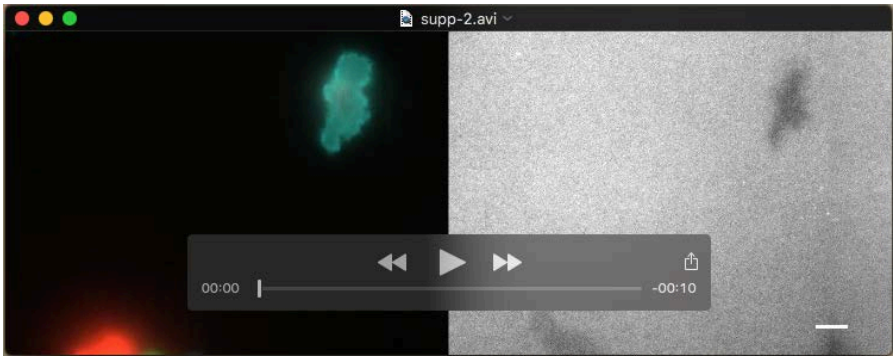
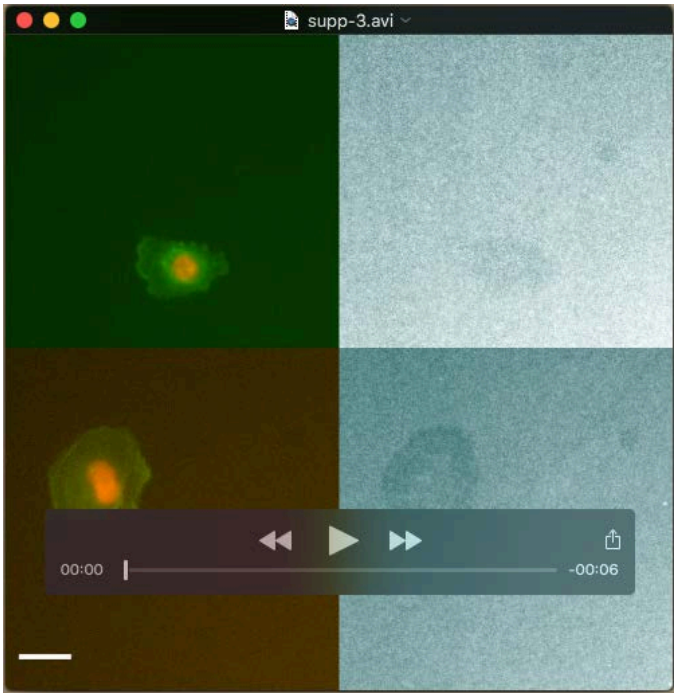
Supplementary Materials



Movie 1. Breast carcinoma cell in G1 degrades fluorescent gelatin.
MDA-MB-231-FUCCI-mCerulean3-Lifeact7 cell in G1 (red nucleus) forming invadopodia (cyan puncta) and degrading the underlying fluorescent gelatin (gray, right panel). Images were acquired every 10 minutes for 25 hours. Scale bar 10 μm.



Movie 2. Breast carcinoma cell in G1, S/G2/M on fluorescent ECM.
MDA-MB-231-FUCCI-mCerulean3-Lifeact7 cell in G1 (red nucleus) assembles invadopodia (green puncta) which degrade the underlying fluorescent gelatin (gray, right panel). Cell in S/G2 assembles invadopodia precursors or degrade ECM. Images were acquired every 10 minutes for 10 hours. Scale bar 20 μm.

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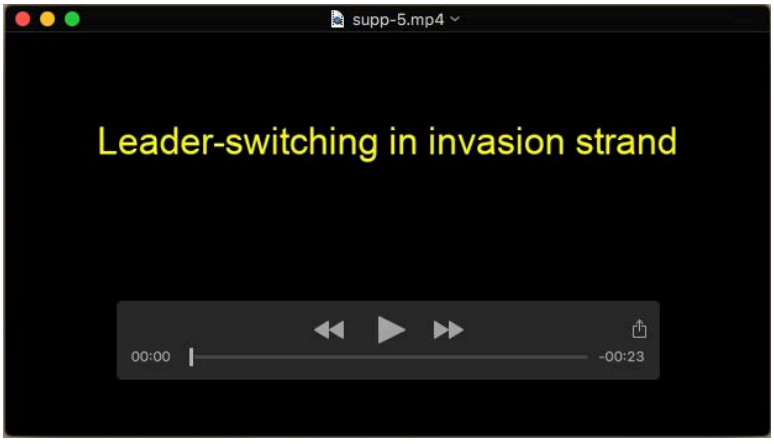
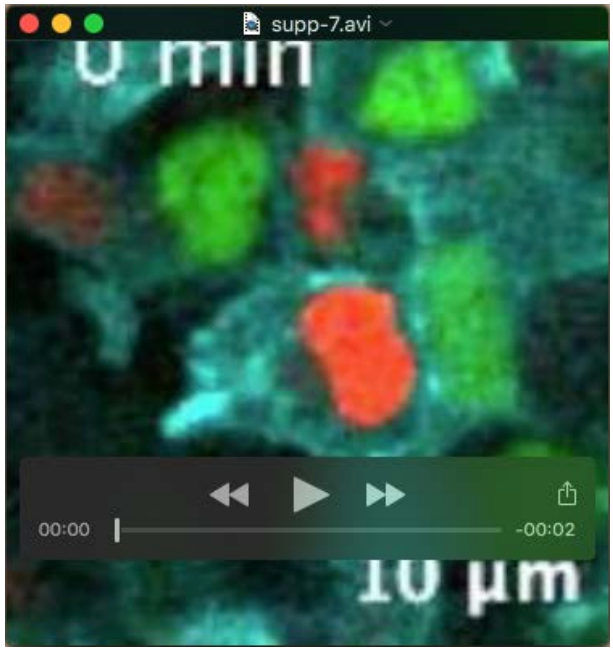
Movie 3. Cells in G1 initiate 3D spheroid invasion.
During 3D spheroid invasion of MDA-MB-231-FUCCI cells in collagen I, cells initiating strand formation are in G1 phase of the cell cycle. Images were acquired every hour over 72 hours. Scale bar 100 µm.

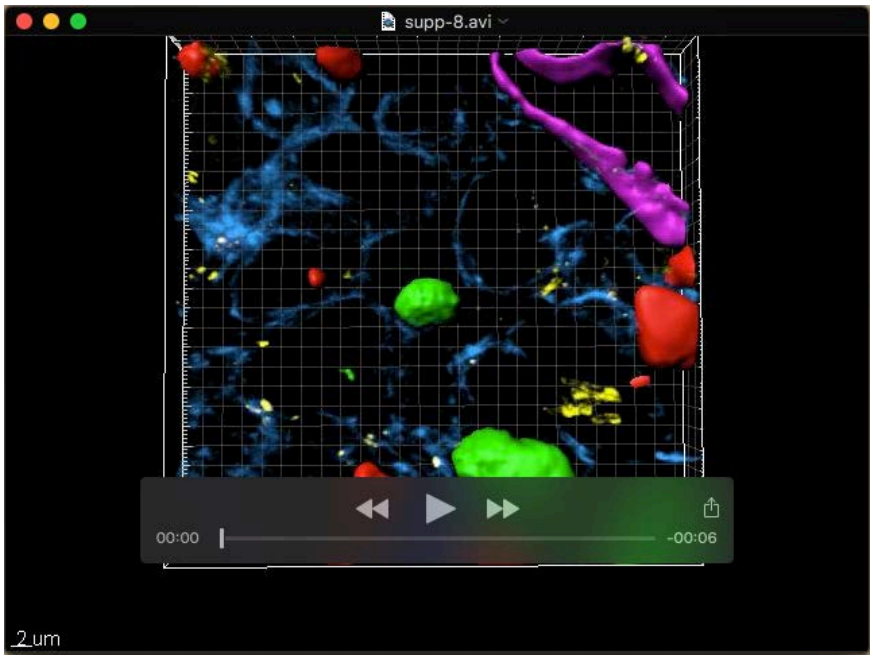


Movie 4. Leader-follower cell switching in 3D spheroid invasion.
3D spheroid invasion of MDA-MB-231-FUCCI cells in collagen I was imaged every hour over 43 hours. Individual red- and green-FUCCI, as well as overlay channels are shown. G1 cell initiates (white arrow) and leads (magenta arrow) the invasion strand; following transition to G2, new leader cells in G1 take over (magenta arrows). Scale bar 50 µm.

Movie 5. Breast carcinoma cell transitioning through G1, S and G2 on fluorescent ECM. MDA-MB-231-FUCCI-mCerulean3-Lifeact7 cell in G1 (red nucleus) forms invadopodia (cyan puncta) and degrades the underlying fluorescent gelatin (green for gelatin and black for degraded gelatin puncta, middle panel) only during G1. Right panel shows live plot of accumulating gelatin degradation. Images were acquired every hour for 50 hours. Scale bar 10 µm.



Movie 6. 4D intravital imaging of orthotopic xenograft tumor cell from Fig. 5A. MDA-MB-231-FUCCI-mCerulean3-Lifeact7 tumor cell in G1 (red nucleus) extends and retracts an actin-rich (cyan) protrusion. Images were acquired every 3 minutes for 30 minutes. Scale bar 10 µm.



Movie 7. Imaris 3D reconstruction of the cryosection in Fig. 5D (top panels).
3D reconstruction of an MDA-MB-231-FUCCI-mCerulean3-Lifeact7 tumor cryosection with isosurfaces for G1 nuclei (red), S/G2 nuclei (green) and blood vessel (magenta). Actin (cyan) and cleaved collagen (yellow) signals are colocalized in the G1 cell (see Fig. 5D, upper panels).

Table S1. List of primers used for gene expression qRT-PCR

Primer name	Sequence 5'->3'	Source
GAPDH F	CCCCTGGCCAAGGTCATCCA	(Fluegen et al. 2017)
GAPDH R	ACAGCCTTGGCAGCGCCAGT	
TKS5α F	TAATCAATGTGACCTGGTCTG	(Iizuka et al. 2016)
TKS5α R	TTGGGGTCCTTCTGGCCAC	
MT1-MMP F	TTGGACTGTCAGGAATGAGG	(Egawa et al. 2006)
MT1-MMP R	GCAGCACAAAATTCTCCGTG	
CTTN F	GGATGGATAAGAATGCGTCAAC	(A. Li et al. 2016)
CTTN R	GTTACTTGTTTTGCTGGTCACAG	
CCND1 F	GCTGCGAAGTGGAAACCATC	Primer Bank ID 77628152c1
CCND1 R	CCTCCTTCTGCACACATTTGAA	
CCNE1 F	GCCAGCCTTGGGACAATAATG	Primer Bank ID 339275820c2
CCNE1 R	CTTGCACGTTGAGTTTGGGT	
CCNA2 F	CGCTGGCGGTACTGAAGTC	Primer Bank ID 166197663c1
CCNA2 R	GAGGAACGGTGACATGCTCAT	
CCNB1 F	AATAAGGCGAAGATCAACATGGC	Primer Bank ID 356582356c1
CCNB1 R	TTTGTTACCAATGTCCCCAAGAG	

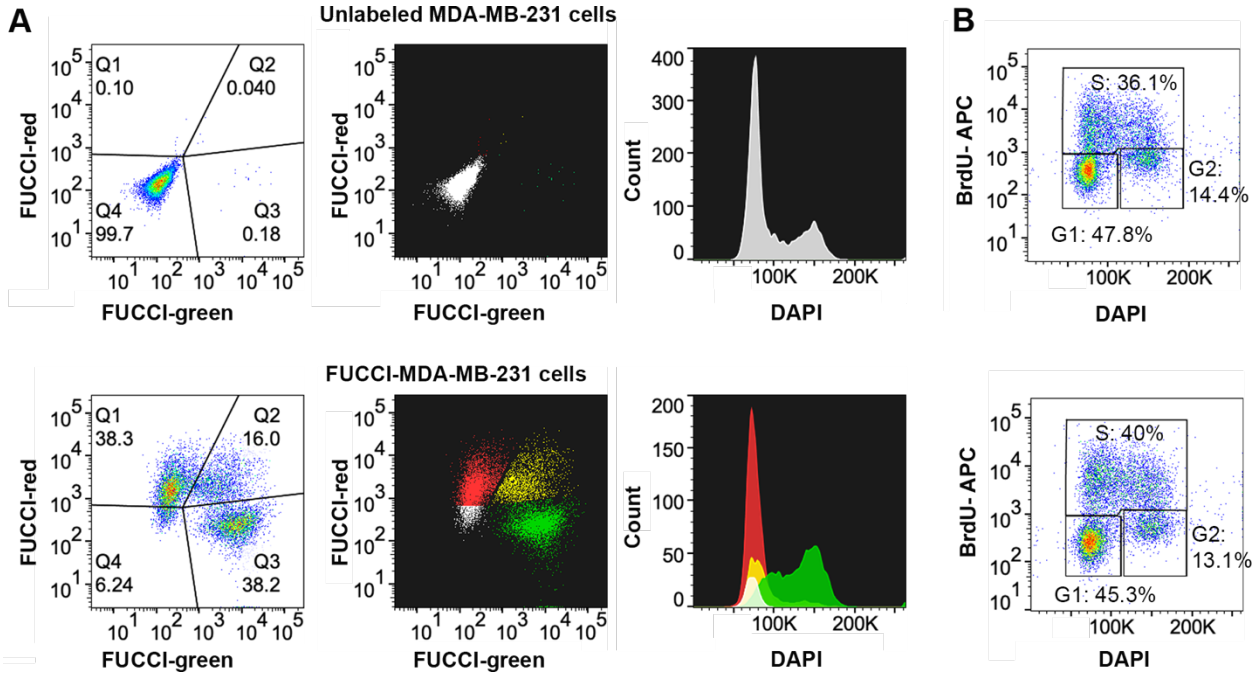


Figure S1. Cell cycle distribution analysis by FUCCI and flow cytometry

A. FUCCI/DAPI flow cytometry of unlabeled control and FUCCI-expressing MDA-MB-231 cells. The gates were selected based on the unlabeled control cells and FUCCI-red and FUCCI-green single-color control cells. The middle panels show the color-coded populations of the FUCCI plot (left). The right panels show the color-coded populations on a DAPI histogram stained for DNA content.

B. BrdU incorporation and DAPI staining measured by flow cytometry for unstained control and FUCCI-Tks5-WT cells. The gates indicate the proportion of cells in each phase.

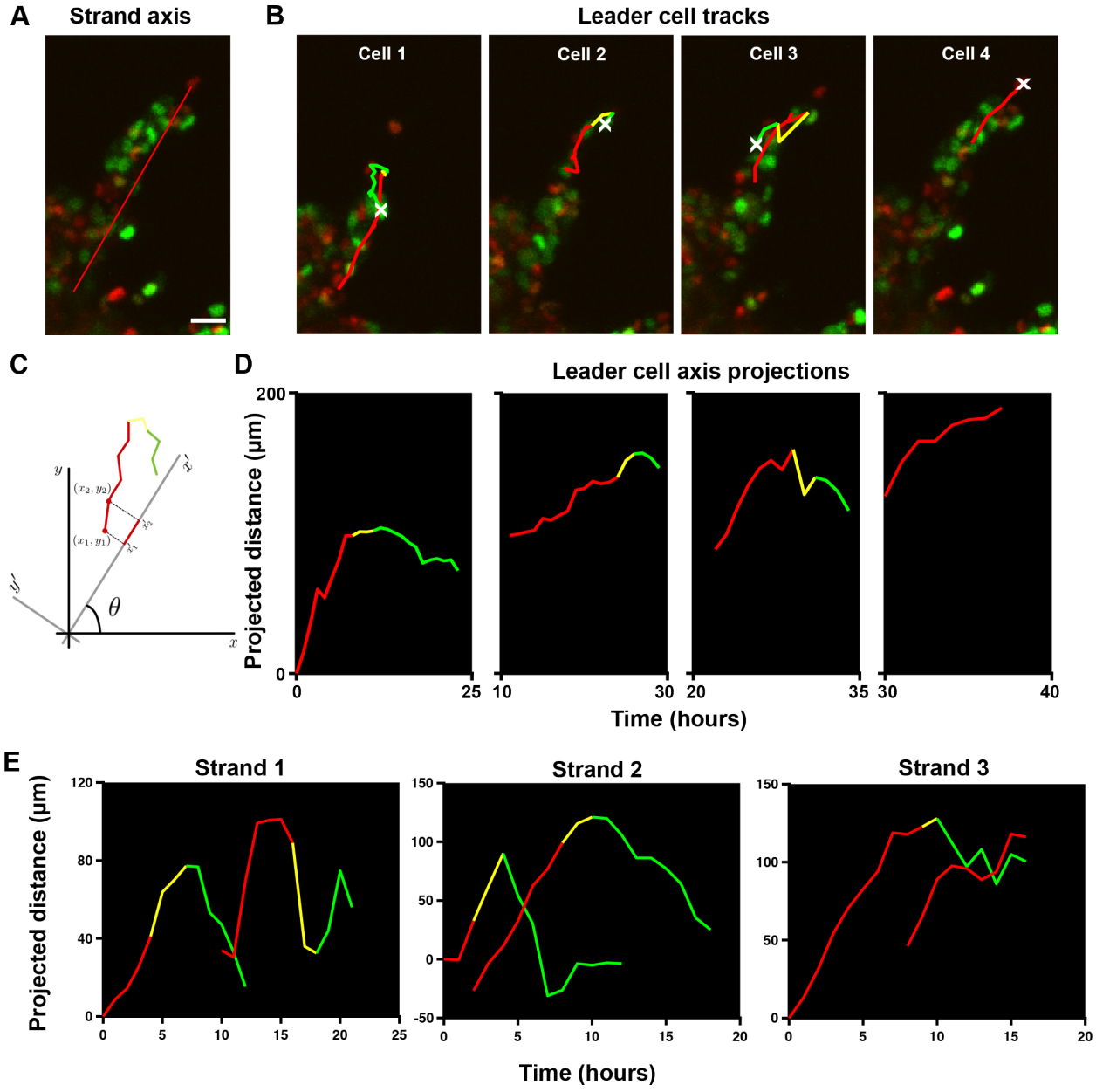


Figure S2. Tracking and analysis of spheroid leader cells

A. Representative image of one invasion strand from the 3D spheroid expressing FUCCI reporters (red- and green- nuclei). The strand axis (red line) is defined by the initial position of the first leader cell and the final position of the last leader cell.
B. Individual tracks of four consecutive leader cells from a given strand. X denotes the end of the track, and the tracks are color-coded corresponding to the cell cycle phase.
C. Schematic of the projection of an individual track onto the strand axis. Unprimed (x, y) and primed (x', y') coordinate systems refer to the image and the strand reference frames, respectively. θ denotes the angle a given strand makes with the horizontal image axis (x-axis).
D. Leader cell tracks presented in Fig. S2B, projected onto the strand axis (Fig. S2A). (see also see Fig. 1G of the main text).

Representative tracks of “leader cell switching” from three separate strands.

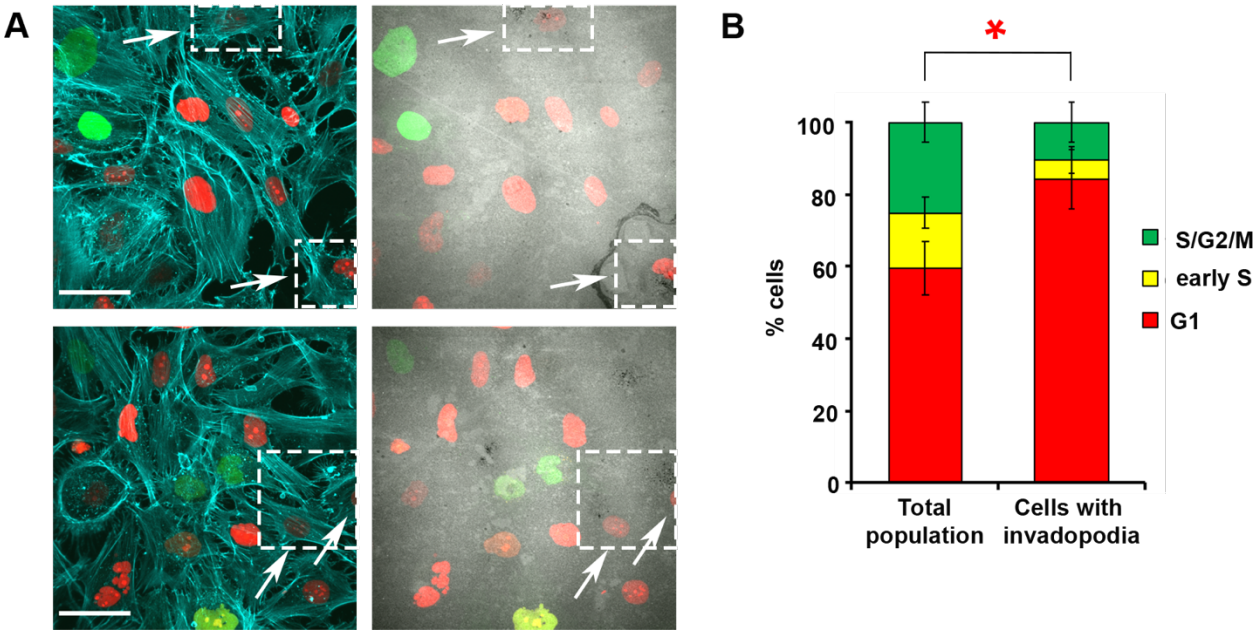


Figure S3. ECM degradation by invadopodia is enriched in the G1 phase of the cell cycle in BT549 cells

A. Representative images of BT549 cells expressing FUCCI reporters (red- and green- nuclei), stained with phalloidin for F-actin (cyan) and plated on fluorescent gelatin (gray). White dashed boxes outline degrading cells. White arrows point to G1 cells degrading the underlying gelatin. Scale bar 50μm.
B. Quantification of the cell cycle distribution in total cells and cells with mature invadopodia. The red asterisk refers to the statistical significance between the G1 populations. Averages and SEMs are shown based on >100 cells from three biological repeats. Statistical significance was calculated using Mann-Whitney U test. *p<0.05

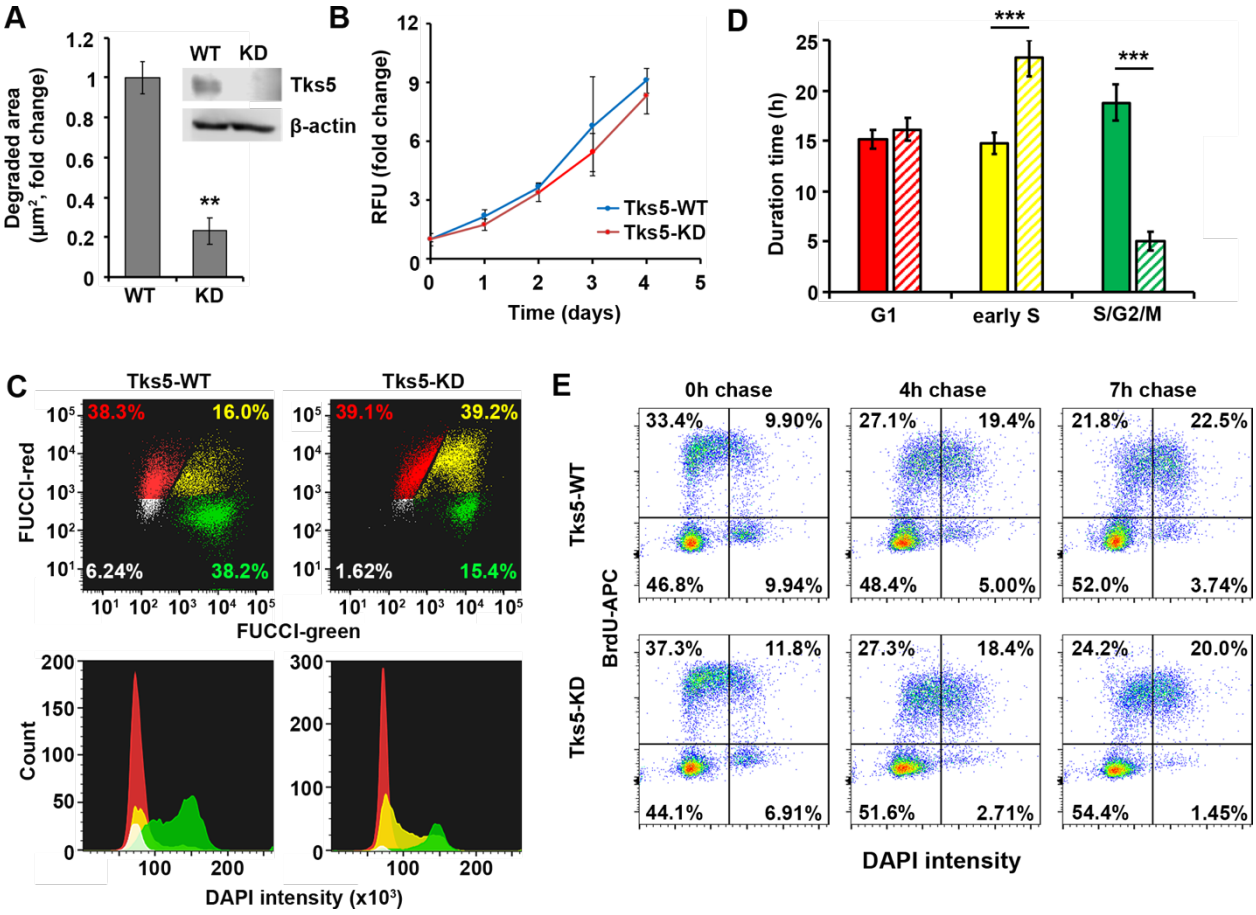


Figure S4. Elimination of invadopodia affects cell cycle progression

A. Western blot analysis shows the efficiency of the knockdown in MDA-MB-231-Tks5-shRNA (Tks5-KD) stable cell line; β-actin was used as a loading control. Bar graph below shows quantification of the degraded area per Tks5-KD cell represented as fold change relative to Tks5-WT control. Averages of degraded area per FOV and SEMs are shown, which include measurements in >100 cells, N_{FOV}=20. Statistical significance was calculated by Student's *t*-test: $^{**}p<0.01$.

B. Proliferation assay of Tks5-WT and Tks5-KD cells. The fluorescence signal of the reduced form of resazurin was measured from Tks5-WT and Tsk5-KD cells over 5 days. The results are represented as relative fluorescence units (RFU) relative to day 0. Average values of three replicates and STDs were shown; each of the timepoints were compared in pairs and showed no statistical difference.

C. FUCCI flow cytometry measurements of Tks5-WT and Tks5-KD cells. Identical gates were applied to both cell populations and the proportions of each quadrant are indicated in the corresponding colors. The cell populations from the FUCCI plot are superimposed onto the DAPI histogram showing DNA content.

D. Live imaging measurements of the duration of cell cycle phases, as defined by the FUCCI reporters (red-G1, yellow-early S, green- late S/G2/M). Solid bars indicate Tks5-WT cells, the striped bars indicate Tks5-KD cells. For each phase, >100 cells

were analyzed, in three biological replicates. Average values and SEMs are shown; statistical significance was calculated by Student's *t*-test: ***$p<0.001$.

E. BrdU pulse chase assay of Tks5-WT control and Tks5-KD. Cells were pulsed with BrdU for one hour, after which BrdU was removed and cells harvested at 0h, 4h and 7h of chase. The samples were stained with anti-BrdU antibody and DAPI for DNA content and analyzed with the flow cytometer. The gates indicate the percentage of cells in each phase of the cell cycle.

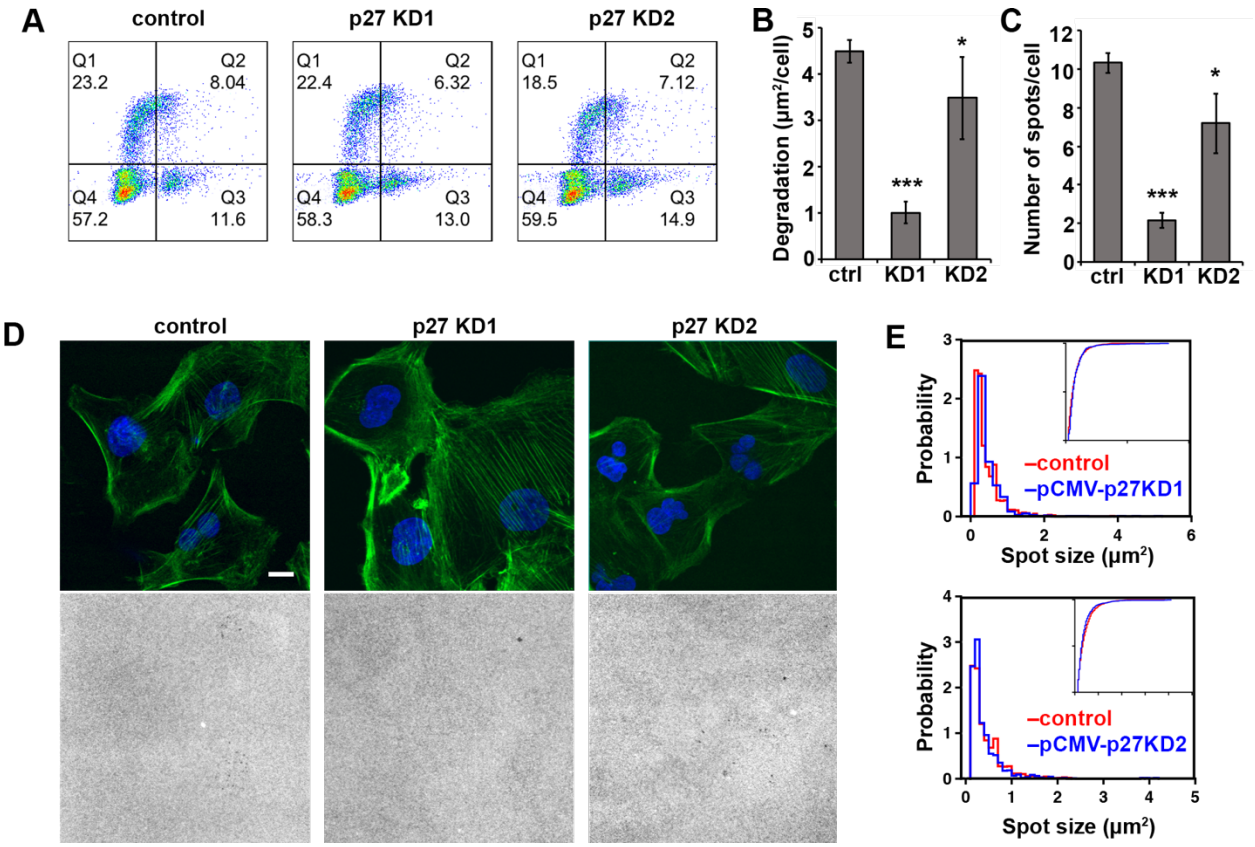


Figure S5. Depletion of p27^{kip1} reduces invadopodia-mediated degradation

A. BrdU-DAPI plots of cell cycle phases in control and two independent p27^{kip1} stable knockdown cell lines (p27KD1, p27KD2).
B. Quantification of the degraded area per cell in control, p27KD1 and p27KD2 cells. Statistical significance between control and each KD was calculated using Mann-Whitney U test. ***p=1.4x10^{-4}, *p=0.032
C. Quantification of the number of degradation spots per cell in control, p27KD1 and p27KD2 cells. Statistical significance between control and each KD was calculated using Mann-Whitney U test. ***p=1x10^{-4}, *p=0.017
D. Representative images of control, p27KD1 and p27KD2 cells plated on fluorescent gelatin (gray). Cell nuclei are marked with DAPI (blue), actin with phalloidin (green). Scale bar 10µm.
E. Probability Distribution Functions (PDFs) and Cumulative Distribution Functions (CDFs, insets) of degradation spot size values for control (red), p27KD1 (blue, upper panel), and p27KD2 (blue, lower panel). Statistical significance between each pair of distributions was calculated using the Kolmogorov-Smirnov test. For control vs p27KD1, p=2x10^{-6}. For control vs p27KD2, p=2.9x10^{-3}.