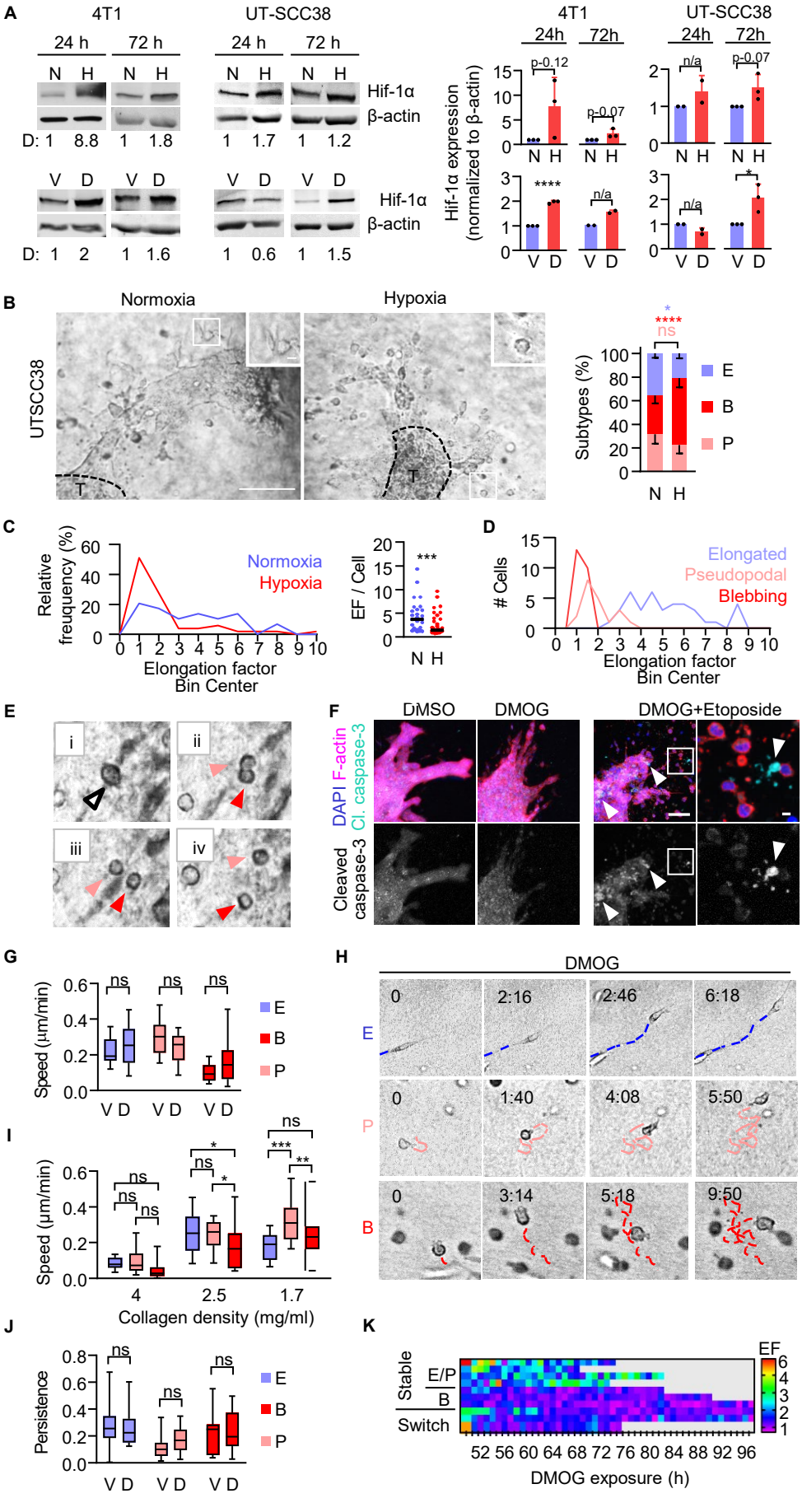
A
4T1
UT-SCC38
24 h
72 h
N H N H
Hif-1α
β-actin
D: 1 8.8 1 1.8
1 1.7 1 1.2
V D V D
D: 1 2 1 1.6
1 0.6 1 1.5
Hif-1α expression (normalized to β-actin)
p-0.12
p-0.07
n/a
****
*
B
Normoxia
Hypoxia
UTSCC38
T
Subtypes (%)
E
B
P
ns
C
Relative freuquency (%)
Normoxia
Hypoxia
Elongation factor Bin Center
EF / Cell
***
D
Cells
Elongated
Pseudopodal
Blebbing
E
i
ii
iii
iv
F
DMSO
DMOG
DMOG+Etoposide
DAPI F-actin Cl. caspase-3
Cleaved caspase-3
G
Speed (μm/min)
H
DMOG
E
P
B
0
2:16
2:46
6:18
1:40
4:08
5:50
3:14
5:18
9:50
I
Collagen density (mg/ml)
4
2.5
1.7
J
Persistence
K
Stable
E/P
B
Switch
EF
DMOG exposure (h)
52 56 60 64 68 72 76 80 84 88 92 96

Figure S1. Morphological and functional mapping of single cell migration modes induced by HIF stabilization. Related to Figure 1. A, Representative Western blot (left panel) and mean+-SD (right panel) of HIF-1α protein content normalized to β-actin loading control in 4T1 and UT-SCC38 cell monolayer culture. D, densitometric analysis. **** P<0.0001, * P=0.028 (unpaired t-test, two-sided), n=2-3 (see all individual experiments in Data Source File). B, Brightfield micrographs of UTSCC38 tumoroids invading into collagen at 96 h (left panel) and frequency of single-cell migration subtypes (right panel). Means ± s.d. (n=5 tumoroids from 1 experiment). **** P<0.0001, * P=0.016, ns P=0.261 (two-way ANOVA). Scale bars, 100 µm (overviews), 10 µm (insets). T, tumoroid; dashed lines, tumoroid edge. C, Elongation factor (EF) distribution of 4T1 single-cell migration subtypes under normoxia and hypoxia in mid-density (2.5 mg/ml) rattail collagen (72 h; 86 cells, n=2). D, EF distribution of migrating 4T1 single-cells (left panel) and overall change of elongation (right panel) after HIF stabilization (72 h; 80 cells, n=2). Horizontal line, median. *** P=0.0002 (Mann-Whitney test, two-sided). E, Cell division of a blebbing-amoeboid migrating cell in collagen 72 h after DMOG treatment. Arrowheads, post-mitotic daughter cells. F, Confocal micrographs of cleaved-caspase 3 (apoptotic marker) in UT-SCC38 tumoroids in 3D collagen 96 h after HIF-stabilization. Etoposide treatment (200 µM) was used as positive control. Insets, blebbing amoeboid-migrating subtypes. Arrowheads, apoptotic cells positive for cleaved-caspase-3. Scale bars, 100 µm (overview), 10 µm (inset). G, Migration speed of individual 4T1 cells in intermediate-density collagen in the presence of DMOG or solvent. Box and whiskers show the median, 25/75 percentile and minimum/maximum (48-72 h; 37-60 cells, n=3). P-values: 0.58 (E); 0.15 (P); 0.27 (B) (unpaired t-test, two-sided). H, Migration velocities of 4T1 single-cells after HIF-stabilization with DMOG in collagen with low (1.7 mg/ml), intermediate (2.5 mg/ml) or high (4 mg/ml) density. Abbreviations: E, elongated; P pseudopodal-rounded; B, blebbing-rounded; N, normoxia; H, hypoxia; V, vehicle control (DMSO); D, DMOG. (48-72 h; 45 to 66 cells per density, n=3). Data are represented as in (G). *** P=0.0001, ** P=0.009, * P<0.025, ns, P≥0.17 (two-way ANOVA). I, Migration persistence. Conditions and data represented as in (G). P values: 0.87 (E); 0.45 (P); 0.99 (B) (unpaired t-test, two-sided). J, Migration paths and related shapes of DMOG-induced 4T1 cells in low-density collagen. Time (h:min). Scale bars, 10 µm. K, Elongation kinetics of individual UT-SCC38 cells during collagen invasion after HIF-stabilization. Rows represent one cell over time. Abbreviations: E, elongated-mesenchymal; P, pseudopodal-amoeboid; B, blebbing-amoeboid; N, normoxia; H, hypoxia; V, vehicle control (DMSO); D, DMOG; n/a, not applicable.

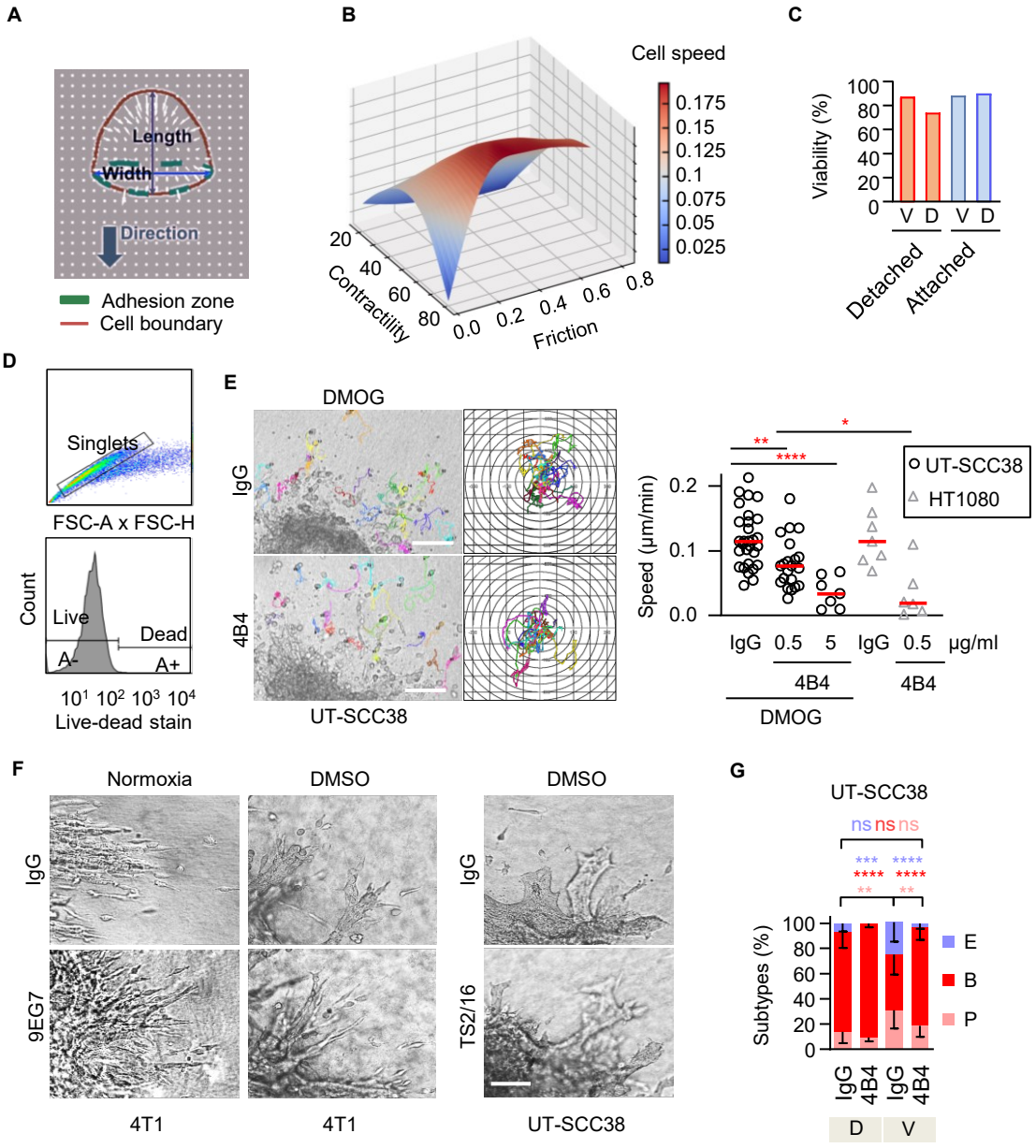
A
Length
Width
Direction
Adhesion zone
Cell boundary
B
Cell speed
0.175
0.15
0.125
0.1
0.075
0.05
0.025
Contractility
Friction
C
Viability (%)
V D V D
Detached
Attached
D
Singlets
FSC-A x FSC-H
Count
Live
Dead
A-
A+
Live-dead stain
E
DMOG
IgG
4B4
UT-SCC38
Speed (μm/min)
UT-SCC38
HT1080
IgG 0.5 5 IgG 0.5 μg/ml
4B4
4B4
DMOG
F
Normoxia
DMSO
DMSO
IgG
9EG7
TS2/16
4T1
4T1
UT-SCC38
G
UT-SCC38
Subtypes (%)
E
B
P
IgG 4B4 IgG 4B4
D
V

Figure S2. Cell viability, migration activity and integrin dependence of phenotypes after HIF stabilization. Related to Figure 2. A, In silico approach for shape simulation of migrating cells. Cell axes (length, width) used for aspect ratio analysis. White arrows, velocity of actin flow. B, Average speed of cells for different contractility and friction simulations represented as landscape plot. C, Cell viability of isolated 4T1 subsets 48 h after treatment in 2D culture, detected by Trypan blue exclusion. D, Flow cytometry gating strategy for selection of single viable cells (A- fraction) for receptor expression and activity analysis. E, Migration paths and trajectories (left panel) and speed (right panel) of individual UT-SCC38 cells after HIF-stabilization with or without β1 integrin blockade (mAb 4B4, 0.5 µg/ml; collagen 1.7mg/ml). For comparison, mesenchymal HT-1080 fibrosarcoma cells were exposed to the same treatment. Data show the median and individual cells (70 cells, n=2). **** P<0.0001, ** P=0.002, * P=0.01 (Mann-Whitney test, two-sided). F, Brightfield micrographs of migrating 4T1 cells in 3D collagen with or without β1 integrin activation during ongoing HIF-stabilization (4T1: 48-72 h, mAB 9EG7, 5 µg/ml; UT-SCC38: 72-96 h, mAb TS2/16, 20 µg/ml). G, Morphology-based scoring of single-cell migration subtypes after interference with β1 integrin activity (adhesion-perturbing mAb 4B4, 10 µg/ml). Data show the means ± s.d. from 49 tumoroids of two independent experiments. **** P<0.0001, *** P=0.0003, ** P<0.005 (two-way ANOVA). Abbreviations: E, elongated; P pseudopodal-amoeboid; B, blebbing-amoeboid; N, normoxia; H, hypoxia; V, vehicle (DMSO); D, DMOG. Scale bars, 100 µm.

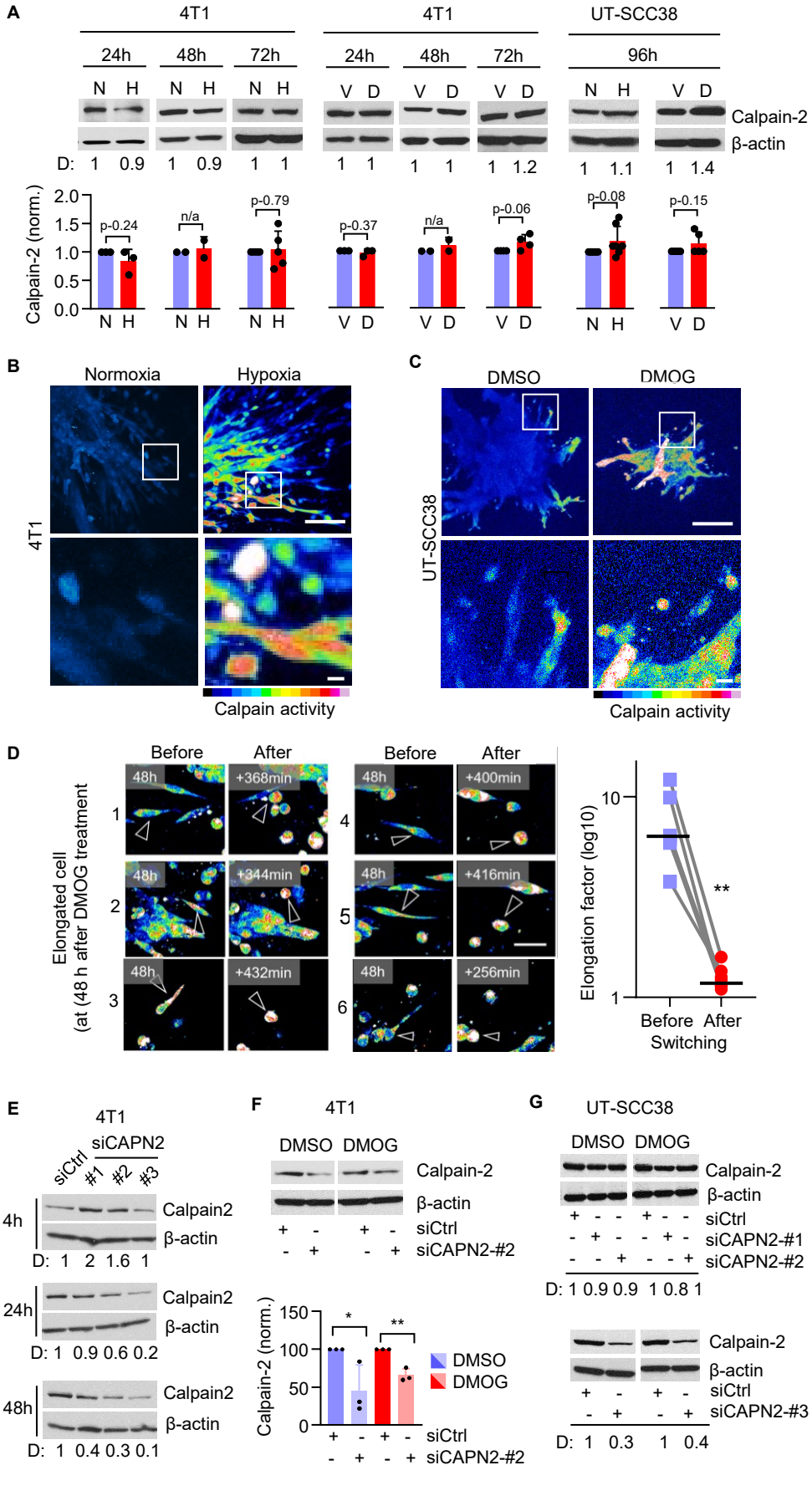
A
4T1
4T1
UT-SCC38
24h
48h
72h
24h
48h
72h
96h
N H N H N H
V D V D V D
N H V D
Calpain-2
β-actin
D: 1 0.9 1 0.9 1 1
1 1 1 1 1 1.2
1 1.1 1 1.4
Calpain-2 (norm.)
p-0.24
n/a
p-0.79
p-0.37
n/a
p-0.06
p-0.08
p-0.15
B
Normoxia
Hypoxia
4T1
Calpain activity
C
DMSO
DMOG
UT-SCC38
Calpain activity
D
Before
After
Before
After
48h
+368min
48h
+400min
48h
+344min
48h
+416min
48h
+432min
48h
+256min
1
2
3
4
5
6
Elongated cell
(at 48 h after DMOG treatment
Elongation factor (log10)
10
1
**
Before
After
Switching
E
4T1
siCAPN2
siCtrl
#1
#2
#3
4h
Calpain2
β-actin
D: 1 2 1.6 1
24h
Calpain2
β-actin
D: 1 0.9 0.6 0.2
48h
Calpain2
β-actin
D: 1 0.4 0.3 0.1
F
4T1
DMSO DMOG
Calpain-2
β-actin
+ - + -
- + - +
siCtrl
siCAPN2-#2
Calpain-2 (norm.)
150
100
50
0
*
**
DMSO
DMOG
+ - + -
- + - +
siCtrl
siCAPN2-#2
G
UT-SCC38
DMSO DMOG
Calpain-2
β-actin
+ - - + - -
- + - - + -
- - + - - +
siCtrl
siCAPN2-#1
siCAPN2-#2
D: 1 0.9 0.9 1 0.8 1
Calpain-2
β-actin
+ - + -
- + - +
siCtrl
siCAPN2-#3
D: 1 0.3 1 0.4

Figure S3. Calpain activity detection and RNA interference in 3D tumoroid invasion culture. Related to Figure 3. A, Calpain-2 protein expression after 24, 48, 72h h (4T1) and 96 h (UT-SCC38) in 2D culture under the indicated conditions. Representative Western blots (top panel) and calpain-2 expression normalized to β-actin and normoxic/vehicle controls (lower panel) showing the mean+-SD (n=2-5) (unpaired t-test, two-sided). D, Densitometry. B,C, Calpain activity (cleaved CMAC intensity) of 4T1 tumoroids in 3D collagen after 72 h culture in normoxia and hypoxia (B) or in UT-SCC38 tumoroids 96 h after HIF-stabilization with DMOG (C). Representative multiphoton micrographs; insets, invasion zone. Scale bar, 100 µm (overview), 10 µm (insets). D) Micrographs of calpain activity in 4T1 cells after 48h of HIF stabilization with DMOG (1 mM). Cultures were incubated with the calpain sensor CMAC (20 µM) 1 hour before imaging. The time difference between images is indicated (left panel). Elongation factor (EF) of individual cells (arrowheads) at the 48-hour time point and after variable time intervals (right panel). Data points represent individual cells. Horizontal line, mean; 7 cells from 2 tumoroid regions. ** P=0.001 (paired t-test, two-sided). Scale bar, 10 µm. E, Calpain-2 protein expression in 4T1 cells after transfection with different siRNA targeting mouse calpain-2 (CAPN2). For molecular and functional studies in 4T1 cells, mouse siCAPN2-#2 was pursued further. D, Densitometry showing normalized calpain-2 levels normalized to β-actin and siCtrl. F, Calpain-2 expression in 4T1 cells 24h after HIF stabilization combined with transient transfection with siCAPN2-#2 compared to siCtrl. Representative Western blot (top panel) and relative change compared to siCtrl determined by densitometry (D) (lower panel). Data represent the mean+SD (n=3) (unpaired t-test, two-sided). G, Calpain-2 protein expression in UT-SCC38 cells 72-96h after HIF-stabilization and transfection with different siRNA targeting human calpain-2. Human siCAPN2-#3 (UT-SCC38) was pursued further in functional studies. D, Densitometry showing normalized calpain-2 levels normalized to β-actin and siCtrl. Abbreviations: N, normoxia; H, hypoxia; V, vehicle (DMSO); n/a, not applicable.

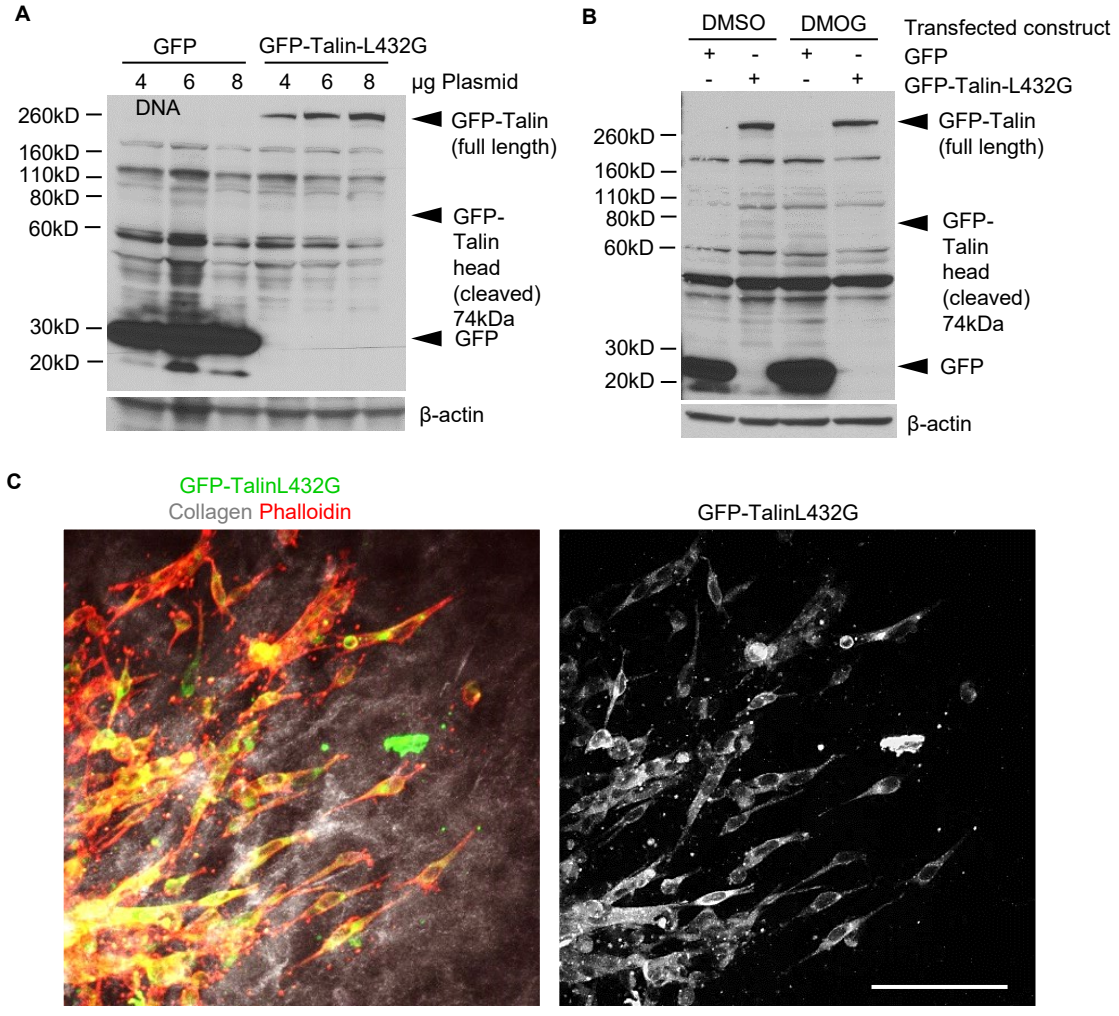

Figure S4. Biochemical and phenotypic analysis of tumor cells after expression of calpain-cleavage resistant talin. Related to Figure 4. A,B, Western blots of GFP expression in 4T1 cells after transient transfection with GFP and GFP-talin-L432G plasmid DNA at 72h in regular 2D culture (A) and after HIF-stabilization (B). GFP located at the N-terminal talin-L432G head region and, if cleaved by calpain-2, represents as GFP-talin head cleavage fragment at 74 kDa. C, Transfection efficiency and transient expression of calpain-uncleavable GFP-talinL432G. Representative multi-photon micrographs of 4T1 tumoroids in 3D collagen (SHG signal) 72h after transfection. Scale bar, 100 µm. Abbreviations: V, vehicle control (DMSO); D, DMOG; PD, PD150606.

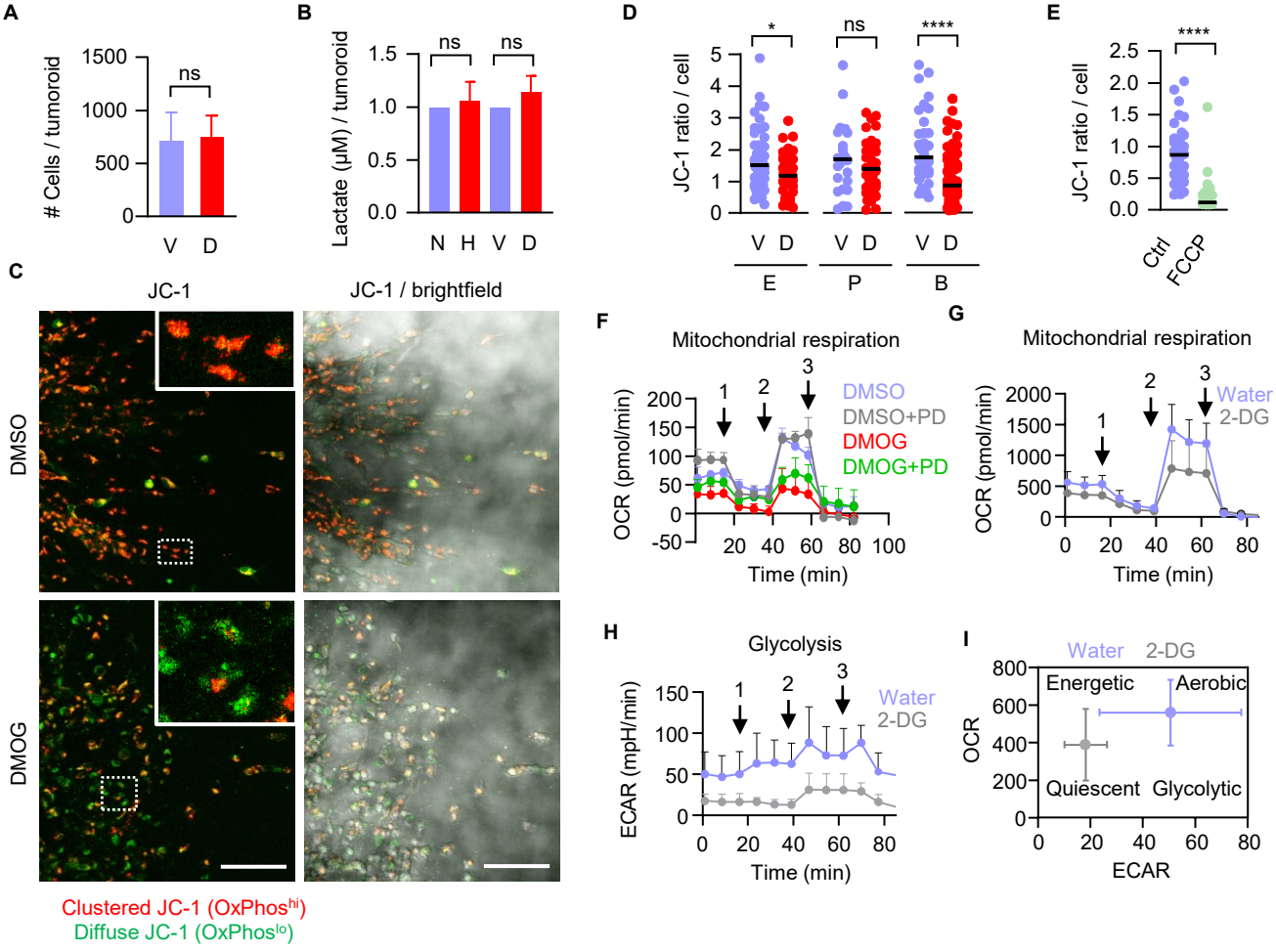
A
Cells / tumoroid
1500
1000
500
0
ns
V D
B
Lactate (µM) / tumoroid
1.5
1.0
0.5
0.0
ns
ns
N H V D
C
JC-1
JC-1 / brightfield
DMSO
DMOG
Clustered JC-1 (OxPhos^hi)
Diffuse JC-1 (OxPhos^lo)
D
JC-1 ratio / cell
5
4
3
2
1
0
*
ns
****
V D V D V D
E P B
E
JC-1 ratio / cell
2.5
2.0
1.5
1.0
0.5
0.0
****
Ctrl
FCCP
F
Mitochondrial respiration
OCR (pmol/min)
200
150
100
50
0
-50
1 2 3
DMSO
DMSO+PD
DMOG
DMOG+PD
20 40 60 80 100
Time (min)
G
Mitochondrial respiration
OCR (pmol/min)
2000
1500
1000
500
0
1 2 3
Water
2-DG
0 20 40 60 80
Time (min)
H
Glycolysis
ECAR (mpH/min)
150
100
50
0
1 2 3
Water
2-DG
0 20 40 60 80
Time (min)
I
Water 2-DG
OCR
800
600
400
200
0
Energetic
Aerobic
Quiescent
Glycolytic
0 20 40 60 80
ECAR

Figure S5. Standardization of cell number, lactate production and single-cell oxidative status in 3D tumoroid culture. Related to Figure 5. A, Cell number of individual 4T1 tumoroids isolated from 3D collagen droplets 48 h after DMSO or DMOG treatment used for Seahorse FX analysis. Data represent experimental means ± s.d. from 5-10 tumoroids per condition and experiment (n=3). ns, P=0.56 (Mann-Whitney test, two-sided). B, Average lactate levels in the supernatant of 4T1 tumoroids in collagen 48 h after HIF-stabilization. Data represent the experimental medians ± s.d. (n=3). Measurements from duplicate tumoroid-collagen droplets were averaged and normalized to the number of tumoroids. ns, P=0.99 (N, H) and P=0.31 (V, D) (Mann-Whitney test, two-sided). C, Representative images (maximum projections; interslice distance, 2 µm) of 4T1 tumoroids in 3D collagen 72h after HIF-stabilization and incubation with fluorescent probe JC-1 60 min prior to microscopy. Scale bars, 100µm. D, Quantification of JC-1 ratio in detached 4T1 single-cell subsets 72 h after HIF-stabilization with DMOG. JC-1 ratiometry was performed as described in Fig. 5G. **** P<0.0001, * P=0.01, ns P=0.18 (unpaired t-test, two-sided). E, Quantification of JC-1 ratio in detached 4T1 single-cell subsets 72 h after normoxic culture treated with FCCP (200nM) or solvent (Ctrl). Data underlie the distribution curve in Fig. 5H. **** P<0.0001 (Mann-Whitney t-test, two-sided). F, Time-dependent oxygen consumption rate (OCR) of 4T1 tumoroids 48 h after HIF-stabilization and 4h incubation with calpain inhibitor PD150606 (50 µM) or DMSO (vehicle control). Arrows, time of addition of (1) oligomycin (2 µM), (2) FCCP (1 µM) and (3) rotenone and antimycin A (2 µM each). Data represent the means ± s.d. with 5-10 tumoroids per condition from one representative experiment (n=2). G,H, Time-dependent oxygen consumption rate (OCR) (G) and extracellular acidification rate (ECAR) (h) of 4T1 tumoroids in 3D collagen invasion culture after HIF-stabilization combined with 2-DG (5 mM). 2DG treatment was initiated 48 h after HIF stabilization and maintained for further 15 h. Arrows and data from 3 independent experiments are represented as in (F). I, Energy map of 4T1 tumoroids showing altered glycolytic (ECAR) and mitochondrial (OCR) activity after glycolysis inhibition with 2DG (5 mM) compared to control cultures. Date were compiled from (G) and (H). Abbreviations: E, elongated; P pseudopodal-rounded; B, blebbing-rounded; N, normoxia; H, hypoxia; V, vehicle control (DMSO); D, DMOG.

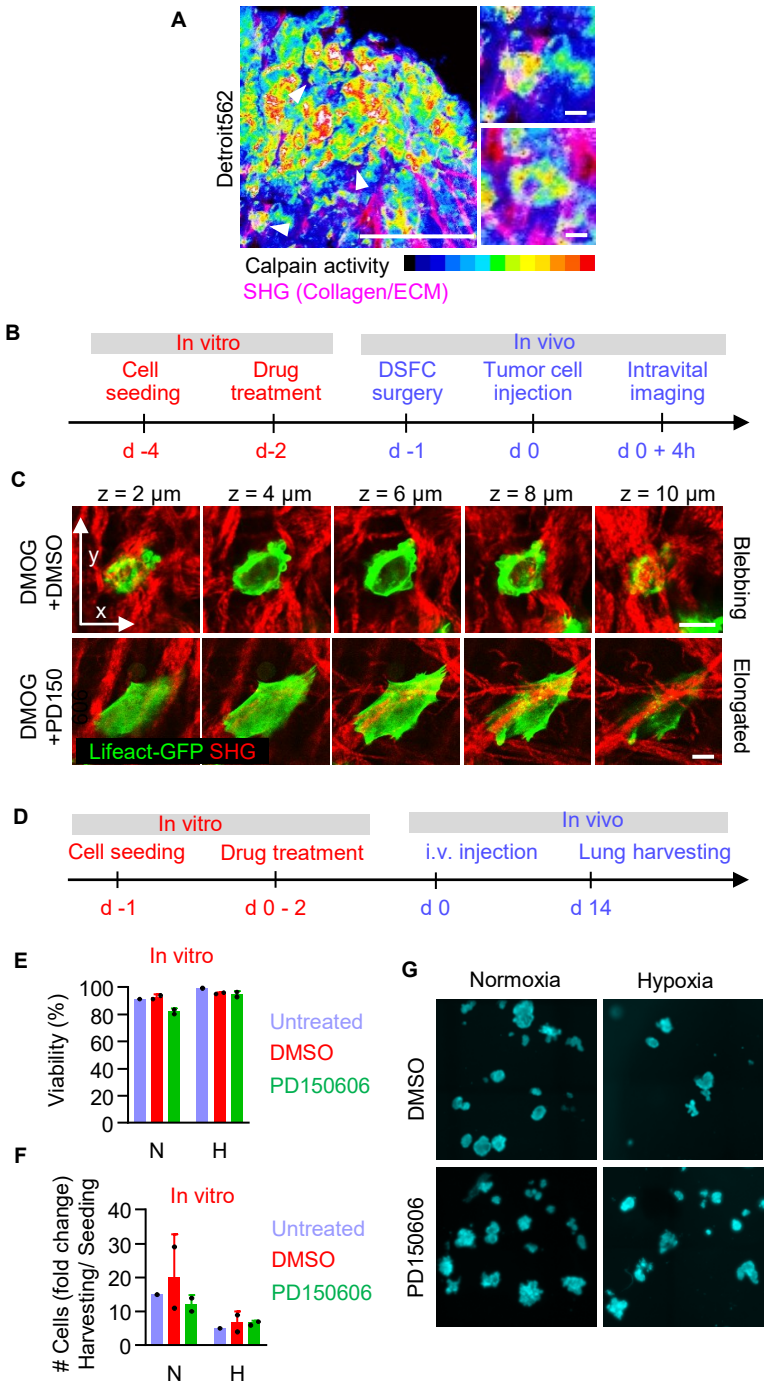
A
Detroit562
Calpain activity
SHG (Collagen/ECM)
B
In vitro
In vivo
Cell seeding
Drug treatment
DSFC surgery
Tumor cell injection
Intravital imaging
d -4
d-2
d -1
d 0
d 0 + 4h
C
z = 2 µm
z = 4 µm
z = 6 µm
z = 8 µm
z = 10 µm
DMOG +DMSO
DMOG +PD150
Blebbing
Elongated
Lifeact-GFP SHG
D
In vitro
In vivo
Cell seeding
Drug treatment
i.v. injection
Lung harvesting
d -1
d 0 - 2
d 0
d 14
E
In vitro
Viability (%)
Untreated
DMSO
PD150606
N
H
F
In vitro
Cells (fold change) Harvesting/ Seeding
Untreated
DMSO
PD150606
N
H
G
Normoxia
Hypoxia
DMSO
PD150606

Figure S6. Calpain activity and function in vivo. Related to Figure 6. A, Calpain activity (cleaved CMAC) in ex vivo orthotopic human Detroit562 HN-SCC tumor xenograft tissue. Insets and arrowheads, invading single cells with round morphology positive for cleaved CMAC. Scale bars, 100 µm (overview), 10 µm (insets). B, Workflow for tumor cell implantation in vivo, including pre-treatment in vitro, injection and imaging of HN-SCC migration morphologies in the mouse dermis for monitoring of migration morphologies. No calpain inhibitor treatment was applied to mice. C, High-resolution z-projection of UT-SCC38 single-cells in the mouse dermis detected by multiphoton microscopy. Collagen interactions of a round cell with blebbing protrusions observed after DMOG pretreatment (top) and an elongated cell with podal protrusions after pre-treatment with DMOG combined with calpain inhibition (PD150606, 100 µM) (bottom). Scale bars, 10 µm. B, Blebbing amoeboid; E, elongated, mesenchymal. D, Workflow for the experimental lung metastasis assay, including pre-treatment in vitro, injection and harvesting of lungs at day 14. E,F,G, Viability (E), cell proliferation rates (F) and colony formation (G) of 4T1 cells after pre-treatment in 2D monolayer culture in vitro prior to tail-vein injection (workflow described in D). Data are mean ± s.d., with points representing independent experiments. Abbreviations: N, normoxia; H, hypoxia.