

Supplementary Information for
Adrenomedullin 2 drives intratumoral cancer cell invasion, intravasation and metastasis.

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Supplementary Methods:

Image acquisition and analysis. A Nikon A1r upright microscope (Nikon) fitted with a temperature regulated enclosure and a range of Nikon microscope objectives (10, 25x (WI)) was used for multicolour imaging of cancer cells and chicken embryo CAM vasculature. Real-time imaging of cancer cell intratumoral invasion or invasion within the metastatic lesions was performed by acquiring 4-dimensional image series of single cancer cells within the CAM tissue. For the in vivo cancer cell invasion imaging a 300 μm image stack was acquired every 20 minutes in 10 μm step size increments for 10-16 hours (10x objective). Image drift was corrected using the ImageJ StackReg plugin (Biomedical Imaging Group, <http://bigwww.epfl.ch/>) prior to tracking analysis. Time 0 was defined as the time of the first image capture. Individual cells were tracked using a built in Volocity Object tracking module. Track velocity was calculated as an average speed of the track. For in vitro time lapse imaging of cell migration, a 300 μm image stack was acquired every 30 minutes in 10 μm step size increments for 10-16 hours (10x objective). For imaging and quantification of the blood vessel diameter contacting cells, 25x z-stack images were acquired (2-5 μm step) and blood vessel diameter or cancer cell-blood vessel contact length (maximum span) was measured using Nikon Elements software built in length measurement module. For quantification of tumour cell intravasation, individual primary tumour z-slices (25x, 1-2 μm step) were analyzed using Nikon Elements software and intravascular tumour cell volume was measured. The intravascular tumor volume was normalized to the total tumor sample volume. Individual, undersaturated 25x optical slices from independent tumours (one slice/tumour) were used to quantify the intensity of ATF2 and 7TCF reporters. Separate channel images were converted into 32-bit grayscale images and average signal intensities were measured using build in ImageJ module. Cells were defined as ATF2^{high} if ATF2/7TCF reporter

ratio was ≥ 3.5 . Cells were defined as 7TCF^{high} if ATF2/7TCF reporter ratio was < 3.5 . MDA-MB-231 breast cancer cells were stained with CellTracker™ Green (C2925, Invitrogen) for intravital imaging experiments according to the manufacturer's protocol.

In vivo detection of vascular leak. On day 6 post-tumour inoculation (day 16 of development), chicken embryos were injected with 50 μ g of fluorescein isothiocyanate (FITC)-dextran (2 MDa; Ex/Em 494/521 nm; Invitrogen, D7137). Two hours post-injection, 10 $\times$ z-stacks (5 $\times$ 5 stitching, 100 μ m total thickness, 10 μ m step size) were acquired. The 7TCF (mCherry) fluorescent reporter signal was used to define the boundaries of the primary tumour. The FITC signal was acquired under non-saturating conditions to enable quantification; consequently, the GFP (ATF2 reporter) signal was not detectable. FITC channel images were converted to 32-bit, and vascular leak was quantified as FITC intensity measured at random locations at the tumour periphery (< 50 μ m), normalized to background signal from tumour-free regions.

ELISA analysis of ADM2 expression. ADM2 protein expression was quantified using a BIOMATIK Human ADM2 ELISA kit (#ELK55270) according to the manufacturer's instructions. Briefly, adherent cells were washed twice with ice-cold PBS and lysed in RIPA buffer (Pierce #89901) supplemented with protease inhibitors (Pierce #1861279) for 30 min on ice with periodic mixing. Cell lysates were clarified by centrifugation at 12,000 $\times$ g for 15 min at 4°C, and the supernatants were collected for analysis. Total protein concentration was determined using a Pierce BCA Protein Assay Kit (Pierce ## 23223 and 23224), and equal amounts of protein were loaded into ELISA wells. ADM2 concentrations were measured using the BIOMATIK ELISA kit following the manufacturer's protocol, including incubation with standards and samples, sequential addition of detection reagents, TMB substrate development, and reaction termination with stop solution. Absorbance was measured at 450 nm using a

microplate reader, and ADM2 levels were calculated from a standard curve and normalized to total protein content.

Measuring the cancer cell extravasation. 25-50x10³ cancer cells were injected intravenously into the chicken CAM artery and allowed to extravasate for 4 hours (HT1080) or 6 hours (MDA-MB-231). 15 min before the assay CAM vasculature was visualized via injection of Lectin-649 and cancer cell extravasation was scored using intravital confocal imaging (25x objective). Random 25x imaging fields (60 µm thick; 2-3 µm step; minimum 4 fields per animal) were acquired, and individual z-stacks were analyzed to quantify the percentage of the cancer cells that extravasated out of the CAM vasculature.

Histology. Lungs and tumors were harvested from mice and fixed for 16 hours in 10% neutral buffered formalin phosphate (Fisher Scientific, Cat No. 23-245684). Tissues were paraffin-embedded and 4-6µm sections were generated. Sections were dewaxed in xylene and rehydrated using graded ethanol to water washes. Heat-induced antigen retrieval was conducted by immersing rehydrated slides in 10mM sodium citrate (pH 6) and heating until boiling occurred. Slides were blocked in 10% normal goat serum (Sigma, 869019-M) with 1% bovine serum albumin (Sigma, A9418) in TBS with 0.4% Triton X-100 for one hour at ambient temperature. For immunohistochemistry, anti-CD31 (Abcam, ab124432, 1:500) was added overnight at 4°C. Endogenous peroxidase was blocked with 3% H₂O₂ for 10 minutes and anti-rabbit-HRP secondary antibody (Dako, P044801-2) was added for 1 hour at room temperature. Color was developed by adding liquid DAB and substrate (Dako, K3468) to sections for 60 seconds. Sections were counterstained with Gill hematoxylin No. 2 (Sigma-Aldrich, GHS232) and blued with 0.02% ammonium hydroxide (Fisher, A669-500) for 10 seconds. Sections were dehydrated, cleared, and then mounted. For immunofluorescence, anti-GFP (Aves, GFP-1020, 1:100) and

anti-mCherry (Rockland, 600-401-P16, 1:500) antibodies were added after blocking overnight at 4°C. Alexa Fluor 546 conjugated goat anti-chicken (Thermo Scientific, A-11040, 1:200) and Alexa Fluor 647 conjugated goat anti-rabbit (Thermo Scientific, A-21246, 1:200) secondary antibodies were added for 1 hour at room temperature in the dark. Samples were mounted with ProLong Gold containing DAPI (Thermo Scientific, P36931). Whole slide scans were generated using Zeiss Axioscan 7 and image snapshots were exported using Zeiss Zen 3.8. Image quantification was done using ImageJ.

qPCR gene expression analysis. Total RNA was isolated from the cultured cells using RNeasy Mini Kit Qiagen (cat. Nos. 74104 and 74106), and then subjected to cDNA synthesis with the PCR BIOSYSTEMS kit. qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) (cat.#1725274). Amplifications were performed following the procedure of a two-step method (95°C for 2:00 min, 1 cycle; followed by 39 cycles of 95°C for 5 sec and 60°C for 10 sec, 65°C for 5 sec). The fold change in mRNA expression levels was calculated using the $2^{-\Delta\Delta C_q}$ method and normalized to Beta actin. Primer sequences were: ATF2 forward primer, 5'-TGG TAG CGG ATT GGT TAG G-3'; ATF2 reverse primer, 5'-TTG GGT CTG TGG AGT TGT G-3'. ADM2 forward primer: 5' CTG AGC CCC ATC TGA AGC C'3; ADM2 reverse primer: 5' CAG CAC TGC GTG TAG ACC AG 3'.

Generation of shear flow using syringe pumps. Cells were seeded in the microfluidic devices and allowed to enter the channels as previously described¹⁹. Shear flow was applied with a syringe pump according to an established protocol. In brief, microbore tubing (Cole-Parmer) was connected to the outlet wells, which drained to separate 50 ml waste collection tubes, enabling verification of equal flow. To generate shear stress, two 50 ml luer-lock syringe (BD) full of media were mounted on two syringe pumps (Harvard Apparatus) and connected via a blunt-tip

needle to microbore tubing. The pump volumetric flow rate adjusted to generate shear stresses of 0.3 dyn/cm^2 (low) or 3 dyn/cm^2 (high) calculated from previously established equations. The volumetric flow rate was verified by measuring the volume in the waste collection tubes at the end of the experiment. Time-lapse imaging was performed using a Nikon A1 confocal microscope equipped with a Plan Apo 10x objective. Cells were imaged with 488 nm and 565 nm laser lines and imaged every 20 minutes for 2-5 hours. Cells were manually tracked using the Manual Tracking plugin in ImageJ (National Institutes of Health, Bethesda, MD). The resulting data were analyzed in MATLAB (v. R2025b; MathWorks, Natick, MA) to compute the average cell velocity.

Supplementary Figures:

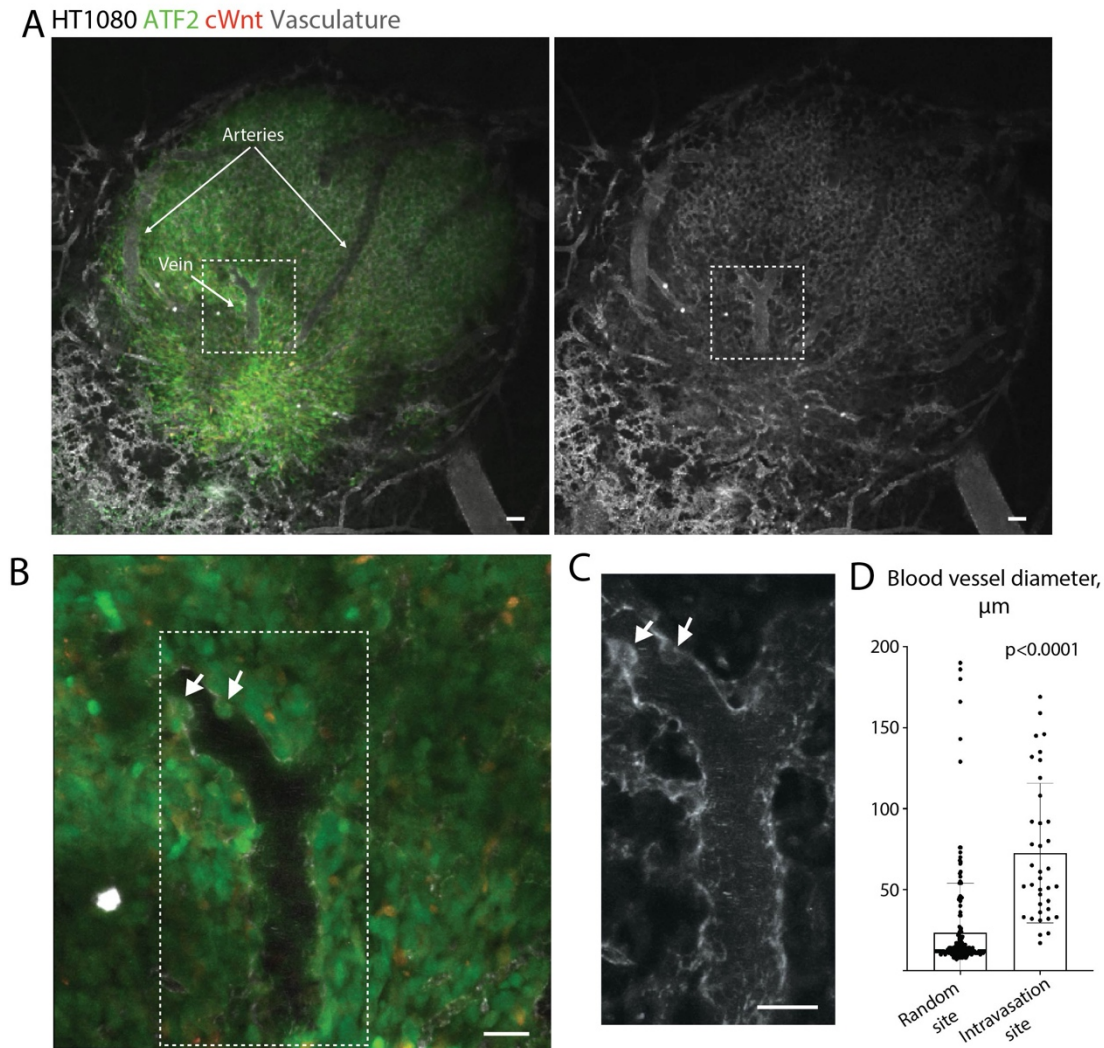


Fig. S1. Cancer cell intravasation preferentially occurs in the large tumor draining veins. (A) The representative image (10x) displays the primary tumor formed by HT1080 DR cells, highlighting a large tumor-draining vein where intraluminal cancer cells can be observed (dashed square). The left panel includes an overlay of the GFP (ATF2^{high}), mCherry (7TCF^{high}), and Lectin 649 (vasculature) channels. The right panel presents only the Lectin 649 (vasculature) channel. (B) A high magnification image (25x) of the area outlined by the dashed square in panel (A), with white arrows indicating intraluminal ATF2^{high} cells. (C) A high magnification image (digital zoom) of the area designated by the dashed rectangle in panel B, showing only the Lectin 649 (vasculature) channel. (D) A plot quantifying the diameter of blood vessels where cancer cell intravasation was observed. The number of animals used for analysis was 13 (D). Statistical comparisons were made using Mann-Whitney test. Scale bars = 50 μm .

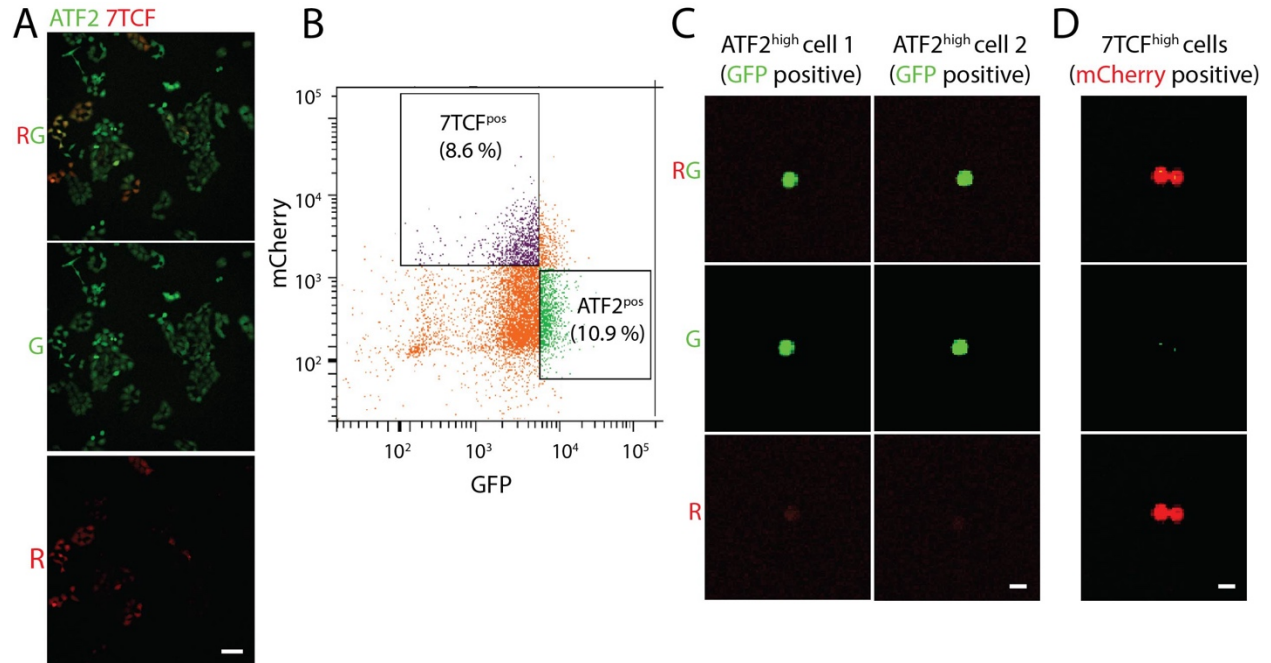


Fig. S2. Isolation of ATF2^{high} and 7TCF^{high} cancer cell populations. (A) Representative images of the cultured HT1080 DR cell line. The upper panel shows the overlay of the ATF2 (GFP) and 7TCF (mCherry) reporter channels, while the lower panels display the ATF2 and 7TCF reporter channels separately. (B) A representative FACS scatter dot plot illustrating the sorting settings used for the isolation of HT1080 DR ATF2^{high} and 7TCF^{high} cell populations. (C to D) Representative images of ATF2^{high} (C) and 7TCF^{high} (D) cancer cells immediately following FACS sorting. Scale bars = 100 μ m (A) and 50 μ m (C, D).

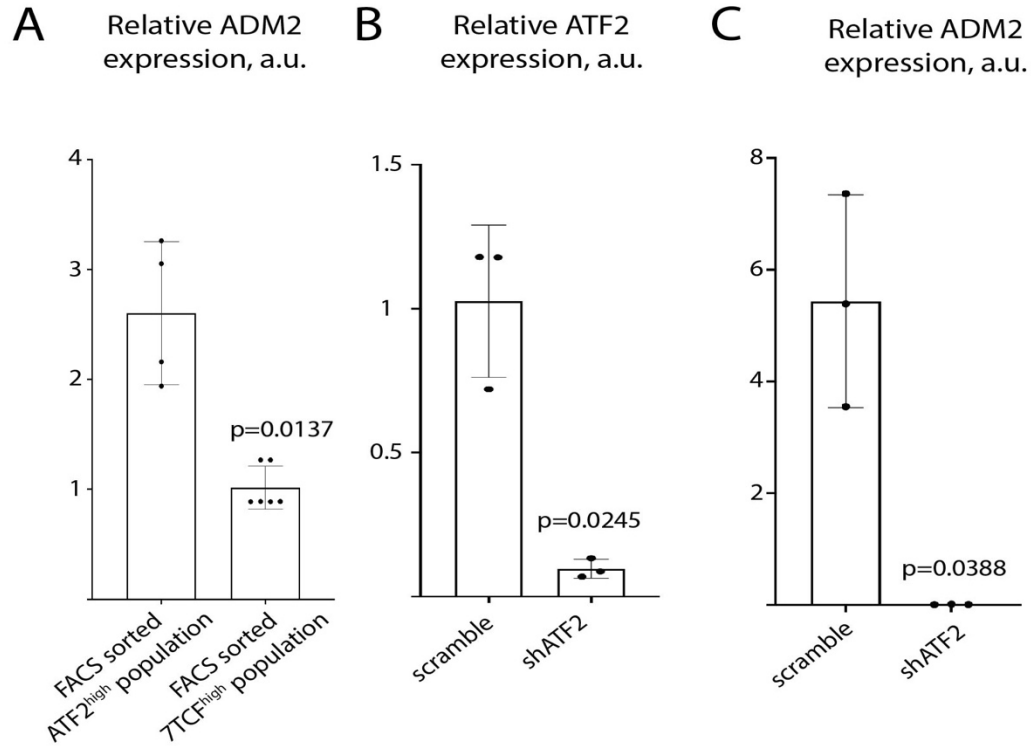


Fig. S3. Analysis of ADM2 and ATF2 gene expression. (A) qPCR analysis of ADM2 expression in FACS-sorted HT1080 DR cell populations with high ATF2 (ATF2^{high}) and high 7TCF (7TCF^{high}) reporter activities. (B) qPCR analysis of ATF2 expression in HT1080 DR cancer cells engineered to express either scrambled shRNA or shRNA that specifically targets ATF2 expression. (C) qPCR analysis of ADM2 expression in HT1080 DR cancer cells engineered to express either scrambled shRNA or shRNA that specifically targets ATF2 expression. Statistical comparisons were made using Welch's t-test test.

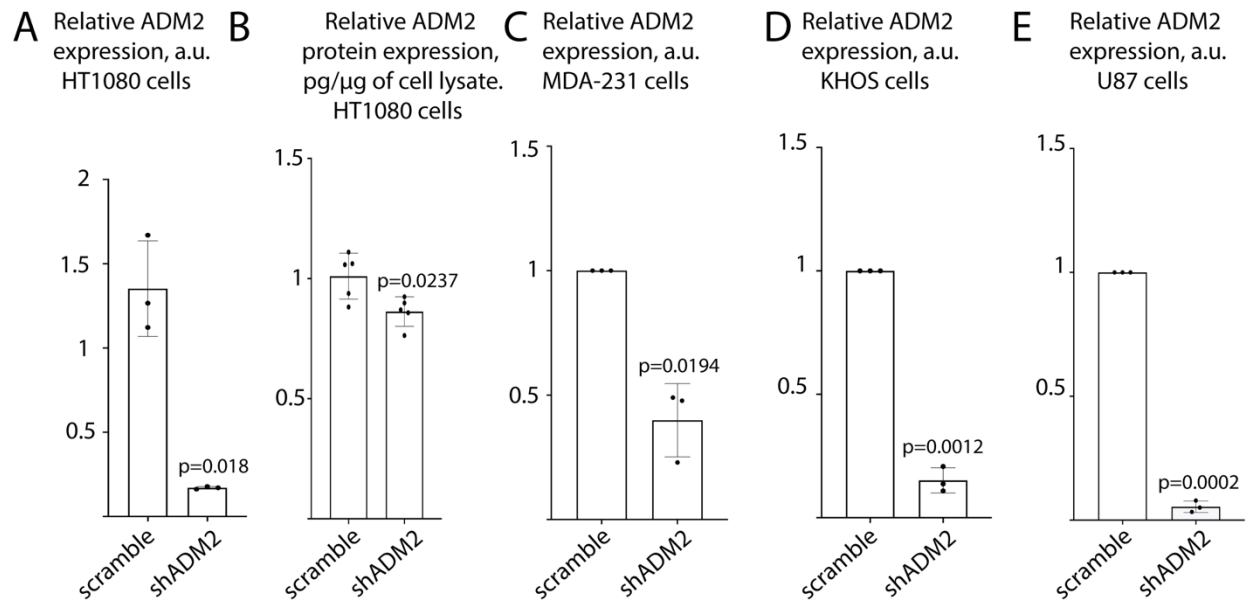


Fig. S4. Generation of cancer cells deficient in ADM2 expression. (A) qPCR analysis of ADM2 expression in HT1080 DR cancer cells engineered to express either scrambled shRNA or shRNA specifically targeting ADM2 expression. (B) ELISA analysis of ADM2 protein expression in HT1080 DR cancer cells engineered to express either scrambled shRNA or shRNA specifically targeting ADM2 expression. (C to E) qPCR analysis of ADM2 expression in MDA-231 (C), KHOS (D), and U87 (E) cancer cells engineered to express either scrambled shRNA or shRNA specifically targeting ADM2 expression. Statistical comparisons were made using Welch's t-test.

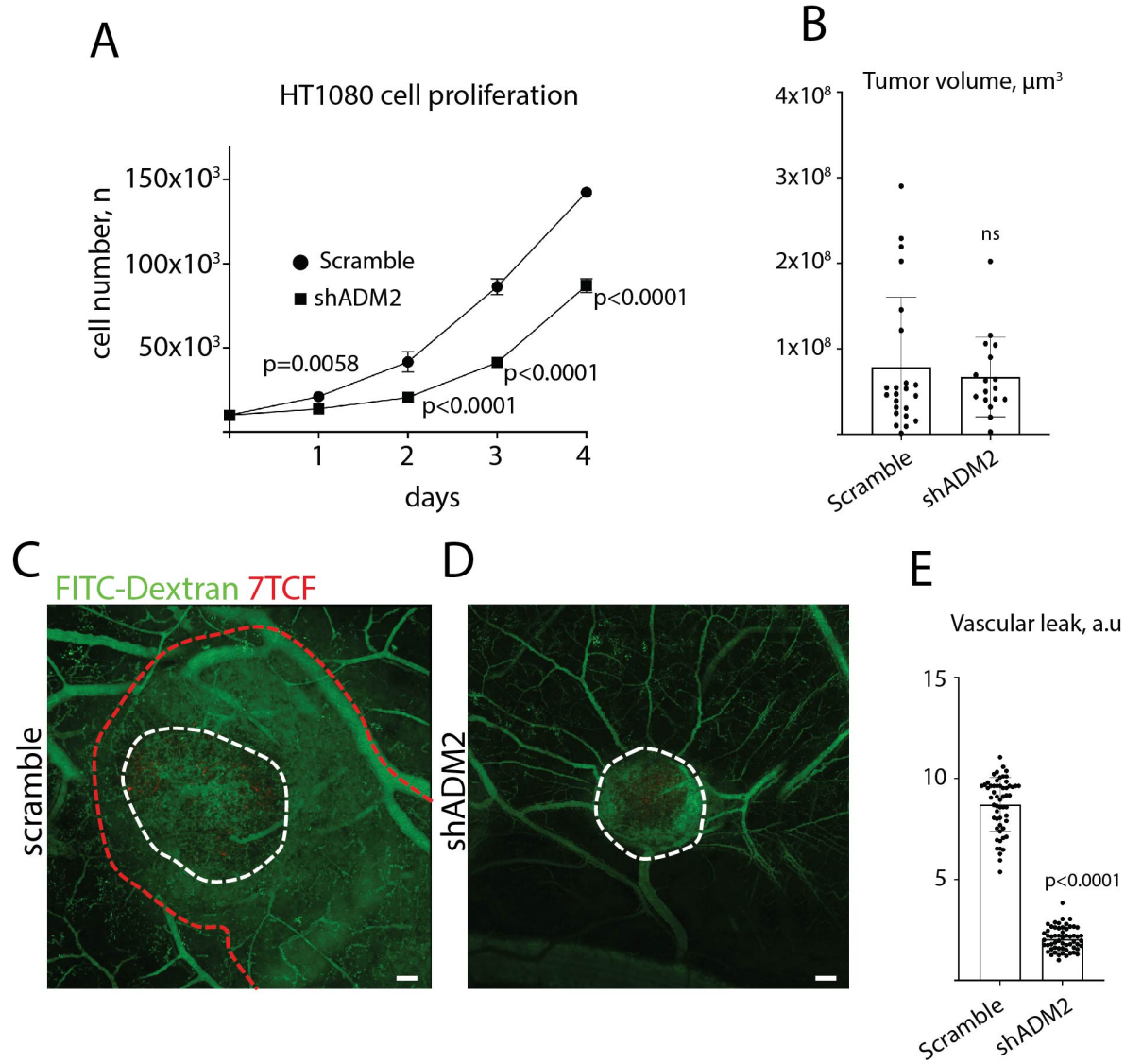


Fig. S5. ADM2 gene expression knockdown decreases primary tumour vascular leak in the chicken CAM model. (A) Analysis of cancer cell proliferation. (B) Quantification of primary tumour volume for tumours formed by HT1080-DR cancer cells engineered to express either scrambled shRNA or shRNA specifically targeting ADM2 expression. (C-D) Representative images showing vascular leak (FITC-dextran, 2×10^6 MW) in the vicinity of primary tumours formed by HT1080-DR cancer cells engineered to express either scrambled shRNA (C) or shRNA specifically targeting ADM2 expression (D), imaged at $10\times$ magnification. White dashed lines delineate the borders of the primary tumours. Red dashed line delineates area of vascular leak. (E) Quantification of average vascular leak for tumours formed by the cell lines shown in (C, D). Statistical comparisons were performed using 2-way ANOVA (A) or the Mann-Whitney test (B, E). The number of animals used for the analyses was 23 and 17 for (B), and 5 and 5 for (E). Scale bar = $200 \mu\text{m}$ (C, D).

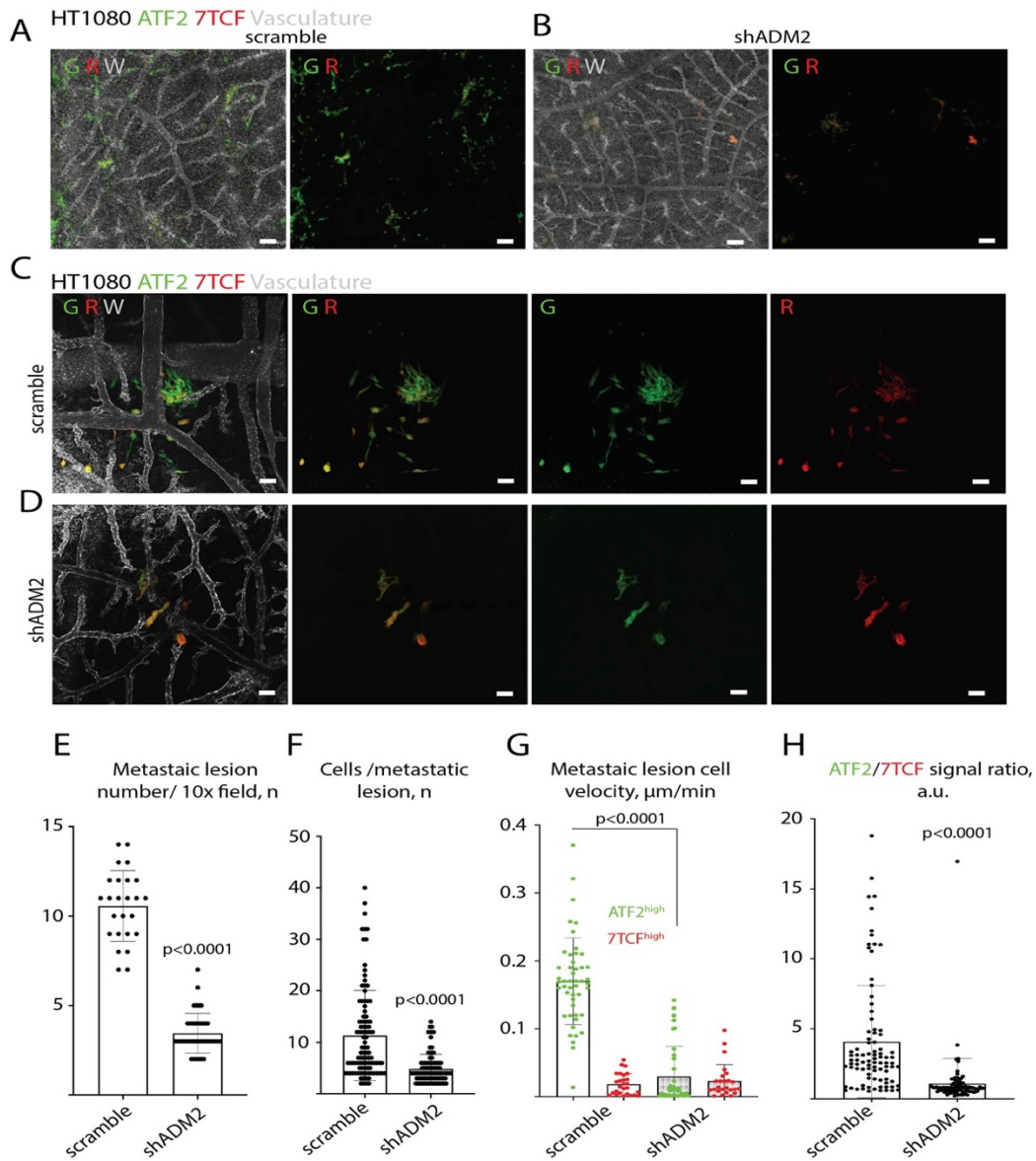


Fig. S6. ADM2 gene expression is necessary for the invasive metastatic lesion formation. (A to B) Representative images showing metastatic lesions (chick CAM, 5 days post injection) formed by HT1080 DR cancer cells engineered to express either scrambled shRNA (A) or shRNA that specifically targets ADM2 expression (B), 10x magnification. (C to D) Representative images showing metastatic lesions formed by HT1080 DR cancer cells engineered to express either scrambled shRNA (C) or shRNA that specifically targets ADM2 expression (D), 25x magnification. Separate panels show either respective channel overlays (ATF2 (GFP); 7TCF (mCherry) or Lectin-49) or the individual ATF2 and 7TCF reporter channels only. (E) Quantification of the average metastatic lesion number/10x field for the metastatic lesions formed by cell lines shown in (A, B). (F) Quantification of the average metastatic lesion size (cell number) for the metastatic lesions formed by cell lines shown in (A, B). (G) Quantification of the average

invasion velocity of ATF2^{high} and 7TCF^{high} HT1080 DR cancer cells within the metastatic lesions formed by cell lines shown in (A). (H) Quantification of the average ATF2/7TCF reporter signal ratio for the cancer cells within the metastatic lesions formed by cell line shown in (A). Statistical comparisons were made using Mann-Whitney test (E-H). The number of animals used for the analyses was 4 and 4 (E, F) or 3 and 3 (G, H). Scale bars = 100 μ m (A, B) or 50 μ m (C, D).

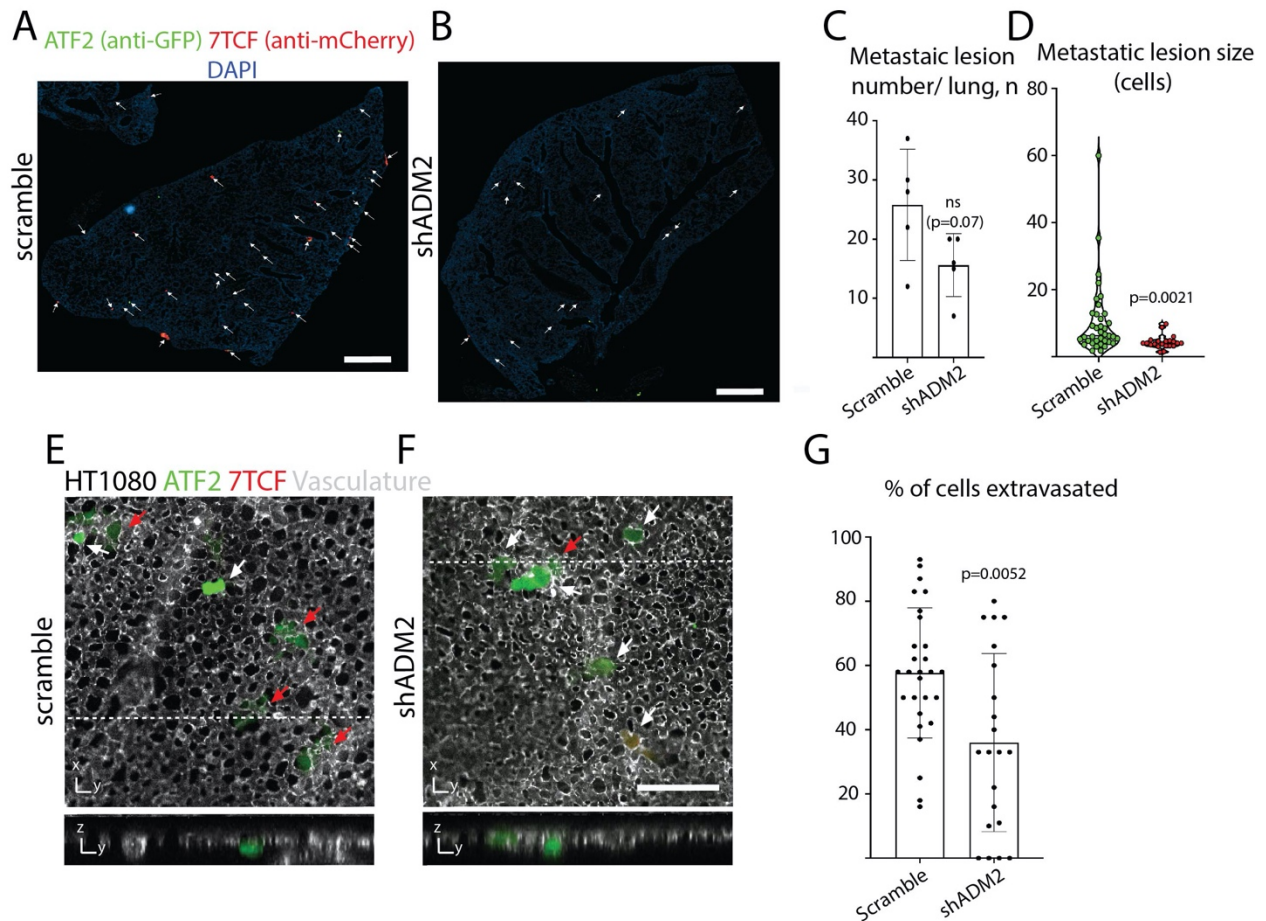


Fig. S7. ADM2 expression is necessary for efficient colonization of mouse lungs and extravasation of HT1080 cells. (A–B) Representative images of mouse lungs colonized by HT1080-DR cells engineered to express either scrambled shRNA (A) or shRNA targeting ADM2 (B). (C) Quantification of the average number of metastatic lesions per lung for tumours formed by the cell lines shown in (A, B). (D) Quantification of the average metastatic lesion size (cell number) for tumours formed by the cell lines shown in (A, B). (E–F) Representative images showing imaging fields (XY view, top panels; ZY view, lower panels) of chicken CAM vasculature with extravasating HT1080 human cancer cells. Cells were engineered to express either scrambled shRNA or shRNA targeting ADM2. Red arrows indicate extravasated cells, whereas white arrows indicate cells remaining within the vascular lumen. Dashed lines indicate the location of the ZY optical section (lower panels). (G) Quantification of cancer cell extravasation for the conditions shown in (E, F). Statistical comparisons were performed using the

Mann–Whitney test (**C**, **G**) or Welch’s *t*-test (**D**). The number of animals used for the analyses was 5 and 5 for (**A–D**) and 4 and 4 for (**E–G**). Scale bars = 1000 μ m (**A**, **B**) and 100 μ m (**E**, **F**).

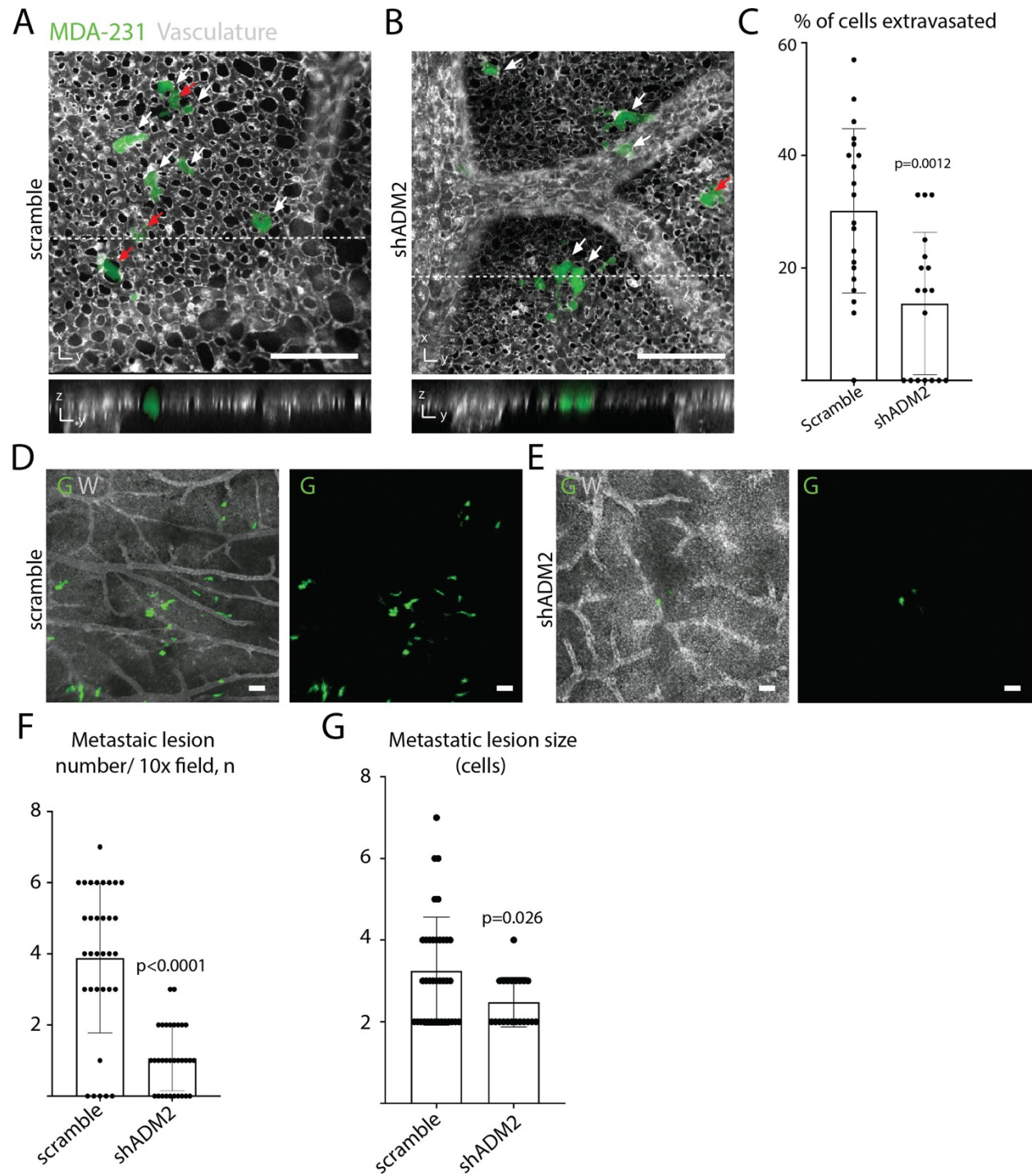


Fig. S8. ADM2 expression is necessary for efficient colonization of chicken CAM tissue by MDA-231 cells. (A–B) Representative images showing imaging fields (XY view, top panels; ZY view, lower panels) of chicken CAM vasculature with extravasating MDA-231 human breast cancer cells. Cells were engineered to express either scrambled shRNA or shRNA targeting ADM2 and stained with CellTracker Green. Red arrows indicate extravasated cells, whereas white arrows indicate cells remaining within the vascular lumen. Dashed lines indicate the location of the ZY optical section (lower panels). (C) Quantification of cancer cell extravasation for the conditions shown in (A, B). (D–E) Representative images showing metastatic lesions (chick CAM) formed by MDA-231 human breast cancer cells engineered to express either scrambled shRNA (D) or shRNA that specifically targets ADM2 expression (E), 10x magnification, 3 days post injection. Cells were stained with CellTracker Green, 72h post injection. (F) Quantification of the average metastatic lesion number/10x field for the metastatic lesions formed by cell lines shown in (A–E). (G) Quantification of the average metastatic lesion size (cell number) for the metastatic lesions formed by cell lines shown in (A–E). Statistical comparisons were performed using the Mann–Whitney test. The number of animals used for the analyses was 3 and 3 for (C) and 3 and 4 for (F–G). Scale bars = 100 μ m.

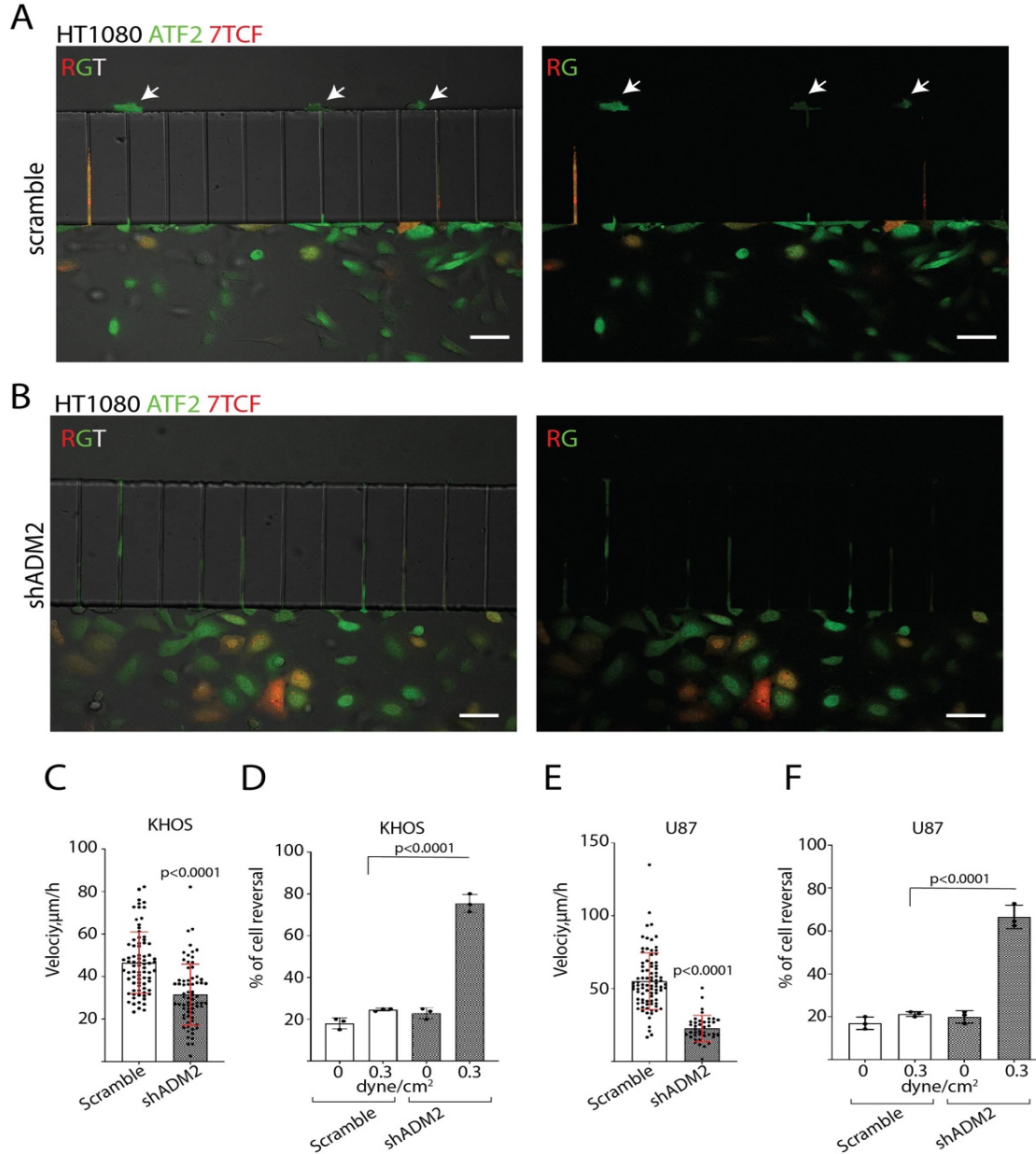


Fig. S9. ADM2 expression is essential for the intravasation of multiple cancer cell lines *in vitro*. (A to B) Representative images depict HT1080 DR cells engineered to express either scramble shRNA (A) or shRNA targeting ADM2 expression (B) within a microfluidic device designed to study cancer cell intravasation *in vitro* ($t=60$ min). The left panels show an overlay of the ATF2 reporter (green, GFP), the 7TCF reporter (red, mCherry), and transparent light channels. The right panels exclusively display the overlay of the ATF2 reporter and 7TCF reporter channels. White arrows indicate individual ATF2^{high} cells that have exited (intravasated) into the upper 2D flow channel. (C) Average velocity of KHOS cancer cells engineered to express either scramble shRNA or shRNA targeting ADM2 during the *in vitro* intravasation assay. (D) The percentage of KHOS cancer cells (human osteosarcoma) engineered to express either scramble shRNA or shRNA targeting ADM2 that reversed their direction of migration when exposed to 0 and 0.3 dyne/cm² shear stress. (E) Average velocity of U87 cancer cells engineered to express either

scramble shRNA or shRNA targeting ADM2 during the in vitro intravasation assay. **(F)** The percentage of U87 cancer cells (human glioblastoma) engineered to express either scramble shRNA or shRNA targeting ADM2 that reversed their direction of migration when exposed to 0 and 0.3 dyne/cm² shear stress. Statistical comparisons were made using Mann-Whitney test (**C** to **E**) or one-way Anova (**D** to **F**). Scale bars represent 50 μ m.

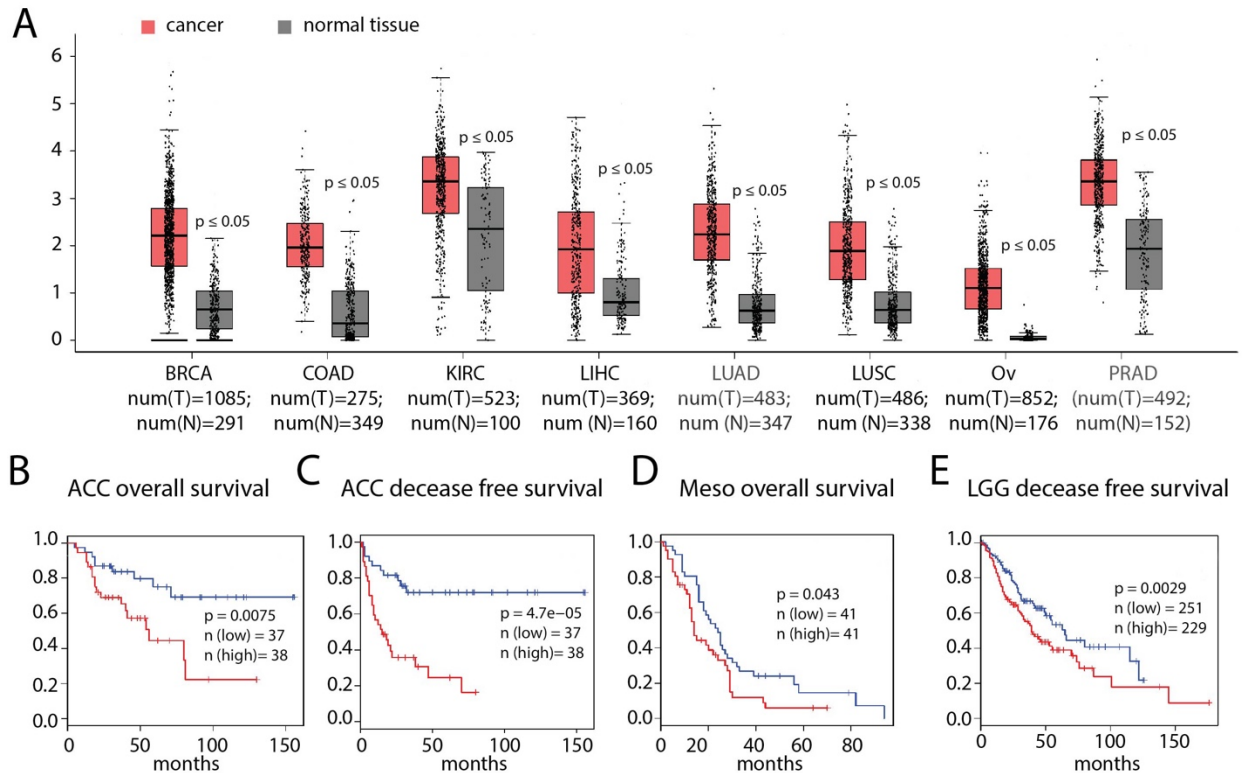


Fig. S10. ADM2 is overexpressed in various human cancers and is associated with poor prognosis. (A) ADM2 mRNA expression (measured in Transcripts per million, TPM) in cancer tissues compared to adjacent normal tissues (data source: Gepia). (B to E) Kaplan-Meier survival curves illustrating overall survival and disease-free survival in patients with different types of human cancers (data source: Gepia). The cancers include ACC (Adrenocortical Carcinoma); BRCA (Breast Invasive Carcinoma); COAD (Colon Adenocarcinoma); KIRC (Kidney Renal Clear Cell Carcinoma); LIHC (Liver Hepatocellular Carcinoma); LGG (Brain Lower Grade Glioma); LUAD (Lung Adenocarcinoma); LUSC (Lung Squamous Cell Carcinoma); Meso (Mesothelioma); Ov (Ovarian Serous Cystadenocarcinoma); PRAD (Prostate Adenocarcinoma). Statistical analyses were performed using a one-way ANOVA (A) or log-rank Mantel-Cox tests (B to E).

Supplementary Tables and videos Legends

Supplementary Tables Legends:

Table S1. List of genes upregulated in ATF2^{high} cells.

Table S2. List of genes upregulated in 7TCF^{high} cells.

Supplementary Videos Legends:

Video S1: ATF2^{high} cancer cells drive intratumoral cancer cell invasion. This time-lapse video presents two independent imaging fields where the invasion of intertumoral cancer cells (HT1080, a human fibrosarcoma) can be observed. Notably, ATF2^{high} cancer cells (green, GFP) rapidly invade within the tumor perivascular regions and along the vasculature, while 7TCF^{high} cancer cells invade significantly less and often appear fragmented. The video consists of single optical slices captured every 20 minutes over a total duration of 13 hours. Red indicates the mCherry (7TCF reporter), and green indicates the GFP (ATF2 reporter). The images were taken with a 10x objective, and the scale bar = 100 µm.

Video S2. In vitro migration of HT1080 DR (ATF2/7TCF reporter) cancer cells.

Representative time-lapse video showing in vitro migration of HT1080 cancer cells (HT1080, a human fibrosarcoma). Notably, multiple migratory ATF2^{high} cancer cells (green, GFP) can be seen within the imaging field, while 7TCF^{high} cancer cells migrate significantly less. Maximum intensity (3D) projection, 30 min/frame, 26.5 h total duration. Red indicates the mCherry (7TCF reporter), and green indicates the GFP (ATF2 reporter). The images were taken with a 10x objective, and the scale bar = 100 µm.

Video S3. ATF2^{high} cancer cells constitute the majority of blood vessel wall contacting (perivascular) cancer cells. This representative time-lapse video captures the perivascular region of a primary tumor formed by HT1080 (a human fibrosarcoma) cancer cells. It is important to note that most of the perivascular cancer cells are ATF2^{high}. The video displays vessel contacting ATF2^{high} cancer cells actively budding into the vascular lumen, indicated by white arrows. Additionally, multiple 7TCF^{high} cancer cell fragments can be seen moving within the tissue, marked by yellow arrows. A red arrow points to an ATF2^{high} cancer cell that can be observed intravasating and disappearing into the bifurcation of a blood vessel. Single optical section, 5 min/frame, 1.5 h total duration. Red indicates the mCherry (7TCF reporter), green indicates the GFP (ATF2 reporter) and white indicates Lectin-649 (vasculature). The images were taken with a 25x objective, and the scale bar = 50 µm.

Video S4. ATF2^{high} cancer cells drive the process of tumor cell intravasation. This representative time-lapse video captures the intravascular cluster of ATF2^{high} cancer cells (HT1080). Note that all the cancer cells within this cell cluster are ATF2^{high}. White arrows point to the individual cancer cell intravasation sites. Yellow arrows point to the 7TCF positive

(mCherry) cell fragments. Multiple other 7TCF cell fragments can be seen within the tumour tissue and vascular lumen. A red arrow points to an ATF2^{high} cancer cell that can be observed detaching the ATF2^{high} cancer cell cluster and been carried away by the blood flow. Maximum intensity (3D) projection, 5 min/frame, 1h 55 min total duration. Red indicates the mCherry (7TCF reporter), green indicates the GFP (ATF2 reporter) and white indicates Lectin-649 (vasculature). The images were taken with a 25x objective, and the scale bar = 100 μ m.

Video S5. ADM2 expression is essential for intratumoral cancer cell invasion. This video presents representative time-lapse recordings that showcase the dynamics of intratumoral cancer cells within primary tumors formed by HT1080 cells, which were engineered to express either scrambled shRNA or shRNA targeting ADM2. In the tumors formed by HT1080 cells expressing scrambled shRNA (upper panel), multiple invasive ATF2^{high} cancer cells can be observed invading the perivascular region. In contrast, there is a significant decrease in intratumoral cancer cell invasion and an increase in 7TCF reporter activity in tumors formed by HT1080 cells expressing anti-ADM2 shRNA (lower panel). Maximum intensity (3D) projection, 20 min/frame, 16.3 h total duration. Red indicates the mCherry (7TCF reporter), and green indicates the GFP (ATF2 reporter). The images were taken with a 10x objective, and the scale bar = 100 μ m.

Video S6. ADM2 expression is essential for cancer cell intravasation *in vitro*. This video showcases representative time-lapse recordings that illustrate the behaviour of HT1080 DR cells, which have been engineered to express either scrambled shRNA (upper panels) or shRNA targeting ADM2 (lower panels). The HT1080 cells invade from the lower seeding channel (no flow) into the upper flow channel (0.3 dyne/cm²) through 3 μ m wide channels. In the case of HT1080 cells expressing scrambled shRNA, ATF2^{high} (green) cells can be seen efficiently migrating towards the flow channel and successfully intravasating. Conversely, in the anti-ADM2 shRNA expressing cells, the cancer cells reverse the direction of their migration. Additionally, there is an increased 7TCF reporter activity (red) observed in the anti-ADM2 shRNA expressing HT1080 DR cancer cells. White arrows indicate individual ATF2^{high} cancer cells that intravasate into the flow channel. The video consists of single optical sections taken at a rate of 10 minutes per frame over a total duration of 2 hours. Red indicates the mCherry (7TCF reporter), while green indicates the GFP (ATF2 reporter). The images were captured with a 10x objective, and the scale bar represents 50 μ m.