Supplementary Material

Supplementary Methods

Immunofluorescence

Immediately after dissection tumour samples were washed is sterile PBS and placed in 1 ml 4% paraformaldehyde (Sigma-Aldrich) for a maximum of 12 h. Following fixation, the samples were transferred to 6% sucrose for 16 h, then 12% sucrose for 24 h and finally into 20% sucrose for 24 h. Following sucrose infiltration, samples were placed in T-12 Peel-A-Way embedding moulds (Polysciences), covered with Shandon Cryomatrix (Thermo Scientific) and set by placing the mould on dry-ice for 10 min. 10μm sections were cut using a cryostat (Leica) and collected onto SuperFrost Plus™ slides (Thermo Scientific). Prior to immunofluorescent staining, slides were thawed at room temperature and a hydrophobic Liquid Blocker Super PAP pen used to create a boundary. Firstly, samples were rehydrated with PBS for 10 min, incubated with 50 mM NH_4CL for 20 min and then blocked for 30 min (blocking buffer: 1% bovine serum albumin, 0.1% Triton X-100, 0.4% Tween20 in PBS). Slides were incubated overnight at 4°C with 100μl primary antibody (rabbit anti-αSMA; Abcam #ab5694; 1:200 diluted in blocking buffer) in a liquid chamber. Samples were washed 3 times with blocking buffer and incubated for 1 h at room temperature in the dark with secondary antibodies (Goat Anti-Rabbit Alexa Flour 594; Invitrogen #A-11012; 1:500 in blocking buffer). Following two washes with PBS and one wash with dH_2O, coverslips were mounted using Mowiol (Sigma) supplemented with DAPI (1:10,000; Invitrogen). Images were acquired using a Nikon Eclipse Ti and CFI Plan-Fluor 10X (N.A.0.3) objective.

H&E staining & Immunohistochemistry

H&E staining was performed on a Leica ST5020-CV5030 automated slide stainer and coverslipper on 4μm sections (see methods section). Additional IHC antibody conditions: mouse anti-BAP1(C-4; Santa Cruz sc-28383) 1:250 and pH9.

Supplementary Figures

Figure S1. *In ovo* kinetics for bioluminescence imaging (BLI).

Figure S2. Additional MESO-12T CAM nodule immunohistochemistry and histology.

Figure S3. Additional MESO-8T CAM nodule immunohistochemistry and histology.

Figure S4. Cq values for housekeeping genes Actin and GAPDH are not affected by 3D growth on CAM.

Figure S5. Additional modalities for MPM-CAM xenograft and vasculature analysis.

Figure S6. Tumour weight versus bioluminescence signal for individual MPM cell lines.

Figure S7. Additional MPM#2, MPM#26 and MSTO-211H CAM nodule immunohistochemistry.

Figure S8. QuPath analysis of an MSTO-211H CAM nodule for Ki-67 staining.

Supplementary Tables

Table S1. Viability at E14 for eggs engrafted with MPM cells at E7**.**

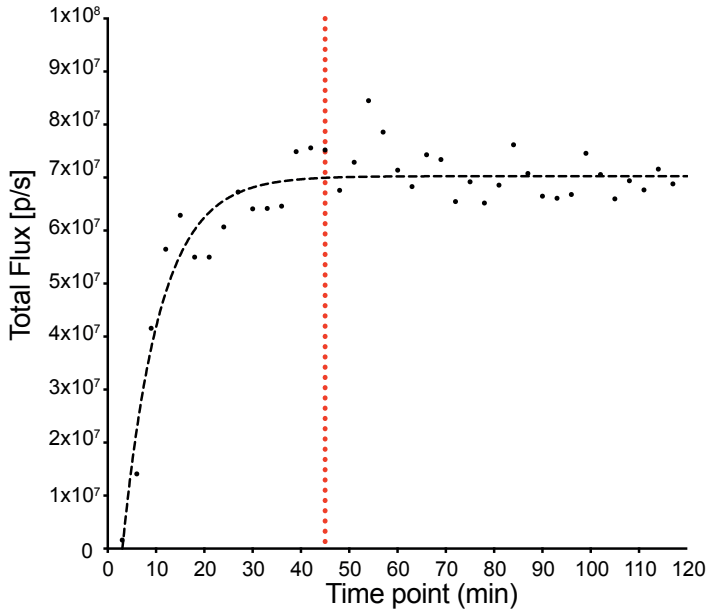

Figure S1. *In ovo* kinetics for bioluminescence imaging (BLI).
Example of *in ovo* kinetics for BLI signals recorded from an engrafted MESO-8T nodule over 2 hours following yolk sac injection of luciferin. A steady state plateau in BLI signal was reached by 45 min for all MPM cell line nodules.

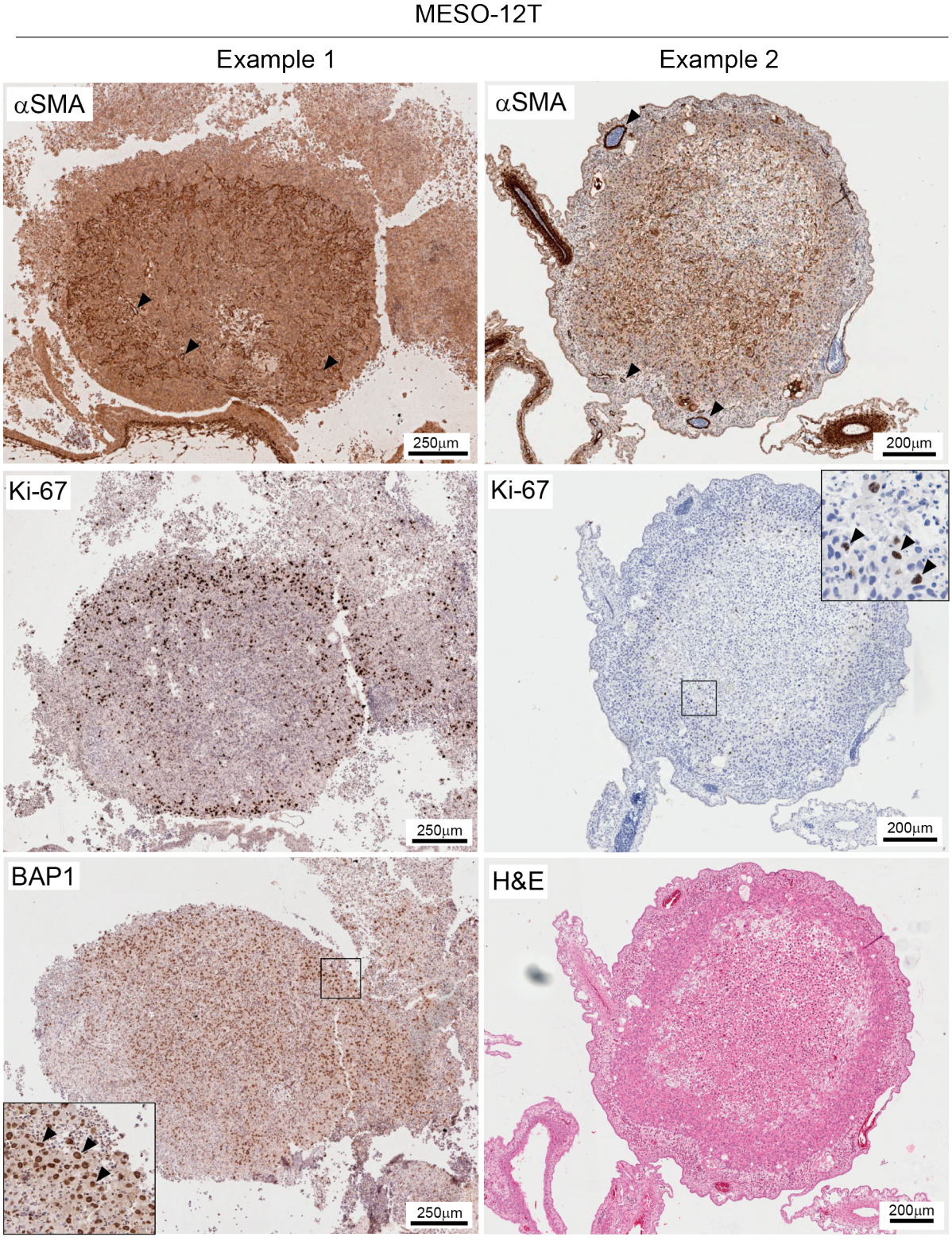

Figure S2. Additional MESO-12T CAM nodule immunohistochemistry and histology. Additional staining on adjacent sections for the examples of MESO-12T nodules shown in Figure 5A. Stain and scale are indicated on each image. Chick fibroblasts and blood vessels are stained with αSMA, proliferating cells are stained with Ki-67, and MESO-12T are positive for nuclear BAP1. H&E, Haematoxylin and Eosin. Arrowheads indicate small blood vessels around the tumour periphery, Ki-67 positive, or BAP1 positive tumour cells.

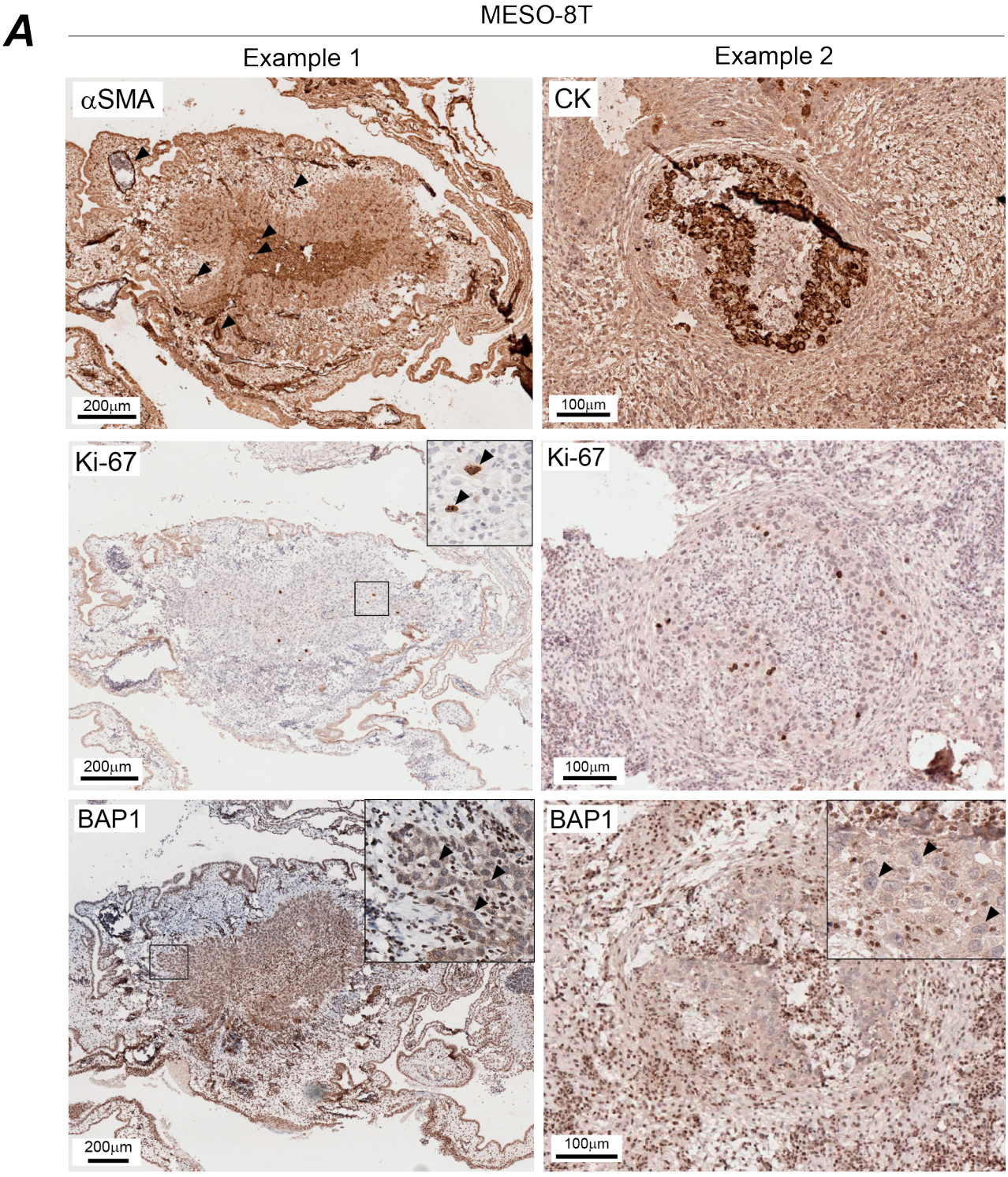

Figure S3. Additional MESO-8T CAM nodule immunohistochemistry and histology.
A, Additional staining on adjacent sections for the examples of MESO-8T nodules shown in Figure 5A and 5D. Chick fibroblasts and blood vessels are stained with αSMA, proliferating cells are stained with Ki-67, and MESO-8T are negative for nuclear BAP1. For example 2, these sections are closer to that shown in Figure 5D and include additional staining for cytokeratin (CK, sc-8018). Arrowheads indicate small intratumoral blood vessels, Ki-67 positive, or BAP1 negative tumour cells. ***B,*** H&E, Haematoxylin and Eosin, staining on an adjacent section for the MESO-8T nodule shown in Figure 5C.

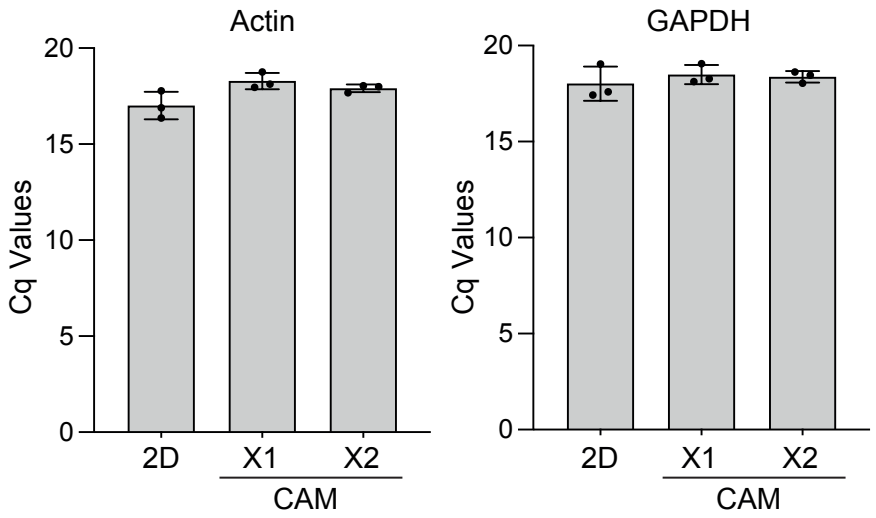

Figure S4. Cq values for the housekeeping genes Actin and GAPDH are not affected by 3D growth on the CAM.

qRT-PCR was used to compare Cq values for Actin or GAPDH expression in MSTO-211H cells grown in 2D culture *in vitro* (n=3) with those for cells grown in 3D culture *in vivo* on the CAM (2 independent experiments X1 and X2, each with 3 nodules). These data were used in calculation of the relative expression for other mRNAs shown in Figure 6.

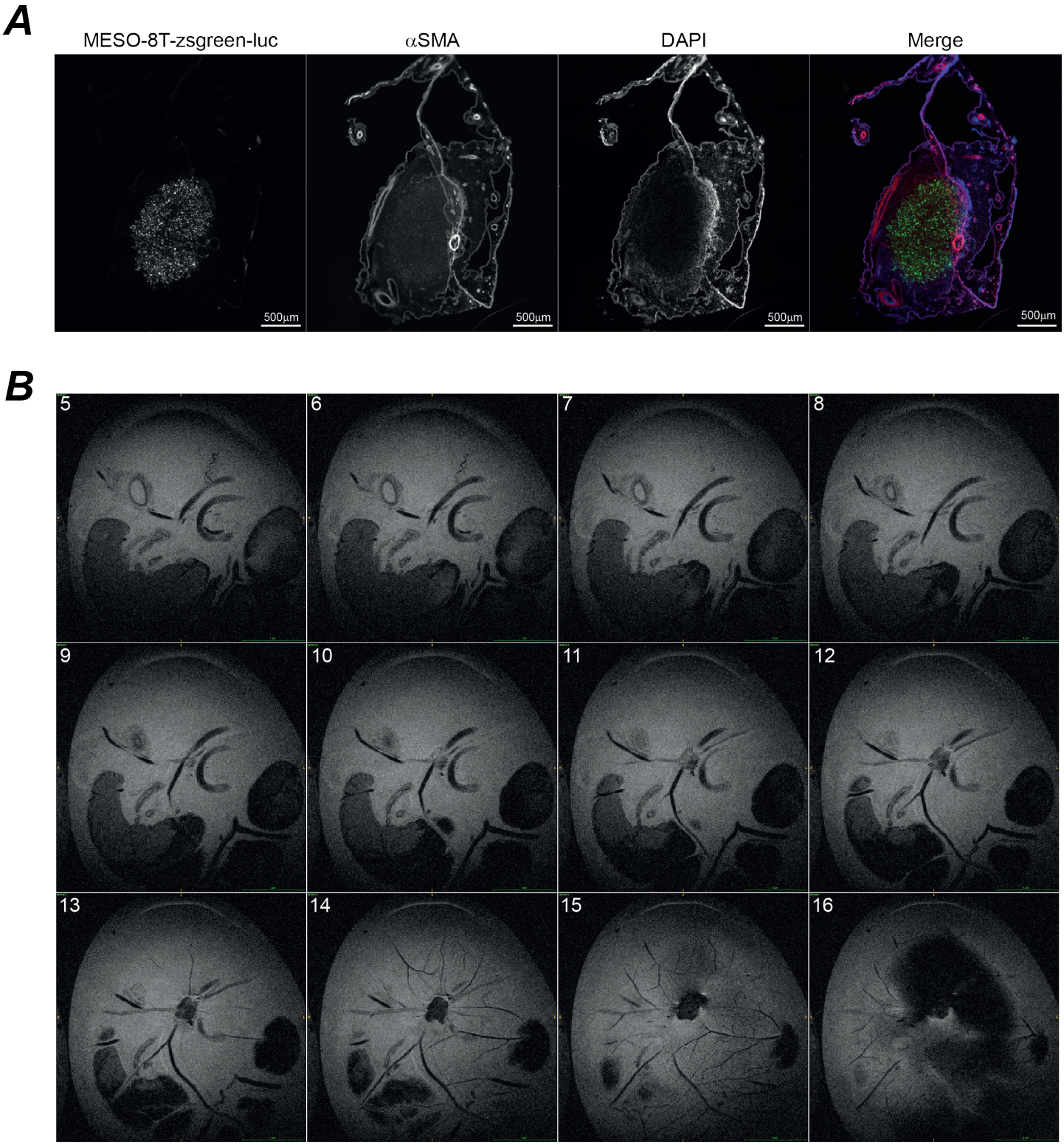

Figure S5. Additional modalities for MPM-CAM xenograft and vasculature analysis. ***A,*** Immunofluorescent staining on a frozen section of MESO-8T tumour nodule, showing tumour adjacent blood vessels stained by αSMA. ***B,*** Representative MR images in the coronal plane where the tumour and the associated vessels appear dark, the CAM appears as grey, while the chick embryo organs are also observed as variable intensities. These images were used to generate the rendered image in Figure 7F. Data acquisition parameters are reported in the methods.

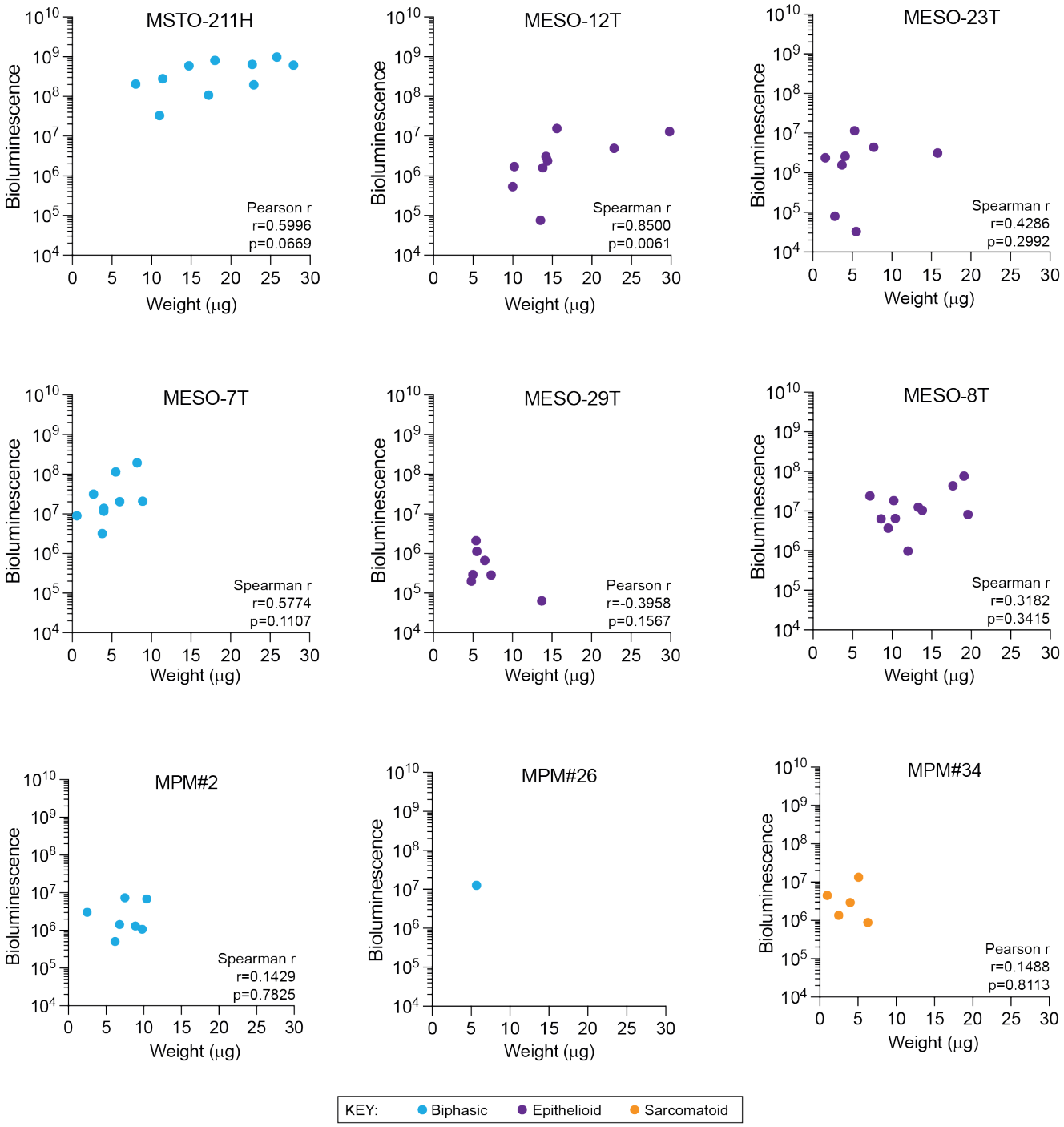

Figure S6. Tumour weight versus bioluminescence signal for individual MPM cell lines. Correlation plots of bioluminescent signal and tumour weight for individual tumours at E14 that were established from 2 million cells for each MPM cell line. Correlations are based on non-transformed data; statistical test, *r* and *P* values are indicated on each graph. These data are compiled in Figure 8C.

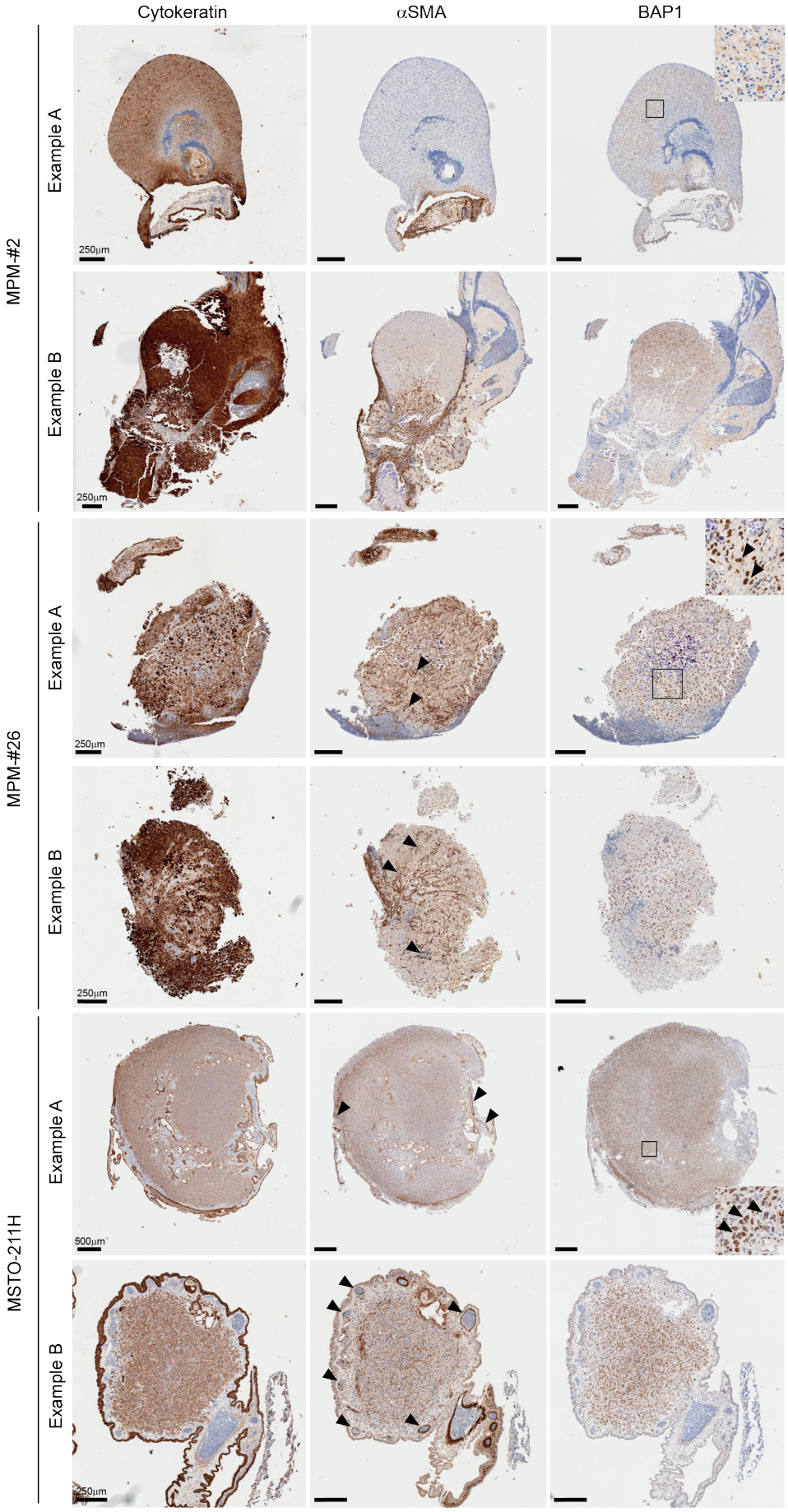

Figure S7. MPM#2, MPM#26 and MSTO-211H CAM nodule immunohistochemistry. Additional staining on adjacent sections for examples of MPM#2, MPM#26 and MSTO-211H CAM nodules in Figure 9A. Chick fibroblasts and blood vessels are stained with αSMA, and tumour cells with cytokeratin (sc-81714). MPM#2 are negative for nuclear BAP1 whilst MPM#26 and MSTO-211H are positive for nuclear BAP1.

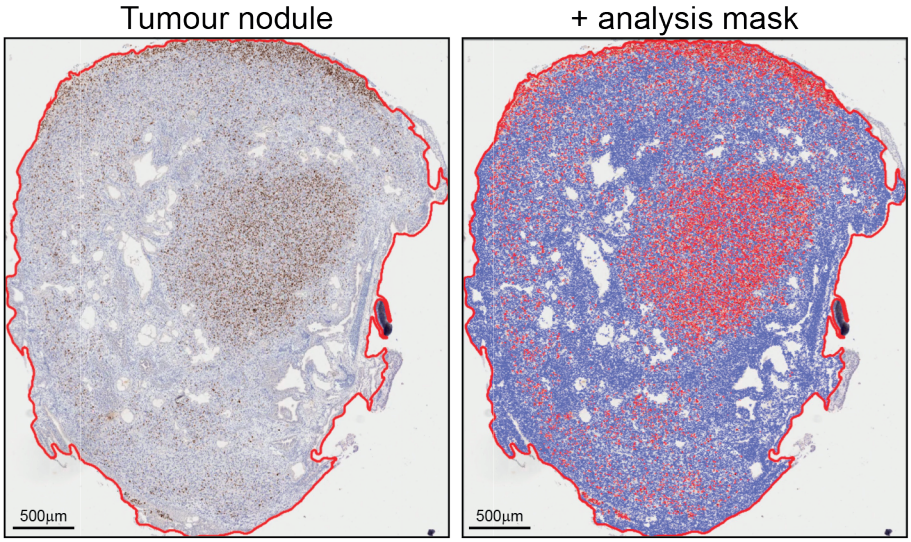

Figure S8. QuPath analysis of an MSTO-211H CAM nodule for Ki-67 staining. Tumour nodule showing Ki-67 staining (left) and with QuPath analysis mask (right); Ki-67 negative tumour or chick infiltrating cells are identified in blue and Ki-67 positive tumours cells in red (17.4%). Scale bars 500µm.

Table S1. Viability at E14 for eggs engrafted with MPM cells at E7. Eggs with surviving chick embryos were used to calculate engraftment rates in Figure 3B.

Embryonic day	7	14	
	n	n	% survival
● MPM#34	8	7	88
● MPM#24	10	9	90
● MSTO-211H	16	13	81
● MPM#2	14	14	100
● MESO-7T	18	12	67
● MPM#26	7	3	43
● MESO-29T	13	10	77
● MESO-12T	13	13	100
● MESO-8T	14	12	86
● MESO-23T	19	16	84

KEY: ● Biphasic ● Epithelioid ● Sarcomatoid