SUPPLEMENTAL INFORMATION

MURILLO ET AL.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse Genotyping

Initial genotyping of all new litters was performed by Transnetyx (Memphis, USA). Assessment of LoxP-p110α recombination was performed by standard PCR using the following primers: forward 5'-CTGTGTAGCCTAGTTTAGAGCAACCATCTA-3'; reverse 5'-CCTCTCTGAAGAGTTCATGTTTGATGGTGA-3'.

Histopathological analysis

Tissue samples were fixed overnight in 10% formalin and subsequently stored in 70% ethanol. Samples were then embedded in paraffin and sectioned. Sections were stained for Haematoxylin and Eosin following standard methods. For other staining, sections standard immunoperoxidase staining was performed. A list of primary antibodies can be found in Table S1. All secondary antibodies were from Vector. Following secondary antibody incubation, sections were incubated with ABC (Vector Laboratories PK-6100) for 30 minutes. Peroxidase activity was detected wit 3,3'-diaminobenzidine (Sigma) following manufacturer´s instructions. For fluorescent detection of endomucin and nestin, Alexa-488 and Alexa-555 secondary antibodies from Life Technologies were used according to manufacture's instructions.

Primary cell culture and in vitro recombination

MEFs were isolated following standard protocol and maintained in DMEM supplemented with 10% FBS. For isolation of endothelial cells, lungs from mice were collected and digested with collagenase (Worthington). The cell suspension obtained was cultured in DMEM:HAMS-F12 (1:1) supplemented with 20% FBS, heparin (100 mg/ml, Sigma) and Endothelial mitogen (AbD Serotec). To select the endothelial cell population, cells were incubated with anti V-Cadherin antibody followed by incubation with a secondary antibody linked to magnetic beads (Invitrogen) according to manufacturer´s instructions. Selected cells were cultured in fresh media. LoxP-p110α recombination in vitro for both MEFs and Endothelial cells was achieved by incubating the cells for 24 hours with 4-OH-Tamoxifen (1mM, Sigma).

Immune system analysis

Tumors were dissected, minced using scissors and digested with collagenase IV (200U/ml; Worthington) and DNase I (0.2mg/ml, Roche) for 30 min at 37C. Tumors were then passed through a 70-µm cell strainer to remove large pieces of undigested tissue. A 70 % / 37 % / 30 % Percoll gradient (GE Healthcare) was then run to remove dead cells and red blood cells. For FACs analysis, leukocyte-enriched cells were collected from the 70-37 % interface, washed once and resuspended in FACS buffer (PBS containing 2% FCS, 2mM EDTA and 0.02% sodium azide). For gene expression analysis cDNA from whole tumour samples or from F4/80 positive sorted-cells was extracted

and analysed using a TaqMan mouse immune array (Life Technologies) following manufacturer instructions. A gene was considered differentially expressed when the normalized expression of it, using both GAPDH and HPRT as housekeeping genes, was different for all samples in one cell line compared with the other one. All antibodies were purchased from BD Pharmingen, eBioscience and Biolegend. Live cell counts were calculated from the acquisition of a fixed number of 10-μm latex beads (Coulter) mixed with a known volume of unstained tumor cell suspension. Nuclear Foxp3 was detected according to the manufacturer's instructions (eBiosience). DAPI (Sigma) was added to the final suspension to exclude dead cells before acquisition on a Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Western blot

Cells were starved for 16 hours prior to any experiment. Cells were stimulated with vehicle, 10 ng/ml of EGF (Sigma), 40 ng/ml of VEGF-2, 40 ng/ml of FGF-2 (Sigma) or 10 ng/ml of PDGF (Sigma) for 10 minutes. Cells were harvested and lysed in a buffer containing 1 % Triton X-100, 0.25 % IGEPAL, 0.25 % sodium deoxycholate, 50 mM Tris-HCl (pH=7.4), 150 mM NaCl and 2.5 mM EDTA plus protease and phosphatase inhibitors (Calbiochem). Protein extracts were separated by SDS-Page electrophoresis and transferred to PVDF membranes. A list of primary antibodies can be found in Table S1. For fluorescence quantification, secondary antibodies linked to fluorochromes were used (Odyssey). Visualization and quantification was performed in an

Odyssey LI-COR system according to manufacturer´s instructions.

Antibodies used

		Company	Reference
Western blot			
	p-AKT	Cell Signaling	9271
	AKT	Cell Signaling	2920
	p-ERKs	Cell Signaling	9101
	ERKs	Cell Signaling	9107
	GRB2	Cell Signaling	3972
	ACTIN	Sigma	A5441
	PARP	Cell Signaling	9542
Immunohistochemistry			
	Endomucin	Santa Cruz	sc65495
	Nestin	BD Biosciences	611659
	Smooth muscle actin	Dako	m0851
	Caspase-3	R&D	af853
	KI-67	Abcam	ab16667
	F4/80	eBiosciences	14-4801-82
Immune Cell analysis			
	F4/80	BioLegend	122610
	Foxp3	eBioscience	12-5773-82
	CD11b	eBioscience	56-0112-82
	GR-1	BD Pharmingen	553127
	CD3	BD Pharmingen	553062

	CD4	BD Pharmingen	553051
	CD45.2	BioLegend	109830
Endothelial cell isolation			
	VE-Cadherin	eBioscience	14-1441-82

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

(A) Mice breeding strategy. (B) New mouse model colony descriptive statistics.

Supplementary Figure 2

Samples from host mice were collected at the end of the experiment and subjected to PCR analysis to assess LoxP-p110α allele recombination.

Supplementary Figure 3

(A) Quantification of bioluminescence emitted by B16F10 cells containing a luciferase expression vector after 1 day of tail vein injection. (B) Bioluminescence images of lungs imaged ex vivo after 26 days of tail vein injection. (C) LLC1 cells were injected in the tail vein and lungs from mice were harvested after 20 days.

Supplementary Figure 4

B16F10 subcutaneous tumour sections stained for Caspase-3 and Ki-67 (A) were quantified using NIS-Elements program (B, C. n=5 per group). The Angiosight module from Leica's Digital Pathology capture & analysis platform was used for the analysis of necrosis (D), vessel density in non-necrotic tissue (E) and average vessel area (F) (n=5 for all groups). (G) B16F10 experimental metastasis lung sections stained for endomucin and smooth-muscle actin. (H) LLC1 subcutaneous tumours stained sections. (I) Quantification of Nestin &

Endomucin intensity staining (n=3). Scale bars represent 100 μm (4A; 4G; 4H, lower panels) and 1000 μm (4H, upper panels), respectively.

Supplementary Figure 5

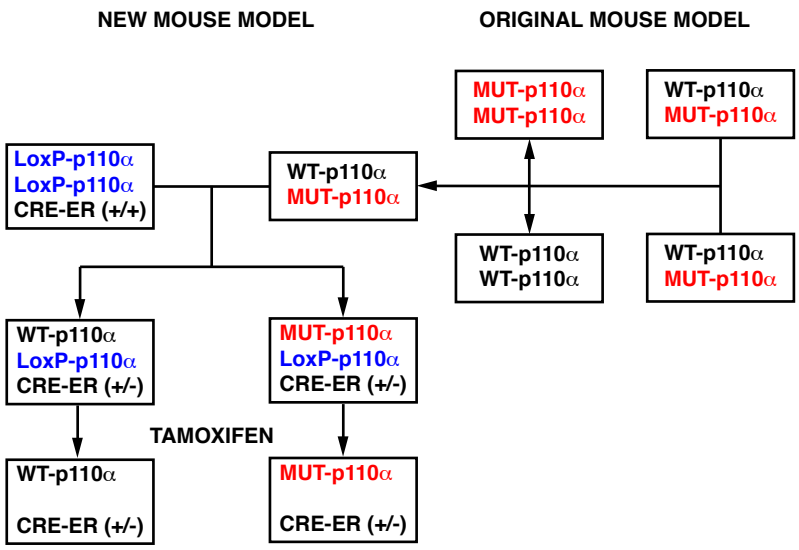
(A-B) Average capillary length was quantified for main figure 5D (n=3 fields per condition). Western blot analysis and quantification of cell protein extracts after 10 minutes stimulation with VEGF-A 40 ng/ml (D) or FGF-2 40 ng/ml (E). (F) Analysis of TUNEL positive cells in matrigel plugs. (G) Analysis of PARP expression and cleavage under different conditions in endothelial cells. Scale bars represent 100 mm.

Supplementary Figure 6

FACs analysis of leukocyte (A), T-cell (B), neutrophil (C), FOXP3+ regulatory T-Cell (D) and F4/80+ CD11b+ cell infiltration (E) (n=10 per genotype). FACS analysis of Major histocompatibility complex (F) and mannose receptor (G) surface expression (n=3 per genotype). ELISA analysis of FGF-2 secreted levels by cells in the tumor (H) (n=3). ELISA analysis of VEGF-A (I) and FGF-2 (J) by tumor cell lines in vitro. F40/80 staining in LLC1 subcutaneous tumors.

Figure S1, Related to Figure 1

A



B

			Weight (grams ± SD)		
	Number	Gender	(14 days)	(28 days)	(42 days)
WT-p110α	158	♂ = 74 (46.8%)	5.64±0.72	9.42±1.42	21.64±2.25
Loxp-p110α CRE-ER	(50.3%)	♀ = 84 (53.2%)	5.61±0.73	9.58±1.40	17.48±1.62
MUT-p110α	156	♂ = 78 (50%)	5.63±0.69	9.37±1.51	21.44±1.52
Loxp-p110α CRE-ER	(49.7%)	♀ = 78 (50%)	5.20±0.63	9.56±1.21	16.73±1.39

Figure S2, Related to Figure 2

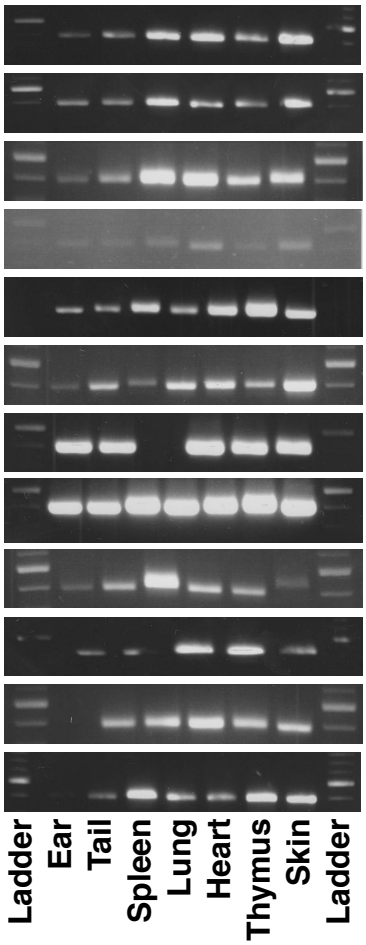
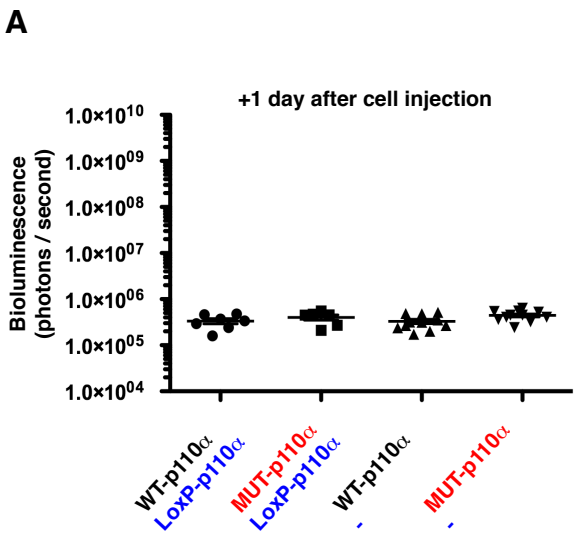
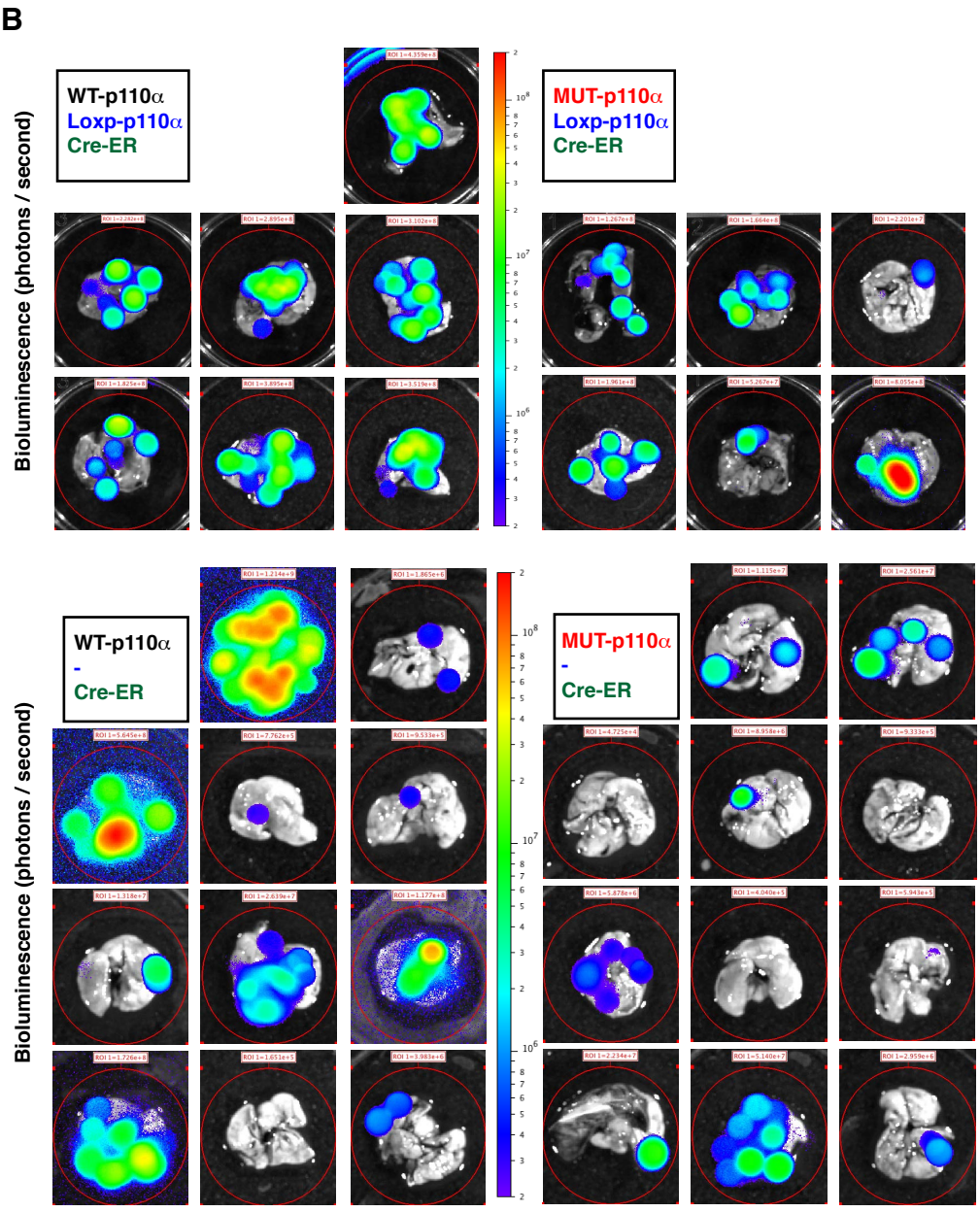


Figure S3, Related to Figure 3





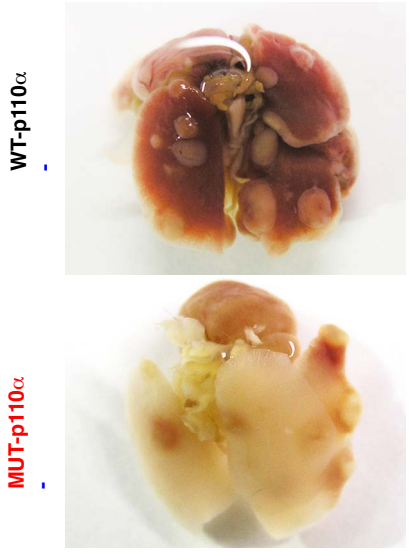
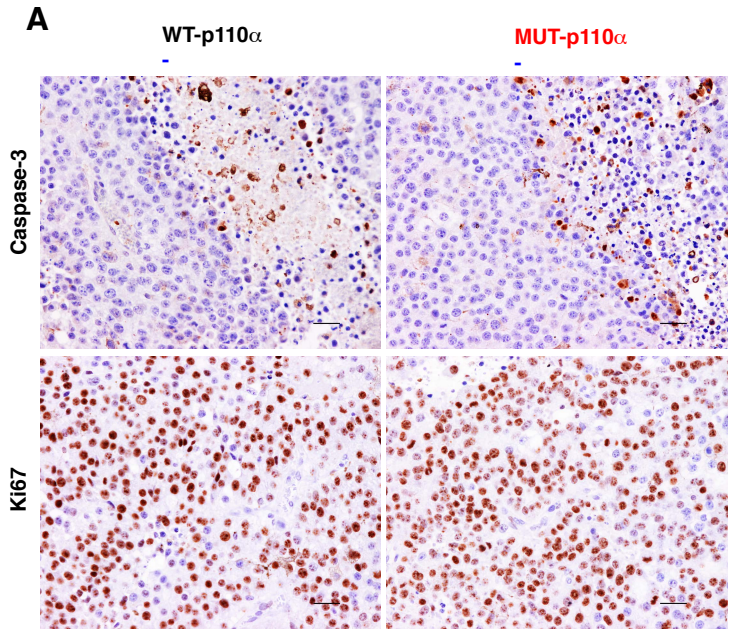
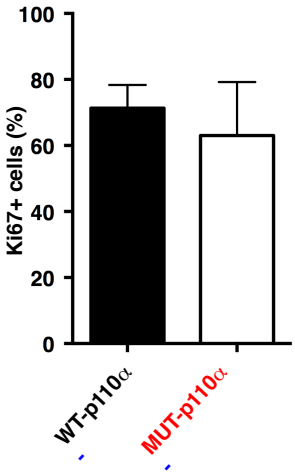
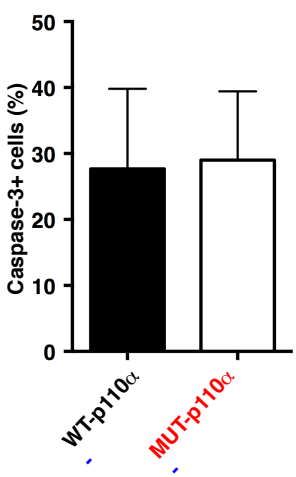
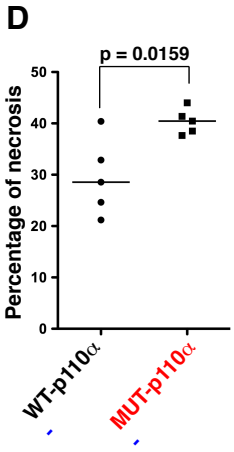


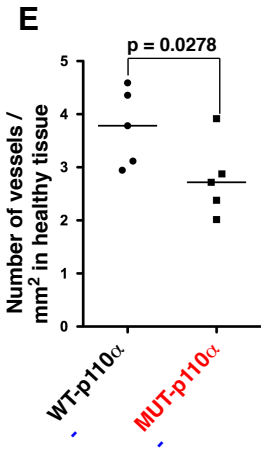
Figure S4, Related to Figure 4

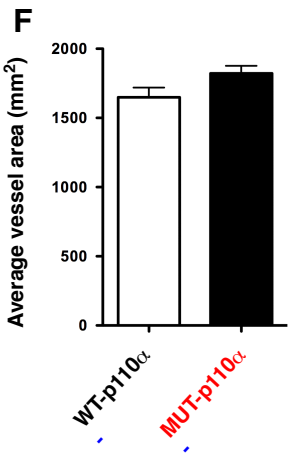


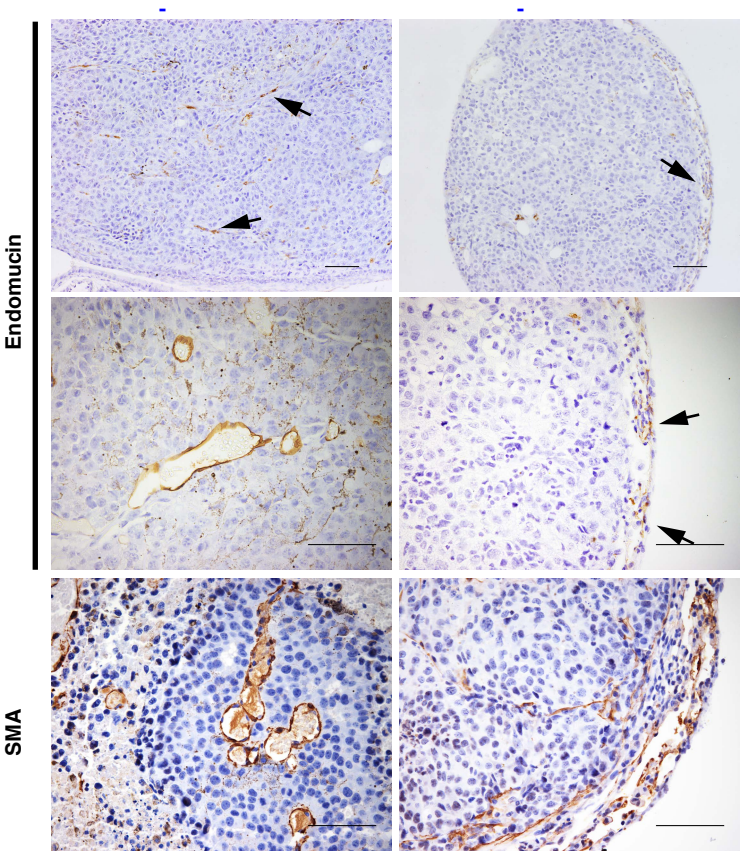


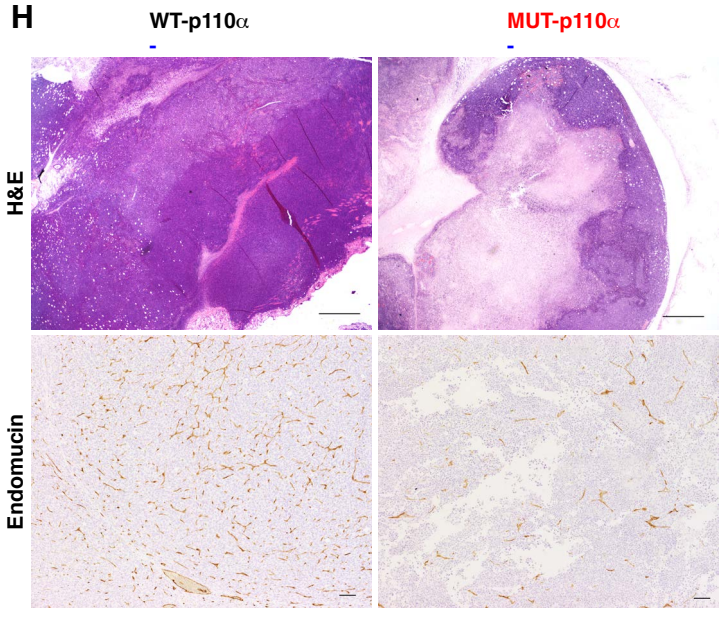












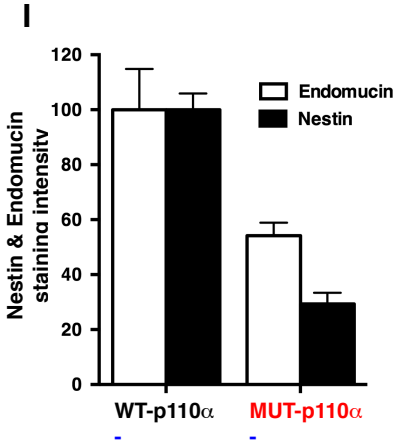


Figure S5, Related to Figure 5

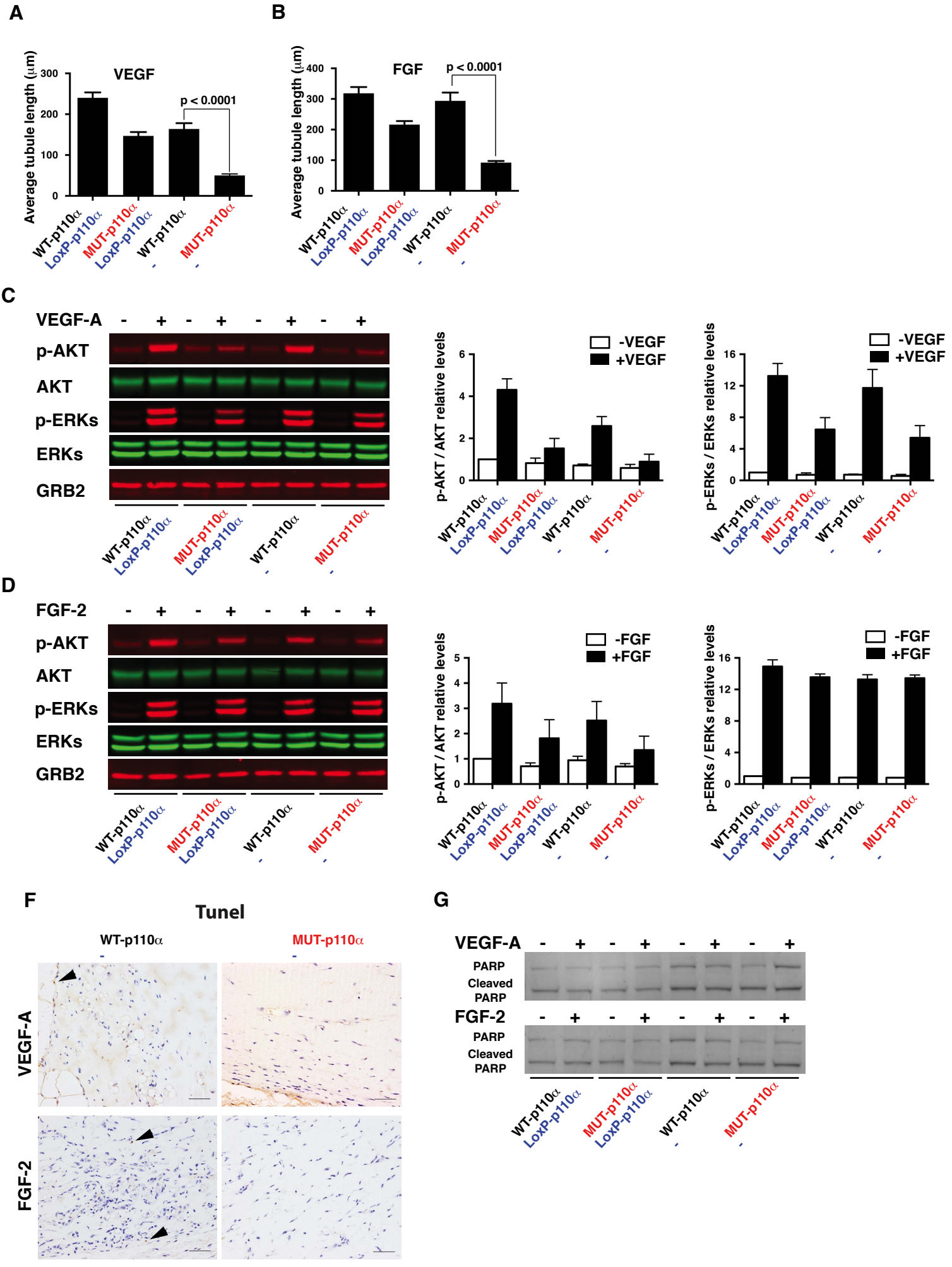


Figure S6, Related to Figure 6