Supplementary Information

Supplementary Table 1: Information on shRNAs.

Target protein	Source	Identifier	Target sequence	KD efficiency
Integrin β5	Sigma-Aldrich	TRCN0000296117 TRCN0000289121 TRCN0000296116	AGCTTGTTGTCCCAATGAAAT CTGAGGGCAAACCTTGTCAAA GGATCAGCCTGAGGATCTTAA	85% 84% 82%
AP2-μ	Sigma-Aldrich	TRCN0000060239 TRCN0000060241	CACCAGCTTCTTCCACGTTAA GCTGGATGAGATTCTAGACTT	94% 95%
Clathrin heavy chain	Sigma-Aldrich	TRCN0000011216 TRCN0000007981	CGGTTGCTCTTGTTACGGATA CGTGTTCTTGTAACCTTTATT	88% 97%
FCHo1	Sigma-Aldrich	TRCN0000162794 TRCN0000164648	CACAACCGCTATTGAGCACTT GCAGGAAGCGATGAAACGTTT	NA NA
FCHo2	Sigma-Aldrich	TRCN0000167218 TRCN0000167925	GCTACAGTATTAAACCAGAAA CCAAAGCTTACTTCAGGCAAA	88% 75%
EPS15	Sigma-Aldrich	TRCN0000007980 TRCN0000007978	CCCAGAATGGATTGGAAGTTT GCAGTGAAACAGCCAACCTTA	86% 79%
EPS15R	Sigma-Aldrich	TRCN0000233084 TRCN0000233083	GAGCATGCCACCGCCTAAATT AGTCTGGCCTCTCGGACATTA	77% 72%
Intersectin 1	Sigma-Aldrich	TRCN0000002009 TRCN0000002010	GCACTAGCTGACATGAATAAT GCAGTTGTTTGATGAGCCGTA	64% 56%
Intersectin 2	Sigma-Aldrich	TRCN0000002385 TRCN0000318540	CCTGGACTGCAAAGAAAGATA GCAGAACGTAAAGCCCAGAAA	75% 84%
Scramble (Negative control)	Addgene	Cat#1864	CCTAAGGTTAAGTCGCCCTCG	NA

Supplementary Table 2. Information on antibodies.

Name	Source	Identifier
Primary antibodies		
Rat anti-integrin β1 antibody (clone 9EG7)	BD Biosciences	Cat#550531
Mouse anti-activated integrin β1 antibody (clone HUTS-4)	Sigma-Aldrich	Cat#MAB2079Z
Mouse anti-integrin αvβ5 antibody (clone P5H9)	R&D Systems	Cat#MAB2528
Mouse anti-alpha adaptin antibody [AP6]	Abcam	Cat#ab2730
Mouse anti-vinculin antibody (clone hVIN-1)	Sigma-Aldrich	Cat#V9131
Mouse anti-paxillin antibody (clone 349/Paxillin)	BD Biosciences	Cat#610051
Mouse anti-talin1 antibody (clone 8D4)	Abcam	Cat#ab157808
Mouse Anti-Vitronectin/S-Protein antibody (clone VN58-1)	Abcam	Cat#ab13413
Rabbit anti-FAK (phospho Y397) antibody (clone EP2160Y)	Abcam	Cat#ab81298
Rabbit anti-Clathrin heavy chain antibody (Polyclonal)	Abcam	Cat#ab21679
Rabbit anti-integrin β5 antibody (clone D24A5)	Cell signaling technology	Cat#3629S
Rabbit anti-FCHo2 antibody (Polyclonal)	Novus Biologicals	Cat#NBP2-32694
Rabbit anti-GAPDH (clone 14C10)	Cell signaling technology	Cat#2118
Rabbit anti-GFP antibody (Polyclonal)	Invitrogen	Cat#A-11122
Mouse anti-mCherry antibody (clone GT857)	Sigma-Aldrich	Cat#SAB2702291
Secondary antibodies		
Alexa Fluor 488-conjugated goat anti-Mouse IgG (H+L) antibody	Thermo Fisher Scientific	Cat#A-11001
Alexa Fluor 568-conjugated goat anti-Mouse IgG (H+L) antibody	Thermo Fisher Scientific	Cat#A-11004
Alexa Fluor 488-conjugated goat anti-Rabbit IgG (H+L) antibody	Thermo Fisher Scientific	Cat#A-11034
Texas Red-conjugated goat anti-Rabbit IgG (H+L) antibody	Thermo Fisher Scientific	Cat#T2767
Alexa Fluor 647-conjugated goat anti-Rat IgG (H+L) antibody	Thermo Fisher Scientific	Cat#A-21247
HRP-linked goat anti-Rabbit IgG (H+L) antibody	Cell signaling technology	Cat#7074
HRP-linked goat anti-Mouse IgG (H+L) antibody	Cell signaling technology	Cat#7076

Supplementary Table S3: The statistics for null hypothesis testing

Figure	Test		Degrees of freedom	Specifics
Fig. 1k	One-way ANOVA		F (10,77) = 140.8	R^2 = 0.9481
Fig. 2d	Kruskal-Wallis test		3	H = 1222
Fig. 2i	One-way ANOVA		F (7,72) = 97.35	R^2 = 0.9044
Fig. 2m	Kruskal-Wallis test	Vitronectin	5	H = 91.28
		Fibronectin	5	H = 67.26
	Mann Whitney test	Gelatin	n/a	U = 720.5
	t-test	PLL	t = 0.1411, df = 70	R^2 = 0.0002843
	t-test	BSA	t = 1.246, df = 73	R^2 = 0.02083
Fig. 3d	One-way ANOVA		F (4, 55) = 0.4478	R^2 = 0.03154
Fig. 3i	t-test		t = 7.761, df = 17.07	R^2 = 0.7792
	One-way ANOVA		F (4, 55) = 120.3	R^2 = 0.8974
Fig. 4d	Mann Whitney test		n/a	U = 2998
Fig. 4g	One-way ANOVA		F (3, 181) = 39.93	R^2 = 0.3983
Fig. 4j	One-way ANOVA		F (8, 225) = 60.44	R^2 = 0.6824
Fig. 5h	Kruskal-Wallis test	Number	3	H = 45.30
	t-test	Size 2D	t = 13.61, df = 21	R^2 = 0.8982
		Size 3D	t = 5.423, df = 8	R^2 = 0.7861
Fig. 6c	Kruskal-Wallis test	U2OS	5	H = 294.2
		A549	5	H = 203.6
		Hela	5	H = 113.3
Extended Data Fig. 3c	Kruskal-Wallis test		3	H = 969.4
Extended Data Fig. 7e	Kruskal-Wallis test		2	H = 58.3
Extended Data Fig. 9c	t-test		t = 11.76, df = 16	R^2 = 0.8963
Extended Data Fig. 10b	One-way ANOVA		F (2, 96) = 25.46	R^2 = 0.3466
*Confidence level is 0.05 for all tests.				

Supplementary Video 1. Dynamics of curved adhesions at nanopillars.

Live-cell fluorescence imaging of U2OS cell expressing ITGβ5-GFP stained with a plasma membrane marker CellMask Red on vitronectin-coated nanopillars at 15 s/frame for ~80 min. The ratiometric images of ITGβ5/membrane (normalized by its mean per cell) are shown in the Parula color scale. Time (in seconds) is indicated. Most curved adhesions at nanopillars are stable for the ~80-min time duration. Meanwhile, few curved adhesions slowly assemble and disassemble at nanopillars. Scale bar, 5 μm.

Supplementary Video 2. Dynamics of FCHo2 accumulations in curved adhesions at nanopillars.

Live-cell fluorescence imaging of U2OS cell co-expressing ITGβ5-GFP and RFP-FCHo2 on vitronectin-coated nanopillars at 15 s/frame for ~20 min. Time (in seconds) is indicated. The FCHo2 accumulations are strong and stable in curved adhesions marked by ITGβ5 accumulations at nanopillars (representative ones are indicated by white circles), suggesting that FCHo2 is an integral component of curved adhesions. Some nanopillars without ITGβ5 accumulation (representative ones indicated by yellow circles) also show FCHo2 accumulations, but these accumulations are usually weak and dynamic, exhibiting frequent assembly and disassembly on a time scale of minutes. Scale bar, 5 μm.

Supplementary Video 3. Z-stack images of cells staying on the top of a 3D matrix of pure collagen fibers.

Confocal fluorescence images of U2OS cells expressing GFP-CaaX (green). Cells were plated on the top of a 3D matrix made of collagen fibers labeled with AF647-collagen. After 72 hours of culture, cells mostly stay on the top of the matrix (magenta). The top 100 μm of the sample was

imaged every 0.5 μm from the bottom to the top. The Z positions are indicated. Scale bar, 10 μm. The corresponding 3D projection (side view) is shown in **Extended Data Fig. 13a** (left).

Supplementary Video 4. Z-stack images of cells embedded in a 3D matrix of vitronectin fibers.

Confocal fluorescence images of U2OS cells expressing GFP-CaaX (green). Cells were plated on the top of a 3D matrix made of vitronectin fibers labeled with AF647-collagen. After 72 hours of culture, cells have infiltrated and are fully embedded in the matrix (magenta). The top 100 μm of the sample was imaged every 0.5 μm from the bottom to the top. The Z positions are indicated. Scale bar, 10 μm. The corresponding 3D projection (side view) is shown in **Extended Data Fig. 13a** (right).

Supplementary Video 5. Z-stack images of curved adhesions in 3D.

Confocal fluorescence images of immunolabeled ITGβ5 (magenta) in U2OS cells expressing FCHo2-GFP (green) embedded in a 3D matrix made of vitronectin fibers labeled with AF647-collagen (grayscale). Curved adhesions, the colocalizations of ITGβ5 and FCHo2 along vitronectin fibers, form extensively. The images were acquired every 0.5 μm from the bottom to the top and the Z positions are indicated. Scale bar, 25 μm.

Supplementary Video 6. Z-stack images of focal adhesions in 3D.

Confocal fluorescence images of immunolabeled vinculin (green) and ITGβ5 (magenta) in U2OS cells embedded in a 3D matrix made of vitronectin fibers labeled with AF647-collagen (grayscale). Focal adhesions, the colocalizations of ITGβ5 and vinculin, are barely observed throughout the cells. Meanwhile, ITGβ5 still extensively accumulates along the ECM fibers. The images were acquired every 0.5 μm from the bottom to the top and the Z positions are indicated. Scale bar, 25 μm.