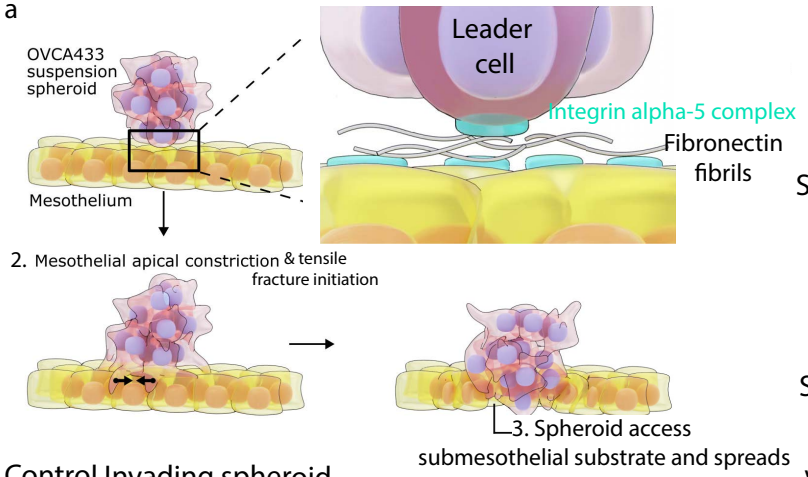

a
Spheroid Paxillin-GFP Mesothelial E-cad tdT
XY
XZ
Spheroid
XY
XZ view of integrin adhesion on mesothelium
Spheroid Paxillin-GFP
b
Spheroid cell Pax-GFP
0 min
5 mins
10 mins
15 mins
20 mins
c
Spheroid E-cad GFP
Mesothelial E-cad tdT
Spheroid
c″
c‴
c
d
Spheroid Ecad GFP intensity (a.u)
Mesothelia Ecad mean intensity (a.u.)
Distance(microns)
e
Rate of Invasion (%)
Cont
TSP-1
f
Mesothelial E-cad tdT
Spheroid
Control
Spheroid ITGA5 KD
g
Rate of Invasion (%)
Control
Spheroid ITGA5 KD
h
Mesothelial E-cad tdT
Spheroid
0 min
240 mins
339 mins
387 mins
603 mins
i
Mesothelial Pax-GFP FN1
Spheroid
j
Mesothelial cells apical region
Paxillin
ITGA5
Paxillin
FN1
Tensin-1
Vinculin
Kank-2
Vinculin
k
Rate of Invasion (%)
Cont
Mesothelial FN1 KD
l
Rate of Invasion (%)
Control
Mesothelial ITGA5 KD
m
ITGA5
GFP
RhoA T19N
Linker
n
Mesothelial
ITGA5 KDR E-cad tdT
Spheroid
ITGA5 KDR + RhoA T19N
o
Rate of Invasion (%)
Mesothelium
ITGA5 KDR
ITGA5 KDR +RhoA T19N

Figure 4

a

1. Intercellular integrin-matrix engagement



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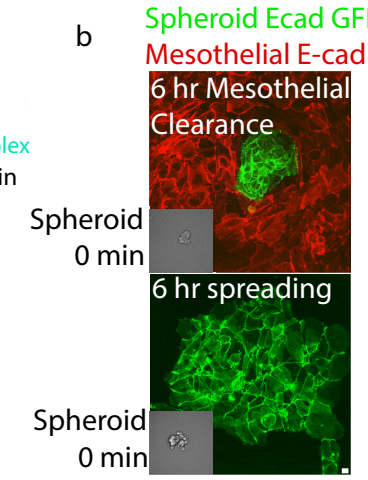

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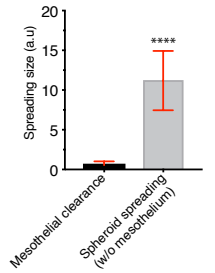

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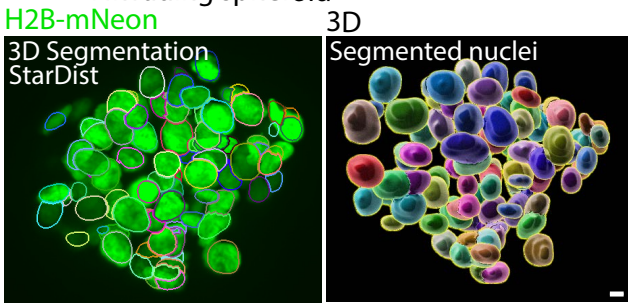

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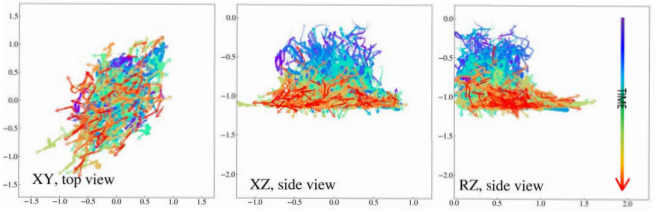

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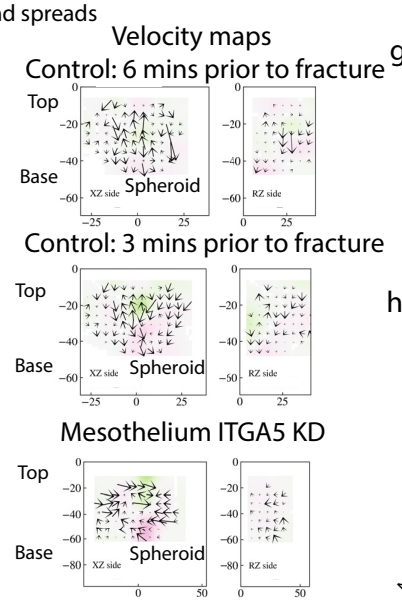

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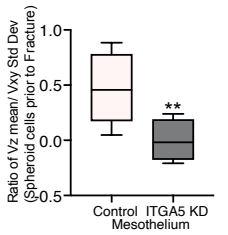

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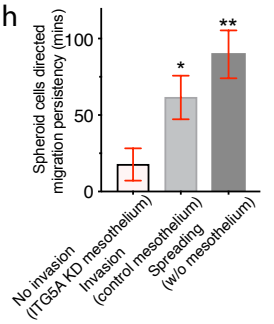

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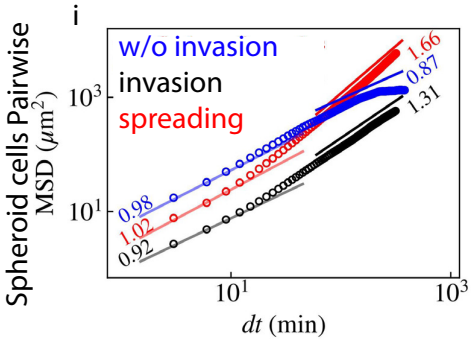

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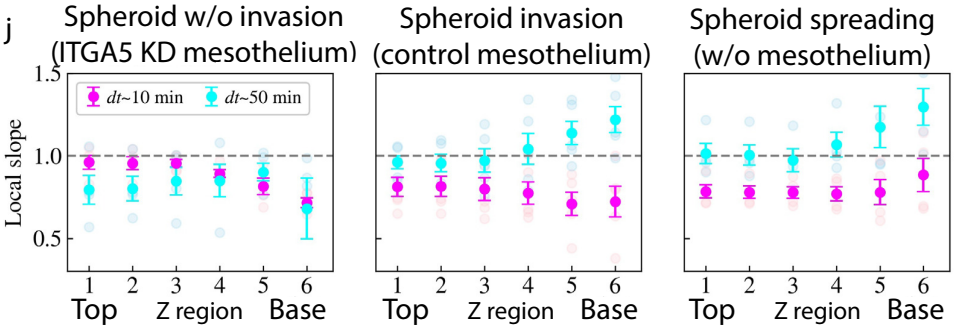

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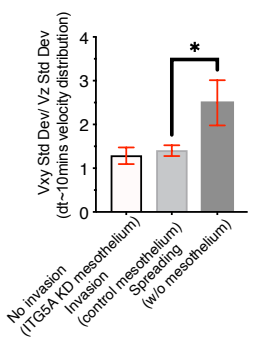

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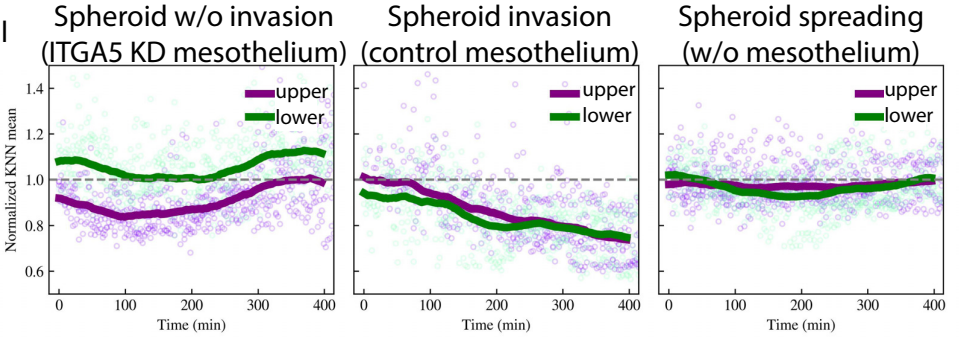

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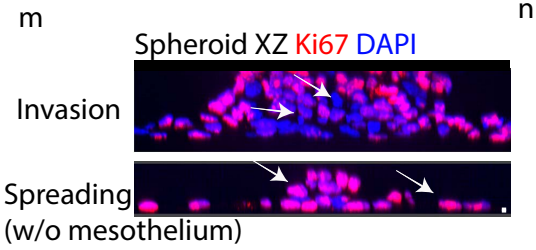

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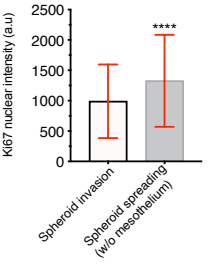

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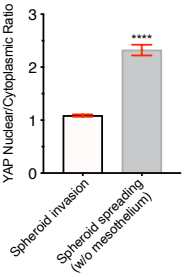
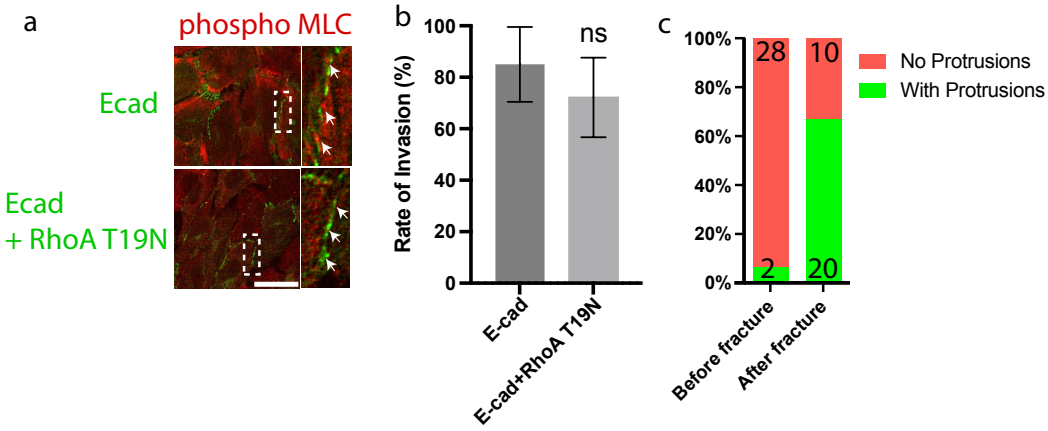
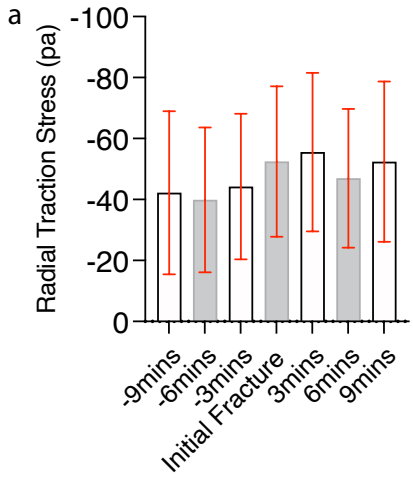


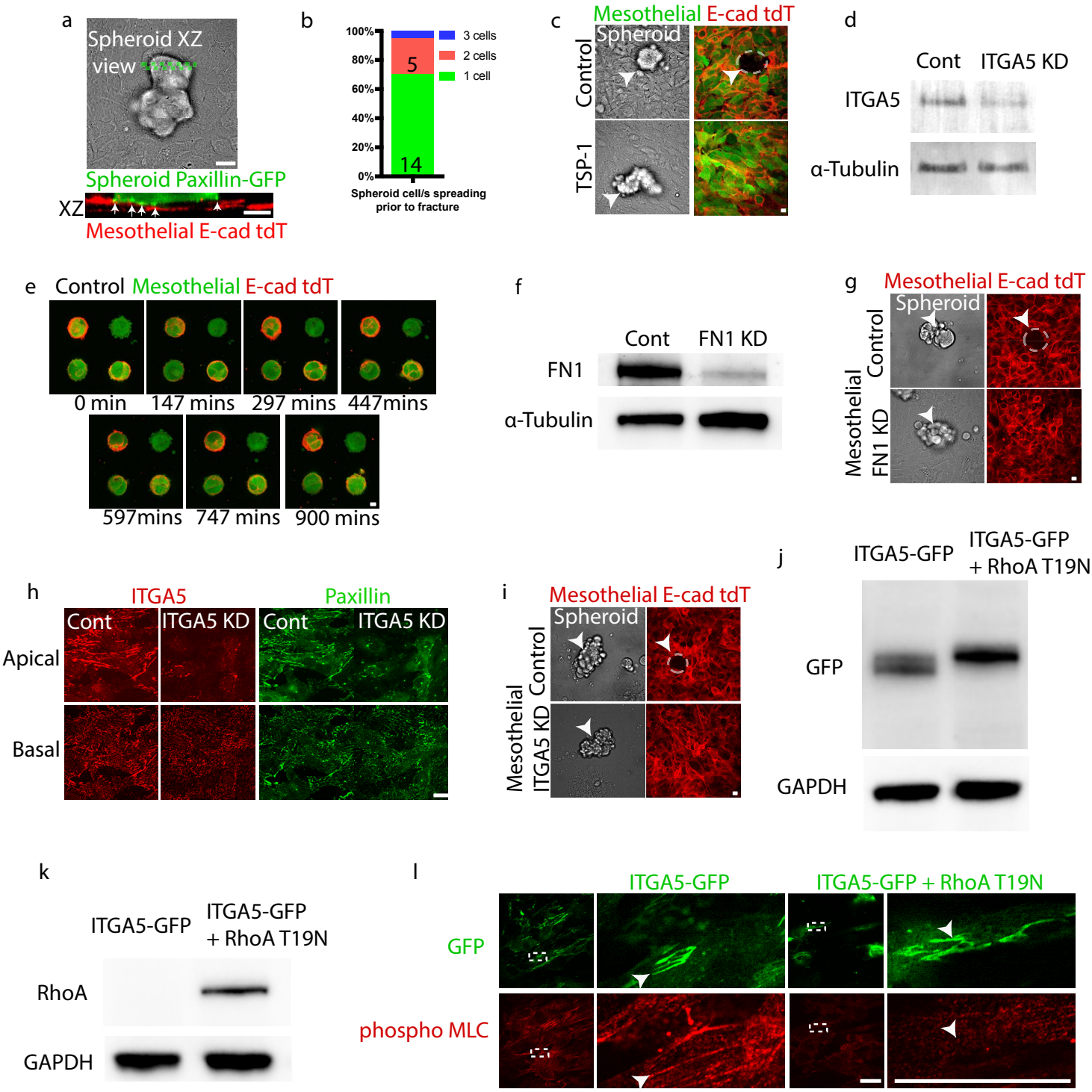
Supplementary Figure 1



Supplementary Figure 2

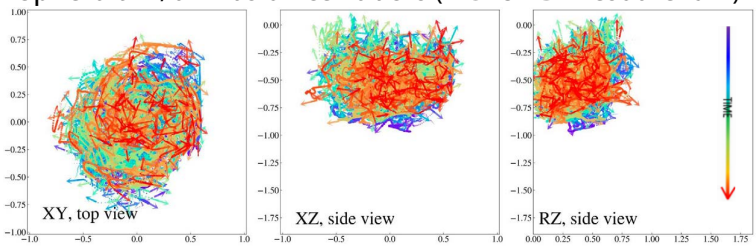


Supplementary Figure 3

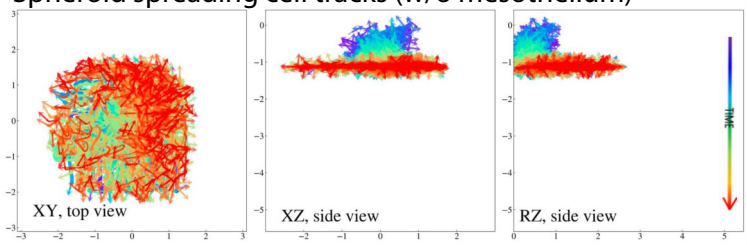


Supplementary Figure 4

a Spheroid w/o invasion cell tracks (ITGA5 KD mesothelium)



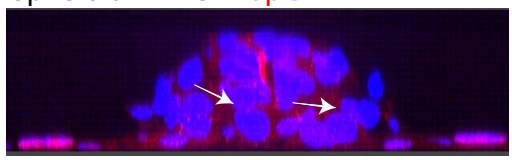
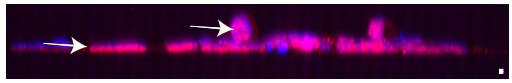
b Spheroid spreading cell tracks (w/o mesothelium)



c

Spheroid XZ view Yap DAPI

Invasion



Spreading (w/o mesothelium)