Supplementary Figures



Figure S1. (a) Representative transmitted light images of (left) an MDA-MB-468 spheroid and (right) an MDA-MB-231 spheroid invading in a collagen I gel at 24 h. Red circle represents invasive distance as described in Materials and Methods. Scale bars = 500 μ m. (b) MDA-MB-468 and MDA-MB-231 multicellular tumor spheroid invasion in 3D collagen I gels at t = 24 h and t = 48 h. Mean values of invasive distance ± SD are shown; n (MDA-MB-468) = 12, n (MDA-MB-231) = 9. (c, d) Transmitted light time-lapse images of spheroids invading in 1 mg/ml 3D collagen I with (c) MDA-MB-468 cells showing round cell invasion and (d) MDA-MB-231 cells showing dynamic interconversion between elongated and round cell morphology of invading cells. Images are (c) 20 min apart starting at t ≈ 7 h or (d) 30 min apart starting at t ≈ 10.5 h after collagen embedding of the spheroids. Arrows indicate emigrating bleb-bearing cells. Scale bars = 50 μ m. Images in (c, d) are obtained from M1 and M2, respectively.



Figure S2. (a, b) Correlative analysis of the occurrence of membrane blebs and actin-driven protrusions in MDA-MB-468 and MDA-MB-231 cells of (a) round cells ($c \ge 0.75$) and (b) elongated cells (c < 0.75) embedded in 3D 1 mg/ml collagen I (Coll) or the composite 1 mg/ml collagen I + 3 mg/ml BME (Coll-BME) matrices. Totals may be more than 100% since some cells have both at least one bleb and at least one actin-driven protrusion. Data set is the same as that used for Fig. 1 in the main text. Data was pooled from 3-4 statistically identical biological replicates. n = 149, 103, 117, 122 for MDA-MB-468 Coll, MDA-MB-468 Coll-BME, MDA-MB-231 Coll, and MDA-MB-231 Coll-BME, respectively. Differences were assessed by t-tests between percents. In this and all subsequent figures in which statistical analysis is performed, the analyses are conducted at α = 0.05, and significant different are marked by * (* p < 0.05, ** p < 0.01, *** p < 0.001) and insignificant by [†]. (c, d) Mean normalized fluorescence intensity of total β 1 integrin staining at individual bleb necks of the same dispersed MDA-MB-231 cells used in the analysis shown in Fig. 4 of the main text plotted against (c) perimeter of the bleb or (d) the width of the bleb neck. Blebs bearing clusters or no clusters are represented by filled and open symbols, respectively. The distributions of bleb perimeters and bleb neck widths of blebs with and without β 1 clusters were compared by KS test and ttest between percents and were found to be statistically equivalent.



Figure S3. (a, c, e, g) Representative confocal fluorescence microscopy maximum projections over $\approx 50 \ \mu m$ of (a, c, e) phalloidin-stained (a) MDA-MB-468 (c) MDA-MB-231, and (e) patient-derived breast cancer organoid and (g) CellMask-stained MDA-MB-231 spheroid at t = 24 h, each in 1 mg/ml collagen I. White squares indicate regions presented in higher magnification in b, f, and g. (b, d, f) Representative images of phalloidin-stained (b) MDA-MB-468, (d) MDA-MB-231, and (f) patient-derived breast cancer cells (red) invading as clusters or individual cells and (green) their immediate collagen environment. (g) Representative images of (red) CellMask stained MDA-MB-231 cells showing polarized bleb bearing regions and (green) their immediate collagen environment. White arrows indicate sites of collagen fiber alignment adjacent to bleb-bearing regions of cells, showing efficient invasion of round bleb-bearing cells into the collagen I matrix accompanied by extensive collagen re-organization. In (b) and (f), dark regions that are apparently devoid of collagen are caused by out of plane portions of the spheroid or organoid. (a, c, e) Scale bar = 200 μ m; (g) Scale bars = 100 μ m on large image and 10 μ m on the small images; (b, f) Scale bar = 50 μ m; (d) Scale bar = 10 μ m.



Figure S4. (a) Comparison of invasion reduction through function blocking antibody (AB) and siRNAmediated integrin knockdown, with two siRNAs used for the MDA-MB-231 cells (siRNA1 and siRNA2). Mean invasion relative to solvent or non-targeting siRNA controls ± SD for MDA-MB-468 and MDA-MB-231 in pure collagen at t = 24 h is shown. n = 11 (MDA-MB-468 function blocking AB and control), 12 (MDA-MB-468 siRNA1 and control), 10 and 11 (MDA-MB-231 function blocking AB and control), 13 (MDA-MB-231 siRNA1 and control) and 13 and 12 (MDA-MB-231 siRNA2 and control). t-tests were used to compare normalized invasion under the function blocking antibody- and siRNA-mediated integrin inhibition (within one cell line) and the resulting inhibitory effects were found to be statistically equal. (b) SDS-PAGE and Western Blot of cell lysates from MDA-MB-231 cells transfected with either control, siRNA1, or siRNA 2 and harvested on either day 3 or day 5. Equal amounts of protein were loaded for each condition. Western Blot was probed against β 1 integrin to show knockdown efficiency, and GAPDH was used as a control to show equal loading. siRNA1 is used at all other points in this paper. (c) Collagen gel contraction by MDA-MB-468 and MDA-MB-231 cells under antibody-mediated integrin inhibition compared to control shown as mean decrease of the gel area \pm SD at t = 24 h. Experiment was performed in biological triplicate. (d, e) β 1 integrin expression of (d) MDA-MB-468 and (e) MDA-MB-231 cells were subjected to fluorescent labeling of cell surface β 1 integrin via sequential incubation with β 1 integrin primary antibody and fluorescently labeled secondary antibody and fluorescence was measured by flow cytometry. Cells treated with secondary antibody only were used as a negative control (left panels). n =10000 cells for each cell line and condition. (f, g) Analysis of changes in cellular morphology and protrusion type of MDA-MB-231 cells embedded in collagen I matrices following siRNA-mediated β1 integrin knockdown in comparison to control group. (f) Histogram of cell circularity of β 1 integrin knockdown cells to control cells. Differences in distributions were assessed by KS analysis (g) Percentage of both β 1 integrin knockdown and control cells bearing at least one protrusion from each class. Actin polymerization-driven protrusions are summarized under "filopodia." Differences in occurrence of each protrusion type were assessed by t-tests. Data was pooled from 3 statistically identical biological replicates. n = 138 and 104 for the control group and the siRNA knockdown, respectively.



Figure S5. (a-c) CellMask images of the 1 mg/ml collagen I-embedded (a) MDA-MB-231 cell (b) MDA-MB-468 cell and (c) the edge of an MDA-MB-231 spheroid shown for each of the three images also shown in Fig. 5 in the main text. (c) White dashed lines show the edge of the spheroid. (d) Representative example of region selection for cluster analysis. Image shows a single confocal slice of a cell with the region of interest (ROI) shown in red. A more complete explanation of the protocol can be found in Materials and Methods in the main text. In (a-d), arrows indicate bleb necks. t = 6 h. Scale bar = 10 μ m. (e) Analysis of integrin distribution on the cell membrane of collagen I-embedded MDA-MB-231 cells immunostained for cell surface total β 1 integrins and CellMask. The measured occurrences of β 1 integrin and CellMask clusters on the bulk cell membrane was compared to values predicted for normal distributions by t-test between percents and were found to be statistically equal. Data was pooled from 3 statistically identical biological replicates. n = 48 (individual, non-overlapping confocal slices were treated as independent samples).



Figure S6. Representative image of an MDA-MB-231 cell stained with (red) phalloidin in a (green) 1 mg/ml collagen I matrix. Image shows polarized bleb formation as well as collagen alignment at bleb necks (white arrows). t = 6 h. Scale bar = 10 μ m.



Figure S7. Representative CFM image of an MDA-MB-231 spheroid triple stained for (red) active β 1 integrin, (green) inactive β 1 integrin and (blue) DAPI implanted into an (orange) 1 mg/ml collagen I matrix showing round, bleb-bearing cells invading. (a) Spheroid core and invading cells. White square shows

region of interest, which is represented at higher magnification below. (b) Higher magnification images collectively show the accumulation of (left) active β 1 integrin, (center) polarized, bleb-bearing cells, and (right) accumulation of collagen overlapping with both the accumulation of active β 1 integrin and the polarized distribution of blebs. White arrows note round, bleb-bearing cells with active β 1 integrin and collagen accumulation. t = 6 h. (a) Scale bar = 100 µm; (b) Scale bar = 10 µm.



Figure S8. Representative example of segmentation for collagen orientation analysis. Measurement regions surrounding a cell for the (a) phalloidin (red) channel and (b) collagen (green) channel. Panel (a) shows the division of the cell into non-blebby regions (#1 and #8) and blebby regions (#2-7). The red lines in (c) indicate the 90° angular window corresponding to collagen alignment for region 5.

Supplementary Table

Table S1. Details of statistical analyses performed	rmed.
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Data	Figure	Test For (Test Used)	Software Used	Test Details ($\alpha = 0.05$)			Meaning of Results
Cell Morphology							
					468 Coll I p	o > 0.05	All dates are equal
	1b	1. Compare distributions by date (KS Test)	XLSTAT	468 Coll-B	ME p > 0.05	excluding one pair	All dates are equal except for
					where p	= 0.02	one date pairing
		2. Compare distributions by matrix (KS Test)	R		p>0.	05	Equal circularity
		1 Compare distributions by date (KS Test)	γιςτατ	221 Coll P		0 > 0.05	All dates are equal
Circularity Parameter	1d	1. Compare distributions by date (KS Test)	ALJIAI	251 COII-B where	n = 0.026	and $n < 0.0001$	one date nairing
				where p = 0.020 a			231 Coll-BME is more round
		2. Compare distributions by matrix (KS Test)	R		p < 0.0	001	than 231 Coll I
		1 Compare distributions by data (KS Tost)	VISTAT	XI STAT		p > 0.05	All dates are equal
	S4f	1. compare distributions by date (KS rest)	ALSIAI	23	31 Scramble	ed p > 0.05	All dates are equal
	-	2. Compare distributions by matrix (KS Test)	R		p < 0.0	001	231 β1 KD is more round than
		1. Compare Occurrence of protrucions by data		400.0-111		n > 0.0F	231 Scrambled
		(Single Factor ANOVA)	Excel	400	II-RMF	p > 0.05	All dates are equal
				400 00	Blebs p < l	0.0001	468 Coll-BME has more
	1c	2. Compare occurrence of protrusions by matrix		A	tin-Driven	p < 0.0001	468 Coll I has more
		(Single Factor ANOVA)	Excel		Both p =	0.037	468 Coll I has more
					Neither p	= 0.037	468 Coll I has more
		1. Compare Occurrence of protrusions by date	Excel	231	Coll I	p > 0.05	All dates are equal
		(Single Factor ANOVA)	LACCI	231 Co	II-BME	p > 0.05	All dates are equal
	1e				Blebs p <	0.0001	231 Coll-BME has more
Protrusions		2. Compare occurrence of protrusions by matrix	Excel	Actin-Driven		p < 0.0001	231 Coll I has more
		(Single Factor ANOVA)			Both p >	- 0.05	231 Coll I has more
		1. Compare Occurrence of protrusions by date		231 6		- 0.001 n > 0.05	All dates are equal
		(Single Factor ANOVA)	Excel	231 Scr	ambled	p > 0.05	All dates are equal
		2. Compare occurrence of protrusions by matrix (Single Factor ANOVA)	Excel	Blebs p = 0 Actin-Driven p Both p = 0		0.001	β1 KD has more
	S4g					p < 0.0001	Scrambled has more
						0.0002	Scrambled has more
		1. Comparison of Percents (Two Sample t-test			Veither p < 0.0001		β1 KD has more
	6i		Statistics	Clustere	ed Blebs	p < 0.0001	Control has more
		between Percents)	Calculator	A68 Coll L		p < 0.0001	β1 KD has more
		 Comparison of Percent of Round Cells Carrying Blebs vs. Actin-Driven Protrusions (One Sample t- test between percents) 	Statistics Calculator	468 COIL1		p < 0.0001	Blebs are more common
	S2a S2b			231		p < 0.0001	Blebs are more common
			culculator	231	BME	p = 0.0380	Blebs are more common
Circularity vs. Protrusions				468	Coll I	p > 0.05	Equal occurrence
		1. Comparison of Percent of Elongated Cells	Statistics Calculator	468 Coll-BME		p > 0.05	Equal occurrence
		Sample t-test between percents)		231 Coll I		p < 0.0001	Actin-driven are more common
				231	BME	p > 0.05	Equal occurrence
Spheroid Invasion and Gel							
Contraction		1. Comparison of invasive distance under various	Statistics		58	n > 0.05	Invasive distance is identical
Impact of β 1		B1 inhibition (Two-Tailed t-test)	Calculator	2:	31	p > 0.05	Invasive distance is identical
		r		2,	Rac	p > 0.05	Invasion is not impacted
	_	1. Comparison of invasive distance under various treatments (Wilcoxon Rank-Sum)		469.6-11	ROCK	p = 0.0014	Invasion is decreased
	за		к	408 COII I	β1 KD	p < 0.0001	Invasion is decreased
					MMPs	p = 0.0034	Invasion is decreased
		1. Comparison of invasive distance under various treatments (Wilcoxon Rank-Sum)			Rac	p = 0.0413	Invasion is decreased
	21			224 6-111	ROCK	p = 0.0095	Invasion is decreased
	30		к	231 COILI	β1 KD #1	p < 0.0001	Invasion is decreased
					DI KD #2	p = 0.0002	Invasion is not impacted
Various Inhibitors		1. Comparison of invasive distance under various treatments (Wilcoxon Rank-Sum)			Rac	n < 0.0001	Invasion is decreased
	3c		_	231 Coll- BME	ROCK	p < 0.0001	Invasion is decreased
			R		β1 KD	p = 0.0002	Invasion is decreased
					MMPs	p < 0.0001	Invasion is decreased
		1. Comparison of function blocking B1 antibody	Statistics Calculator	468	siRNA1	p > 0.05	Invasion is impacted equally
		to β 1 siRNA knockdown (t test between percents)		231	siRNA1	p > 0.05	Invasion is impacted equally
	S4a			231	siRNA2	p > 0.05	Invasion is impacted equally
		2. Comparison of SIKNA #1 to SIKNA #2 (t-test between percents)	Calculator	23	31	p > 0.05	Invasion is impacted equally
Gel Contraction	S4c	1. Comparison of Gel Contraction under β 1	R	23	31	p = 0.0265	KD decreases contraction
L		antibody inhibition (Wilcoxon Rank-Sum)		46	00	p = 0.0284	KD decreases contraction

Data	Figure	Test For (Test Used)	Software Used	т	est Details (Meaning of Results	
Occurrence of β 1 Clusters							
Removal of Outlier	NA	1. Removal of outlying bleb based on bleb perimeter (Two Tailed Grubb's Test for Outliers)	Excel	p = 0.0023			Bleb is an outlier
				ß1	Skew	s=1.223	Distribution is normal
		1. Determination of Skew and Kurtosis of	Excel	p1	Kurtosis	k = 3.690	Distribution is normal
		distributions (Skew and Kurtosis)		CellMask	Skew	s = 0.764	Distribution is normal
				Centrask	Kurtosis	k = 2.301	Distribution is normal
		2. Comparison of Occurrence of Clusters by Date	Statistics Calculator	β	1	p > 0.05	All dates are equal
β1 vs CellMask Clusters at		(Two Sample t-test between percents)		Cell	Лask	p > 0.05	All dates are equal
the Bleb Neck - 468	4d	3. Comparison of Occurrence of β1 Clusters vs CellMask Clusters (One Sample t-test between Percents)	Statistics Calculator		p < 0.00	001	β 1 has more clusters
		4. Comparison of actual vs expected Occurrence	Statistics	β	1	p < 0.0001	There are more β1 Clusters than expected
		between Percents)	Calculator	Cell	Лask	p = 0.023	There are more CellMask Clusters than expected
				ß1	Skew	s = 0.715	Distribution is normal
		1. Determination of Skew and Kurtosis of	Excel	pı	Kurtosis	k = 0.814	Distribution is normal
		distributions (Skew and Kurtosis)		CollMack	Skew	s = 0.207	Distribution is normal
				Cellividsk	Kurtosis	k = -0.001	Distribution is normal
		2. Comparison of Occurrence of Clusters by Date	Statistics	β	1	p > 0.05	All dates are equal
		(Two Sample t-test between percents)	Calculator	Cell	Лask	p > 0.05	All dates are equal
		3. Comparison of Distribution by Date (Single Factor ANOVA)		ß1	Skew	p > 0.05	All dates are equal
β1 vs CellMask Clusters at the Bleb Neck - 231	4d		Excol	pī	Kurtosis	p > 0.05	All dates are equal
			EXCE	CollMack	Skew	p > 0.05	All dates are equal
				Сспиназк	Kurtosis	p > 0.05	All dates are equal
		4. Comparison of Occurrence of β1 Clusters vs CellMask Clusters (One Sample t-test between Percents)	Statistics Calculator	p = 0.0012			$\beta 1$ has more clusters
		5. Comparison of actual vs expected Occurrence of β1 and CellMask Clusters (One Sample t-test between Percents)	Statistics	β1		p = 0.028	There are more β1 Clusters than expected
			Calculator	CellMask		p > 0.05	There are equal CellMask Clusters to expected
β1 vs CellMask Clusters on	S5e	1. Comparison of actual vs expected Occurrence of β 1 and CellMask Clusters (One Sample t-test between Percents)	Statistics	β1		p > 0.05	There are equal β1 Clusters to expected
the Bulk Membrane - 231			Calculator	CellMask		p > 0.05	There are equal CellMask Clusters to expected
Dependence of Bleb Size	S2c	1. Dependence of bleb perimeter on the Occurrence of β1 clusters (Two-Tailed t-test)	Excel	p > 0.05			β1 Clusters occur independently of perimeter
231	S2d	1. Dependence of bleb neck width on the Occurrence of β1 clusters (Two-Tailed t-test)	Excel	p > 0.05			β1 Clusters occur independently of neck width
		1. Determination of Skew and Kurtosis of distributions (Skew and Kurtosis)	Excel	β1 CellMask	Skew	s=0.895	Distribution is normal
					Kurtosis	k = 1.416	Distribution is normal
					Skew	s = 0.324	Distribution is normal
β1 vs CellMask Clusters at the Bleb Neck - 231 Spheroids					Kurtosis	k = 0.253	Distribution is normal
	4d	2. Comparison of Occurrence of Clusters by Date (Two Sample t-test between percents)	Statistics	β1		p > 0.05	All dates are equal
			Calculator	CellMask		p > 0.05	All dates are equal
		3. Comparison of Occurrence of β1 Clusters vs CellMask Clusters (One Sample t-test between Percents)	Statistics Calculator		p < 0.00	001	β1 has more clusters
		4. Comparison of actual vs expected Occurrence	Statistics	β1 CellMask		p < 0.0001	There are more β1 Clusters than expected
		of	Calculator			p = 0.0001	There are more CellMask Clusters than expected

$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$	Data	Figure	Test For (Test Used)	Software Used	т	est Details	(α = 0.05)	Meaning of Results
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$					04	Skew	s = 0.682	Distribution is normal
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$			1. Determination of Skew and Kurtosis of	Fueel	рı	Kurtosis	k = 1.003	Distribution is normal
$ \begin{array}{ $			distributions (Skew and Kurtosis)	Excel	T - 11 -	Skew	s = 0.906	Distribution is normal
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$					Talin	Kurtosis	k = 1.604	Distribution is normal
$ \begin{array}{ $			2. Comparison of Occurrence of Clusters by Date	Statistics	β	1	p > 0.05	All dates are equal
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$			(Two Sample t-test between percents)	Calculator	Та	lin	p > 0.05	All dates are equal
A. Comparison of actual vs expected Occurrence of \$1 and Tain Clusters (Incusters (Incusters (Incusters Clone Sample Ltest) between Percents)Statistics Calculator $\beta \perp$ $r = 0.0001$ There are more $\beta \perp$ (Latters the expected of \$1 and Tain Clusters (Incusters Clone Sample Ltest) 	β1 vs Talin Clusters at the Bleb Neck - 231	5c	3. Comparison of Occurrence of β1 Clusters vs Talin Clusters (One Sample t-test between Percents)	Statistics Calculator	p > 0.		05	$\beta 1$ has equal clusters to Talin
$ \begin{array}{ c c c c c } \hline \below end Percents \\ \hline \below end$			 4. Comparison of actual vs expected Occurrence of β1 and Talin Clusters (One Sample t-test between Percents) 	Statistics Calculator	β1		p < 0.0001	There are more β1 Clusters than expected
Active β is smartine β is smart in β is and β in β i					Та	lin	p = 0.0001	There are more Talin Clusters than expected
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$			5. Comparison of actual vs expected Occurrence of β1 and Talin Co-clusters (One Sample t-test between Percents	Statistics Calculator	p = 0.0025		025	There are more β1 and Talin Co- clusters than expected
βI ws Vinculin Clusters at the Bleb Neck - 2311. Determination of Skew and Kurtosis of distributions (Skew and Kurtosis) $\Sigma cell$ $\frac{\rho \mu}{kurtosis}$ $k = 1.614$ Distribution is normal $\frac{1}{kurtosis}$ βI ws Vinculin Clusters at the Bleb Neck - 2312. Comparison of Occurrence of Clusters by Date $\frac{1}{2}$ βI $p > 0.05$ All dates are equal $\frac{1}{kurtosis}$ βI ws Vinculin Clusters fore Sample 1-test between percents) βI $p > 0.05$ All dates are equal $\frac{1}{kurtosis}$ βI $p > 0.05$ All dates are equal $\frac{1}{kurtosis}$ βI $p < 0.0011$ $p < 0.0011$ $p < 0.0011$ $p < 0.0011$ There are more βI Clusters tha $\frac{1}{kurtosis}$ $p < 0.0011$ There are more βI Clusters tha $\frac{1}{kurtosis}$ βI $p < 0.0011$ $p < 0.0011$ There are more βI Clusters tha expected $\frac{1}{kurtosis}$ $p < 0.0011$ There are more βI Clusters tha expected $\frac{1}{kurtosis}$ There are more βI Clusters tha expected $\frac{1}{kurtosis}$ βI $p < 0.0011$ There are more βI Clusters tha expected $\frac{1}{kurtosis}$ $p < 0.0011$ There are more βI Clusters tha expected $\frac{1}{kurtosis}$ βI βI $p < 0.0011$ There are more βI Clusters $\frac{1}{kurtosis}$ $p < 0.0011$ There are more βI Clusters $\frac{1}{kurtosis}$ βI βI $p < 0.0011$ There are more βI Clusters $\frac{1}{kurtosis}$ $p < 0.0011$ There are more βI Clusters $\frac{1}{kurtosis}$ βI <td></td> <td></td> <td></td> <td></td> <td>01</td> <td>Skew</td> <td>s = 1.000</td> <td>Distribution is normal</td>					01	Skew	s = 1.000	Distribution is normal
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$			1. Determination of Skew and Kurtosis of	Evcol	рт	Kurtosis	k = 1.614	Distribution is normal
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$			distributions (Skew and Kurtosis)	Excel	Vinculin	Skew	s = 1.107	Distribution is normal
$ \begin{array}{ c c c c c c c c c c c c c c c c c c c$					vinculin	Kurtosis	k = 2.161	Distribution is normal
βi vs Vinculin Clusters at the Bleb Neck - 231 $(Two Sample t test between percents)CalculatorVinculinp > 0.05All dates are equal\beta 1 has more clusters than\gamma burner in\gamma burner in\gamma burner in\gamma burner in\gamma burner in\beta 1 has more clusters than\gamma burner in\gamma burn$			2. Comparison of Occurrence of Clusters by Date	Statistics	β	1	p > 0.05	All dates are equal
β1 vs Vinculin Clusters at the Bleb Neck - 231S. Comparison of Occurrence of β1 Clusters vs Vinculin Clusters (one Sample t-test between between Percents)Statistics Calculator $p < 0.001$ There are more β1 Clusters than vinculin There are more β1 Clusters than expected CalculatorSc			(Two Sample t-test between percents)	Calculator	Vinc	culin	p > 0.05	All dates are equal
Active β_1 vs inactive β_1 vs inactive β_1 4. Comparison of actual vs expected Occurrence of β_1 and Vinculin Clusters (One Sample t-test between Percents)Statistics Calculator β_1 $p < 0.001$ There are more β_1 Clusters that expected of β_1 and Vinculin Clusters (One Sample t-test between Percents)Statistics Calculator β_1 $p < 0.001$ There are more β_1 clusters that expected of β_1 and Vinculin Clusters (One Sample t-test between Percents)Statistics Calculator $p < 0.001$ There are more β_1 and Vinculin Clusters (One Sample t-test between Percents)Statistics Calculator $p < 0.001$ There are more β_1 and Vinculin Clusters (One Sample t-test between Percents)Statistics Calculator $p < 0.001$ There are more β_1 clusters that expected of Clusters by Date CalculatorStatistics Calculator $p < 0.001$ There are more β_1 clusters that expected of Clusters by Date CalculatorStatistics Calculator $p < 0.001$ There are more β_1 clusters that expected of Clusters by Date CalculatorStatistics Calculator $p > 0.05$ All dates are equal Clusters that expected β_1 $p > 0.05$ All dates are equal Clusters that expected β_1 $p > 0.05$ All dates are equal β_1 $p < 0.05$ All dates are equal β_2 $p < 0.05$ All dates are equal β_2 $p < 0.05$ All dates are equal β_1 $p < 0.05$ All dates are equal β_2 $p < 0.05$ All dates are equal β_2 $p < 0.05$ All dates are equal β_2 $p < 0.05$ All dates are equal β	β1 vs Vinculin Clusters at the Bleb Neck - 231	5c	3. Comparison of Occurrence of β1 Clusters vs Vinculin Clusters (One Sample t-test between Percents)	Statistics Calculator	p < 0.0001		001	β1 has more clusters than Vinculin
Active β 1 vs inactive β 1 vsInactive β 1			 Comparison of actual vs expected Occurrence of β1 and Vinculin Clusters (One Sample t-test between Percents) 	Statistics Calculator	β1		p < 0.0001	There are more β 1 Clusters than expected
S. Comparison of actual vs expected Occurrence of \$\beta\$ and Vinculin Co-clusters (One Sample t-test between Percents)Statistics Calculator $p < 0.001$ There are more \$\beta\$ and Vinculin Co-clusters than expected Co-clusters than expectedActive \$\beta\$ 1 and Vinculin Co-clusters (One Sample t-test between Percents)1. Determination of Skew and Kurtosis of distributions (Skew and Kurtosis) $Active β 1$SkewKurtosisβ = 0.533$Distribution is normalKurtosis1. Determination of Skew and Kurtosis)1. Determination of Skew and Kurtosis)ExcellActive β 1$SkewKurtosis$\beta$ = 0.533$Distribution is normalKurtosis2. Comparison of Occurrence of Clusters by DateCellMask Clusters on Occurrence of Active β 1StatisticsCalculatorActive β 1$p > 0.05$All dates are equalInactive β 1$3. Comparison of Occurrence of Active β 1StatisticsCalculatorp = 0.001Active β 1 has more clusters theCalculator4. Comparison of Occurrence of Active β 1$StatisticsCalculatorp > 0.05$All dates are equalInactive β 1$5. Comparison of Occurrence of Active β 1$StatisticsCalculatorp > 0.05Inactive β 1$6. Comparison of Occurrence of Active β 1$StatisticsCalculatorp > 0.05Inactive β 1$6. Comparison of Occurrence of Active β 1$StatisticsCalculatorp = 0.001There are more Active β 1$6. Comparison of actual vs expected Occurrenceof β 1 and CellMask Clusters (One Sample t-test$					Vinculin p > 0.05		p > 0.05	There are equal Vinculin Clusters to expected
Active β_1 vs inactive β_1 vs Bieb Neck - 2311. Determination of Skew and Kurtosis of distributions (Skew and Kurtosis)ExcelActive β_1 Skew $\overline{s} = 0.608$ Distribution is normal Distribution is normal 			5. Comparison of actual vs expected Occurrence of β1 and Vinculin Co-clusters (One Sample t-test between Percents	Statistics Calculator	p < 0.0001			There are more β1 and Vinculin Co-clusters than expected
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5. Comparison of Occurrence of Active $\beta1$ Clusters vs Inactive $\beta1$ Clusters (One Sample t-test between Percents)Statistics Calculator $p = 0.011$ Active $\beta1$ has more clusters that Inactive $\beta1$ Clusters than expected 0 Clusters than expected 0 Clusters to a clusters that between Percents)Active $\beta1$ has more clusters that Inactive $\beta1$ 6. Comparison of actual vs expected Occurrence of $\beta1$ and CellMask Clusters (One Sample t-test between Percents)Active $\beta1$ $p < 0.0001$ There are more Active $\beta1$ Clusters than expectedActive $\beta1$ vs Inactive $\beta1$ against Collage I Borgering is P kluwer1. Comparison of Pearson's R Values between Active $\beta1$ and Cellman in and Collage n and Inactive $\beta1$ and Collagen (Wilcown Parks Sum)R $p = 0.0016$ Active $\beta1$ is more correlated with collagen accurulation that with collagen accurulation that collagen (Wilcown Parks Sum)	CellMask Clusters at the Bleb Neck - 231		4. Comparison of Occurrence of Inactive β1 Clusters vs CellMask Clusters (One Sample t-test between Percents)	Statistics Calculator	p > 0.05			Inactive β1 has equal clusters to CellMask
$ \begin{array}{ c c c c c } \hline Active \ \beta 1 \ vs \ Inactive \ \beta 1 \ and \ Collagen \ 1. \ Comparison \ of \ Pearson's \ R \ Values \ between \ Percents \ 1. \ Comparison \ of \ Pearson's \ R \ Values \ between \ Percents \ 1. \ Comparison \ of \ Pearson's \ R \ Values \ between \ Percents \ 1. \ Comparison \ of \ Pearson's \ R \ Values \ between \ Pearson's \ R \ Values \ between \ Pearson's \ R \ Values \ Pearson's \ R \ R \ Pearson's \ R \ Pearson's \ R \ R \ R \ Pearson's \ R \ R \ R \ R \ R \ R \ R \ R \ R \ $			5. Comparison of Occurrence of Active β1 Clusters vs Inactive β1 Clusters (One Sample t-test between Percents)	Statistics Calculator	p = 0.011			Active $\beta 1$ has more clusters than Inactive $\beta 1$
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	Active β1 vs Inactive β1 against Collagen I	6f	1. Comparison of Pearson's R Values between Active β1 and Collagen and Inactive β1 and	R	p = 0.0016			Active β 1 is more correlated with collagen accumulation than

Data	Figure	Test For (Test Used)	Software Used	Test I	Details (α	= 0.05)	Meaning of Results
Collagen Pulling							
		1. Data resampling to determine normality (Bootstrap Resampling)	R	Cell-Free Gels		Distribution is Normal	
				β1 KD		Distribution is Normal	
				Non-Blebby Region		Distribution is Normal	
Collagen Density	8e			Blebby Region		Distribution is Normal	
		2. Analysis of Density of Collagen I in Bleb Bearing Regions of Cells as compared to other regions (Two Tailed t-test)	D	Blebby to Non-blebby		p < 0.0001	Blebby Regions have higher
				Blebby to $\beta 1$ KD		p < 0.0001	Blebby Regions have higher
			n	Blebby to Cell Free		n < 0.0001	Blebby Regions have higher
						p<0.0001	collagen density
	8f	1. Data resampling to determine normality (Bootstrap Resampling)		Cell-Free Gels		Distribution is Normal	
			D	β1 KD		Distribution is Normal	
			ĸ	Non-Blebby Region		Distribution is Normal	
Collagen Alignment				Blebby Region		Distribution is Normal	
		2. Analysis of Collagen Alignment in Bleb Bearing Regions of Cells as compared to other regions (Two Tailed t-test)	R	Blebby to Non-blebby		p = 0.0166	Blebby Regions have more
				Blebby to $\beta 1$ KD		p = 0.0023	Blebby Regions have more
				Blebby to Cell Free		n < 0.0001	Blebby Regions have more
						p<0.0001	aligned collagen I
468 Refers to MDA-MB-468							
231 Refers to MDA-MB-231							

Supplementary Movies



Movie 1. Transmitted light images of an MDA-MB-468 spheroid showing round cell invasion into a 1 mg/ml collagen I gel. Images are taken starting at t = 2.25 h after collagen embedding of the spheroid. Frames were taken every 5 minutes. Length of movie is 6.25 hours. Movie is played back at 7 frames/s. Frames from this movie are also shown in Figs. S1c and Fig. 2 in the main text. Scale bar = 25 μ m.



Movie 2. Transmitted light images of an MDA-MB-231 spheroid showing invasion of cells with dynamically changing morphology into a 1 mg/ml collagen I gel. Images are taken every 5 minutes starting at t = 7.75 h after collagen embedding of the spheroid. Length of movie is 6.25 hours. Movie is played back at 7 frames/s. Frames from this movie are also shown in Fig. S1d and Fig. 2 in the main text. Scale bar = 25 μ m.



Movie 3. Confocal reflectance images of the collagen matrix surrounding the MDA-MB-468 spheroid shown in M1 (at a slightly different location on the spheroid). Locations of apparent lack of collagen I that develop over the time course of the movie are caused primarily by out of plane spheroid and invading cells limiting excitation light reaching the imaging plane. Images are taken every 5 minutes starting at t \approx 2.25 h after collagen embedding of the spheroid. Length of movie is 6.25 hours. Movie is played back at 7 frames/s. Frames from this movie are shown in Fig. 2 in the main text. Scale bar = 25 μ m.



Movie 4. Confocal reflectance images of the collagen matrix surrounding the MDA-MB-231 spheroid shown in M2. Locations of apparent lack of collagen I that develop over the time course of the movie are caused primarily by out of plane spheroid and invading cells limiting excitation light reaching the imaging plane. Images are taken are taken every 5 minutes starting at t \approx 7.75 h after collagen embedding of the spheroid. Length of movie is 6.25 hours. Movie is played back at 7 frames/s. Frames from this movie are shown in Fig. 2 in the main text. Scale bar = 25 μ m.



Movie 5. MDA-MB-231 cell labeled with (red) CellMask and embedded in (green) fluorescently labeled collagen I imaged shortly after completion of collagen gelation (t = 36 min after collagen embedding). The cell shows initial attachment to and pulling of a collagen fiber by a single bleb (white arrows). Images were taken every 10 seconds. Length of movie is 560 seconds. Movie is played back at 7 frames/s. Frames from this movie are also shown in Fig. 7A in the main text. Scale bar = 10 μ m.



Movie 6. MDA-MB-231 cell also shown in V4 shown over a longer time period starting immediately after completion of collagen gelation (t = 81 min after collagen embedding). The cell shows persistent attachment of collagen fibers at the site of multiple recurrent blebs (bracketed region). Images were taken every 10 seconds. Length of movie is 28.5 minutes. Movie is played back at 7 frames/s. Frames from this movie are also shown in Fig. 7B in the main text. Scale bar = 10 μ m.



Movie 7. MDA-MB-231 cell labeled with (red) CellMask and embedded in (green) fluorescently labeled collagen I imaged starting at t = 6 h after cells were embedded in collagen. The cell shows how bleb formation becomes increasingly confined to a particular cell region over time and how the coincident collagen reorganization is spatiotemporally coordinated with the recurrent bleb formation. Images were taken every 10 seconds. Length of movie is 12.5 minutes. Movie is played back at 7 frames/s. Frames from this movie are also shown in Fig. 8B in the main text (rotated by 180°). Scale bar = 10 μ m.



Movie 8. MDA-MB-231 cell labeled with (red) CellMask and embedded in (green) fluorescently labeled collagen I imaged starting at t = 5.75 h after cells were embedded in collagen. The cell shows the strongest collagen reorganization at the site of polarized, recurrent clustered blebs. Images were taken every 9.7 seconds. Length of movie is 30 minutes. Movie is played back at 7 frames/s. Frames from this movie are also shown in Fig. 8C,D in the main text. Scale bar = $10 \mu m$.