SUPPLEMENTARY MATERIAL
FOR

FAM96A is a Novel Pro-Apoptotic Tumor Suppressor in Gastrointestinal Stromal Tumors

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Supplementary Materials and Methods

Materials

The following substances were used: oxaliplatin (GRY-Pharma GmbH, Kirchzarten, Germany), staurosporine (Merck, Darmstadt, Germany), imatinib mesylate (Novartis, Basel, Switzerland), Polybrene (Sigma-Aldrich, Taufkirchen, Germany), and puromycin (Sigma-Aldrich).

Purification of Interstitial Cells of Cajal (ICCs), ICC Stem Cells (ICC-SCs) and 'Fibroblast-Like Cells' (FLCs) from Mouse Corpus+Antrum Tunica Muscularis

ICC, ICC-SC and FLC were identified by flow cytometry in the hematopoietic marker-negative fraction of dissociated gastric corpus+antrum *tunica muscularis* as $Kit^{+}Cd44^{+}Cd34^{-}$, $Kit^{low}Cd44^{+}Cd34^{+}$ and $Kit^{-}Cd44^{-}Cd34^{-}$platelet-derived growth factor α (Pdgfra)$^{+}$ cells, respectively, and isolated by fluorescence-activated cell sorting (FACS) using previously published protocols[1, 2] with modifications (see **Supplementary Table 1** for detailed antibody information). Briefly, intact gastric corpus+antrum muscles were incubated with allophycocyanin (APC)-anti-Kit antibody (clone: ACK2) at 4°C for 3 h, then dissociated with collagenase digestion and trituration and filtered. Single-cell suspensions were incubated in 100 µl with anti-mouse Cd16/32 antibody (Fc block). Hematopoietic cells were identified with phycoerythrin (PE)-cyanine (Cy) 7-coupled anti-mouse Cd11b, anti-Cd45 and anti-F4/80 antibodies. Cells were also labeled with APC-anti-Kit (clone 2B8, which recognizes an epitope distinct from the epitope detected by ACK2), PE-anti-Pdgfra, eFluor 450- or fluorescein isothiocyanate (FITC)-anti-Cd34 and APC-Cy7-anti-mouse/human Cd44. Additional aliquots of the same samples were labeled in an identical manner but with isotype controls for the anti-Pdgfra or the anti-Pdgfra plus the anti-Cd34 or the anti-Pdgfra plus the anti-Cd34 plus the anti-Cd44 antibodies. Our experimental design was extensively verified by using single-stained and fluorescence-minus-one controls as well as by sorting and detection of the ICC markers Kit and Ano1 and the FLC markers Pdgfra and Kcnn3 by real time reverse transcription—polymerase chain reaction (RT-PCR; not shown). Samples were analyzed using a Becton Dickinson (BD Bioscience, Franklin Lakes, NJ, USA) LSR II flow cytometer and FlowJo software (Treestar, Ashland, OR, USA) (see **Supplementary Figure 1** for results). Cells were sorted using a Becton Dickinson FACS Aria II Cell Sorter configured to match the LSR II cytometer (excitation wavelengths of the lasers utilized: 407 nm, 488 nm and 635 nm).

Yeast Two-Hybrid Screen

The *Caenorhabditis elegans CED-4* gene was cloned as a fusion construct with the *GAL4* DNA-binding domain into the *pAS2.1* bait plasmid (Clontech, Mountain View, CA, USA) and transformed into the Y190 (HIS3, lacZ) yeast reporter strain by the lithium acetate method. A yeast strain with stable CED-4 expression was subsequently transformed with 50 µg mouse T-cell two-hybrid cDNA library (Matchmaker 2, Clontech) and the screen was performed according to the manufacturer's protocol. To analyze the interaction between FAM96A and APAF1 protein in yeast, *APAF1$_S$ΔCARD* (amino acids [aa] 92–end) was fused with the *GAL4* DNA-binding domain in *pAS2.1*.

Cell Lines

HEK293T human embryonic kidney cells and RKO human colorectal carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Germany), 1% L-glutamine and 1% penicillin/streptomycin. GIST48 cells were cultured in F-10 medium supplemented with 18% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 7.5 mg/500 ml Bovine Pituitary Extract (BD Biosciences, Franklin Lakes, NJ) and 1.25 mg/500 ml Mito^{TM} plus Serum Extender (BD Biosciences). GIST882 cells were cultured in RPMI 1640 containing 15% heat inactivated FBS, 1% L-glutamine and 1% penicillin/streptomycin. All cell lines were cultured in an atmosphere of 5% CO_2 at 37°C.

Glutathione-S-Transferase (GST) Pull-down Assays

Mouse (m) and human (h) *FAM96A* were cloned into the *pDEST 15* vector (Life Technologies), expressed as an N-terminal GST fusion protein in *Escherichia coli* BL21-codon plus bacteria (Agilent Technologies, Santa Clara, CA) using standard methods and subsequently sequence-verified by mass spectrometry (T. Kempf and M. Schnölzer, DKFZ Heidelberg, Germany). *hAPAF1$_S$ΔWD40* (coding for aa 2-421) was cloned into the bacterial expression vector, *pGEX-KG* (GE Healthcare, Buckinghamshire, United Kingdom), and expressed as an N-terminal GST fusion protein in *Escherichia coli* BL21-codon plus bacteria (Agilent Technologies) using standard methods. Bacterially expressed GST or GST fusion proteins were purified after binding to glutathione-sepharose 4B beads according to the manufacturer's instructions (GE Healthcare, Freiburg, Germany). The following *FAM96A* full-

length and deletion mutants were *in vitro*-translated and ^{35}[S]-methionine-labeled using the TNT-T7 coupled reticulocyte lysate system (Promega, Mannheim, Germany) as described by the manufacturer: human and mouse FAM96A, full-length (aa 1–160), mFAM96A-C1 (aa 1–55), mouse FAM96A-C2 (aa 1–139), mFAM96A-C3 (aa 55–139) and mFAM96A-C4 (aa 55–160). The ^{35}[S]-labeled proteins were incubated with GST or GST-hAPAF1$_S\Delta$WD40-coupled beads in binding buffer (25mM HEPES pH 7.1, 125mM potassium acetate and 1mM DTT) for 4 h at 4°C on a rotating wheel. The beads were washed three times with binding buffer and resuspended in Laemmli buffer. The eluted proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent autoradiography. 10% of the *in-vitro* translated proteins were run as input control.

Expression Constructs

PCR-amplified products of either N-terminal Flag-tagged or untagged mouse and human full-length *FAM96A* cDNAs (aa 1-160), N-terminal Flag-tagged human *AVEN* (full-length) and N-terminal Flag-tagged mouse *FADD* (full-length) were cloned into the mammalian expression vector, pcDNA3.1(-) Zeo (Invitrogen, Darmstadt, Germany). Human full-length *APAF1$_S$* (aa 1-1194) cloned into the mammalian expression vector, *pcDNA3.1/myc-His(-)A*, was kindly provided by X. Wang (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center at Dallas, Dallas, TX).

Western Blot Analysis

Cells were lysed in 10mM Tris-HCl (pH 7.4), 1mM EDTA (pH 8.0), 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM dithiothreitol (DTT) and a protease inhibitor cocktail (Roche). The lysates were sonicated and centrifuged for 10 min at 15,700 *g* at 4°C. The proteins separated by SDS-PAGE electrophoresis were transferred onto 0.45-µm nitrocellulose membranes (Whatman, Dassel, Germany) using a TE77X semi-dry transfer unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA). To prevent nonspecific antibody binding, the membranes were blocked in TBS-T (0.1% Tween in 1xTBS) supplemented with 5% skim milk powder for 1 h at room temperature before incubation in TBS-T plus skim milk with primary antibodies. which were applied overnight at 4°C at the following dilutions: anti-Flag antibody (M2, Sigma-Aldrich; 1:1,000), anti-Myc antibody (9B11, Life Technologies; 1:1,000), goat anti-β-Actin antibody (C-11, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:10,000), mouse anti-β-Actin antibody (Santa Cruz; 1:3,000), polyclonal rabbit anti-APAF1 antiserum

(BD Biosciences; 1:1,000), polyclonal rabbit anti-FAM96A antiserum raised in our laboratory (1:250; see below), monoclonal rabbit anti-ferritin heavy chain (H-FT, Abcam; 1:600), monoclonal mouse anti-IRP1 (295B, kindly provided by R. Eisenstein (Madison, WI, USA); 1:3,000), and monoclonal mouse anti-transferrin receptor (TfR; H68.4, Santa Cruz; 1:1,000). The membranes were then further washed with blocking buffer and incubated with peroxidase-conjugated secondary anti-mouse (1:1,000) or anti-rabbit (1:2,000) IgG antibodies (GE Healthcare, Freiburg, Germany) in phosphate-buffered saline (PBS; pH 8.1) plus 5% skim milk and 0.1% Tween 20 for 1 h at room temperature. The protein bands were detected by chemiluminescence using an ECL kit (GE Healthcare, Freiburg, Germany).

Generation of Rabbit Anti-FAM96A Antisera

Mouse full-length FAM96A protein (mFAM96A aa 1–160) was expressed in bacteria as a GST fusion protein. 500 µg of recombinant GST-mFAM96A protein was purified and injected into rabbits (Pineda, Berlin, Germany). For affinity purification, purified GST-mFAM96A was coupled to glutathione-agarose beads (Sigma-Aldrich). After pre-clearing with GST-agarose beads, the antisera were incubated with the GST-mFAM96A-coupled beads, eluted with PBS (pH 11.0) and dialyzed in PBS (pH 7.0). The protein concentrations of the different fractions were determined, and the purity of the protein fractions was tested by SDS-PAGE followed by immunoblotting. In an alternative approach, antiserum was retrieved from rabbits immunized with the FAM96A-specific peptide C-YDLIRTIRDPEKPN (hFAM96A aa 41-54) and was affinity-purified using the peptide coupled to a column. Results obtained with the two rabbit anti-FAM96A antisera were indistinguishable. The antisera's specificity and reactivity with human FAM96A were verified by Western immunoblotting (**Supplementary Figure 2**).

Co-Immunoprecipitation

HEK293T cell lysates were prepared by adding 1 ml ice-cold lysis buffer (20mM Hepes-KOH (pH 7.5), 10mM KCl, 1.5mM $MgCl_2$, 1mM EGTA and 1mM EDTA supplemented with protease inhibitors (1mM DTT, 12.5 µl PMSF (100mM), 1 µg/ml leupeptin and 2 µg/ml aprotinin)). After homogenization and centrifugation at 15,700 *g* for 15 min, the lysates were pre-cleared by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology) for at least 2 h. Meanwhile, 1 µg of the appropriate antibody per mg protein was coupled to agarose beads for 4 h at 4°C in TBS/3% bovine serum albumin (BSA). The coupled beads were washed three times with lysis buffer, centrifuged for 3 min at 830 *g* and the immune

complexes were collected overnight at 4°C. The immune complexes were washed three times with lysis buffer prior to boiling in Laemmli sample buffer for 5 min at 95°C. The immunoprecipitates were analyzed by SDS-PAGE and visualized by immunoblotting.

For co-immunoprecipitation of FAM96A with the monoclonal anti-APAF1 antibody 2E12 (Abnova, Heidelberg, Germany), HEK293T cells were lysed in Triton-Lysis buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% Triton X-100). After pre-clearing with 50 µl magnetic beads (µMACS Protein G, Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at 4°C, 2 mg lysate was incubated with 2 µg of antibody (anti-APAF1 or control isotype anti-FLIP antibody Dave 2 (Pierce Antibodies, Thermo Fisher Scientific, Rockford, IL)). The beads were washed four times with lysis buffer and separated from the supernatant by magnetic force, as described by the manufacturer. Proteins were eluted in 40 µl Laemmli-SDS buffer and further processed as described above. 5 or 10% of total cellular lysate served as input control.

Confocal Immunofluorescence Microscopy

HEK293T cells (either wildtype or transfected with the indicated constructs) were grown on glass coverslips and processed 24 h after transfection. Briefly, the cells were fixed in 2% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in 100mM PBS for 10 min. After washing, the coverslips with cells were blocked for 60 min in PBS containing 3% BSA and then incubated for 1 h with one or more of the following primary antibodies diluted 1:100 in blocking buffer: mouse anti-Myc monoclonal antibody (mAb 9B11, Life Technologies), rat anti-APAF1 mAb (18H2, Alexis, Exeter, UK) and affinity-purified rabbit anti-mFAM96A polyclonal antiserum (self-raised, 1:10 dilution). After washing the slides three times with blocking buffer for 10 min each, the slides were incubated with the appropriate secondary fluorochrome-conjugated antibodies (Alexa Fluor [AF] 488-goat anti-mouse IgG, AF546-goat anti-rabbit IgG or AF488-goat anti-rat IgG; all from Life Technologies) applied at 1:1,000 dilution at room temperature for 1 h. After washing three times in blocking buffer, the coverslips were mounted with 10 µl of VectaShield (Vector Laboratories, Burlingame, CA) containing 1 µM TOPRO-3 (Life Technologies) for DNA staining. Images were obtained using a Leica TCS SL confocal microscope equipped with an HCX PL APO oil-immersion objective (Leica; 63x; numerical aperture: 1.32); and the images were analyzed with Leica Confocal Software (LCS, Leica Microsystems, Heidelberg, Germany).

Northern Blot Analysis

Tissue-specific expression of *FAM96A* mRNA was examined by hybridizing commercial mouse and human Northern blot membranes (RNWAY Laboratories, Seoul, Republic of Korea; Human Tissue Combination B, 20 µg of total RNA per lane; mouse parenchymal tissue, 2 µg of polyA$^+$ mRNA per lane) with a ^{32}[P]-labeled *mFAM96A* cDNA probe encompassing the complete coding sequence.

Lentiviral Constructs for FAM96A Overexpression and Short Hairpin RNA (shRNA)-Mediated Knock-Down

Human and murine *FAM96A* cDNA including the deletion mutant *mFAM96A* aa 1-55 were cloned into the *LeGoiG2* vector (www.lentigo-vectors.de). For the lentiviral knock-down of human *FAM96A* in RKO cells, *pLKO.1* shRNA constructs and the corresponding non-targeting controls were purchased from Sigma-Aldrich. The shRNA sequences were as follows: Non-targeting control shRNA: 5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTG-3' (sense and antisense underlined); and human *FAM96A* shRNA: 5´-CCGGGTGGAAGTTCAGGAGATAAATCTCGAGATTTATCTCCTGAACTTCCACTTTTTG-3´.

Lentiviral Transduction

HEK293T cells (2x10^6) were transfected with packaging vectors (1.625 µg *p8.91* and 0.875 µg *pMD2.G*) and 2.5 µg of the overexpression or shRNA constructs for the production of lentiviral particles. 2x10^5 target cells (RKO, GIST48 and GIST882) were infected 48 hours later by "spin" infection. To this end, filtered (0.45 µm; Corning Inc., NY) HEK293T medium plus polybrene (8 mg/ml) were added to the cells, then the cell suspension was immediately centrifuged for 1 h at 340 *g* and 32°C. The medium was exchanged 4-6 hours later, and the infection was repeated the next day. Puromycin (2 µg/ml) was used to select for stably infected cells. Alternatively, transduced, GFP-expressing cells were sorted using a FACSAria instrument (BD Biosciences, Heidelberg, Germany).

Cell Death Assays

RKO cells were seeded in a 6-well plate ($1x10^5$ cells/well) and transfected with 1 µg of either *hFAM96A* or *pcDNA3.1* (empty-vector control). Apoptosis was induced by ultraviolet light (UV) irradiation (70-150 mJ/cm^2 UV radiation (Stratalinker 1800 UV Crosslinker, Stratagene, Amsterdam, The Netherlands). Alternatively, apoptosis was induced by exposing $2x10^5$ RKO cells to 100µM oxaliplatin for 20 hours before fixation. After 6 hours, the cells were harvested, washed with PBS and fixed with ice-cold 70% ethanol overnight at 4°C.[3] The fixed cells were stained for 20 min with propidium iodide (PI, 50 µg/ml) in 38 mM sodium citrate plus 5 µg/ml RNase A at room temperature in the dark. The apoptotic sub-G1 RKO cell fraction was quantified by flow cytometry analysis (FACSCalibur, BD Biosciences) using the CellQuest Pro software (BD Biosciences). GIST cells were seeded at $2x10^5$ cells/well in a 6-well plate 24 hours prior to the induction of apoptosis using 1µM staurosporine for 16 hours (GIST48) or 10µM imatinib mesylate for 24 hours (GIST882). The cells were harvested and stained for 20 min at room temperature with PI (50 µg/ml) in PBS supplemented with 3% FBS or 7-Aminoactinomycin D (7-AAD) (Life Technologies). PI- or 7-AAD-positive cells were quantified by flow cytometry.

Caspase-9 Activity Assays

Caspase-9 activation was measured using the Caspase-Glo® 9 Assay from Promega (Madison, WI, USA) according to the manufacturer´s protocol. Briefly, $5x10^5$ RKO cells per well were plated in 6-well plates 24 hours before UV irradiation. 6 hours later, the RKO cells were harvested for the assay in 1xPBS. Alternatively, $3x10^5$ GIST48 cells per well were plated in 6-well plates 24 hours before transient transfection using Lipofectamine® LTX (Life Technologies). 30 hours later, the GIST48 cells were incubated with staurosporine for 16 hours and harvested in 1xPBS. Cells were washed with 1xPBS and resuspended in 400 µl 1xPBS. 50 µl of the cell suspension per well were pipetted into Thermo Fisher (Waltham, MA, USA) Microlite 2+ Luminescence Microtiter 96-well plates with white walls, together with 50 µl of the Caspase-Glo® 9 reagent. After 1 hour, luminescence of each sample was quantified as relative light units in a plate-reading luminometer (LUMIstar Galaxy, BMG Labtech, Ortenberg, Germany).

Zebrafish Experiments

UV Irradiation and Cell Death Detection

UV irradiation of sphere-stage embryos was performed as previously described.[4] For the detection of apoptotic cells, terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) was performed using the In Situ Cell Death Detection Kit (Roche) as described previously.[5]

Morpholino Injections

Antisense morpholino oligonucleotides (MO; Gene Tools, Philomath, OR) were dissolved in water to a final concentration of 1mM. For the injections, MOs were further diluted to 0.3 pmol/nL in 1xDanieu's Buffer,[6] and 1.5 nL per embryo was injected at the 1-2-cell stage as described previously.[7] The MO sequences were as follows: *zFAM96A* MO: 5'-CGGACACAAGCTCCATGATCCGGT C-3'; *zFAM96A* 5mm MO control (containing 5 mismatches indicated by lowercase letters): 5'-CGcACAgAAGCTgCA TcATCCcGT C-3'; and *tp53* MO: 5'-AGAATTGATTTTGCCGACCTCCTCT-3'.

Immunoblotting

The embryos were injected with 75 pg *FAM96A-Myc* mRNA together with either *FAM96A* MO or *FAM96A* 5mm MO and grown until the shield stage (early gastrula, 6 hpf). Embryonic extracts were generated as described previously.[8] The protein samples were separated by SDS-PAGE on 10% acrylamide/bisacrylamide gels and blotted onto Hybond P membranes (GE Healthcare, Buckinghamshire, United Kingdom). Immunoblotting was performed using either an anti-Myc antibody (9E10; Roche) or an anti-pan-cadherin antibody (Sigma-Aldrich).

CGH Database

The Progenetix CGH database (http://www.progenetix.org/cgi-bin/pgHome.cgi) used in this study was originally described by Baudis and Cleary. At the time of last access (June 2014) it contained data from 31,915 tumor samples including 334 GISTs.[9, 10]

Quantification of mRNA from GIST Tissues by Real-Time RT-PCR

Extraction of RNA from frozen and paraffin-embedded tissues

Total RNA was extracted from frozen tissue sections using Trizol (Life Technologies) following the manufacturer's protocol. Briefly, 50-120 mg tissue was extracted with 1 mL Trizol, homogenized and purified by phenol/chloroform extraction. Alternatively, RNA was isolated from frozen tissue sections using RNeasy spin columns from Qiagen (Hilden, Germany) according to the manufacturer's instructions.

Total RNA from formalin-fixed, paraffin-embedded tissue was extracted using the method described by Rupp and Locker[11] and modified by Specht et al.[12] Briefly, unstained 10-µm tissue sections were prepared, and the paraffin was removed by extracting twice with 1 mL xylene for 10 min. This step was followed by rehydration by 5 min washes with 100%, 90% and 70% ethanol diluted in RNase-free water. After the final wash, the tissue sections were scraped into tubes, resuspended in 20 µL RNA lysis buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA pH 8.0, 2% SDS pH 7.3 and 500 µg/mL proteinase K (Sigma-Aldrich)) and incubated at 60°C overnight. The RNA was purified by phenol/chloroform extraction and precipitated with an equal volume of isopropanol plus 0.5 µL 20 mg/mL glycogen at –20°C. After centrifugation (30 min; 4°C; 15,700 *g*), the RNA pellet was washed with 70% ethanol and dissolved in 20 µL DEPC-H_2O.

Reverse Transcription

Total RNA extracted from frozen or formalin-fixed, paraffin-embedded tissue sections was reverse-transcribed using Superscript II reverse transcriptase (200 U/µL; Life Technologies). The reaction conditions were as follows: 65°C, 5 min; 42°C, 90 min and 90°C, 15 min. Alternatively, 1 µg total RNA was converted to cDNA using iScript reverse transcriptase (BioRad, Munich, Germany) according to the manufacturer's specifications.

Quantitative Real-Time RT-PCR

Experiments were performed using TaqMan chemistry and an Applera 5700 apparatus (Life Technologies) as described previously.[12] The labeled probes and primers were as follows (Eurofins MWG Operon, Ebersberg, Germany): *FAM96A-forward*: 5´-TTGCTCTTTGGCGACTCTTATT-3´; *FAM96A-reverse*: 5´-CTGCCACTCGCTCTTTGTCATT-3´; and *FAM96A probe*: 5´-TTATTGATGTCTTCTTCTG TTGAGTGGGTTCCTTCC-3´. The PCR reactions were performed using TaqMan Universal PCR Master Mix (Life Technologies). The PCR conditions were as follows: denaturing at 95°C for 15 s, hybridization and elongation at 60°C for 1 min; 60 cycles for paraffin-RNA and 50 cycles for cryo-RNA. Alternatively, mRNA expression was measured with an iCycler instrument (BioRad) using

standard protocols. The PCR reaction mixtures consisted of 12.5 µL 2× ABsolute SYBR Green Fluorescein MIX (Fisher Scientific, Schwerte, Germany), 0.5 µL of each target primer (10µM each) and 5 µL diluted cDNA template (1:50) in a reaction volume of 25 µL. The thermal cycling conditions employed for the iCycler were as follows: an initial denaturation/polymerase activation step of 15 s at 95°C and 40 cycles of 30 s at 95°C and 30 s at 58°C. dsDNA-specific fluorescence was measured at the end of each extension phase. *FAM96A* mRNA expression was normalized to *β-actin* (*ACTB*; primers: forward: 5´-GCGGGAAATCGTGCGTGACATT-3´; reverse: 5´-GATGGAGTTGAAGGTAGTTTCGTG-3´).

Immunohistochemical Analysis of GISTs and Multi-Tumor Tissue Microarrays (TMA)

53 de-identified, surgically resected GIST tumors were retrieved from the surgical pathology files of the Institute of Pathology, University of Erlangen, Germany. A TMA was constructed from formalin-fixed, paraffin-embedded tissue blocks by standard techniques.[13] Briefly, tissue cylinders with a diameter of 2.0 mm were punched from representative areas of each donor tissue block and introduced into a recipient paraffin block using a home-made semiautomated tissue arrayer. TMA sections were mounted on charged slides (SuperFrost™Plus, Menzel GmbH, Braunschweig, Germany). Hematoxylin- and eosin-stained TMA sections were used for reference histology.
Slides were subjected to 5 min heating at 120°C in Tris-EDTA-Buffer pH 8.5 in a pressure cooker. Endogenous peroxidase activity was quenched by 5 min incubation with Peroxidase-Blocking Solution (catalog no. s2023, Dako, Hamburg, Germany) at room temperature, then the slides were washed with buffer (catalog no. 3006, Dako) for 5 min. The sections were incubated with affinity-purified rabbit anti-mFAM96A polyclonal antiserum (self-raised, 1:20 dilution) for 30 min and washed again for 5 min. The slides were subsequently incubated with a labeled polymer-horseradish peroxidase (EnVision, catalog no. K5007, Dako) for 30 min and washed for 5 min. Antibody binding was visualized using 3,3'-diaminobenzidine in chromogen solution (DAB+ Substrate, catalog no. K5007, Dako). Tissue sections were counterstained with hematoxylin (catalog no. 1051750500, Merck, Darmstadt, Germany).

20 de-identified, surgically resected GIST tumors archived as formalin-fixed, paraffin-embedded blocks at the Mayo Clinic (see **Supplementary Table 2** for demographic and clinicopathological information) were sectioned at 4-6 µm thickness and processed for FAM96A immunohistochemistry using Leica BOND-III fully automated immunohistochemistry stainer and the following reagents: Epitope Retrieval Solution 1 (Leica AR9961), Bond Antibody Diluent (Leica AR9352), Polymer Refine Detection Kit (Leica DS9800), Dako X0909

Protein Block, Leica DAB Kit and hematoxylin counterstain. The rabbit polyclonal anti-human FAM96A antibody HPA040459, a Prestige Antibody® developed and validated by the Human Protein Atlas project (www.proteinatlas.org) (Sigma-Aldrich, St. Louis, MO; (immunogen: RIMEEKALEVYDLIRTIRDPEKPNTLEELEVVSESCVEVQEINEEEYLVIIRFTPTVPHCSL), was applied at 1:150 dilution for 45 min. Glandular cells of the stomach were used as internal positive controls; additional positive controls included squamous epithelium of the cervix and normal breast tissue. Blind scoring was performed by Dr. Jason T. Lewis, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA. Genotyping of the same tissues for common *KIT* and *PDGFRA* mutations was performed by PCR and sequencing by the Molecular Anatomic Pathology Laboratory Research Service, Department of Pathology and Laboratory Medicine, Mayo Clinic, Rochester, MN, USA (Director: Dr. Andre M. Oliveira).

Double Immunofluorescent Labeling of Normal Human Gastric Tissues for KIT and FAM96A

5 de-identified gastric tissues from bariatric surgeries were fixed with 4% paraformaldehyde, incubated in 30% sucrose overnight and frozen in OCT embedding compound (Sakura Finetek, Torrance, CA, USA). 12 µm cryosections were blocked in 1% BSA/0.3% TritonX-100 in PBS and double-labeled with mouse monoclonal anti-human KIT antibody (clone: 0.N.183; isotype: IgG_{2a}; 5 µg/mL; US Biological, Salem, MA, USA) and rabbit polyclonal anti-FAM96A antibody (HPA040459; IgG; 4 µg/mL; Sigma-Aldrich; see details above) at 4°C overnight without previous antigen-retrieval. Secondary antibodies (AF594-goat polyclonal anti-mouse IgG and AF488-chicken polyclonal anti-rabbit IgG) were applied at 10 µg/mL for 60 minutes at room temperature. Slides were mounted with SlowFade Gold with DAPI (Life Technologies, Carlsbad, CA, USA). Wide-field fluorescent images were captured with a Nikon Eclipse TS-100F microscope (Nikon Instruments, Melville, NY, USA) equipped with Jenoptik MF Cool digital camera (Jenoptik AG, Jena, Germany). In the antibody preabsorption experiments, the FAM96A antibody was incubated with 10-fold molar excess of recombinant human FAM96A protein (H00084191-P01; Novus Biologicals, Littleton, CO, USA) at 4°C overnight before application to the tissue sections.

Gene Expression Studies in Sorted Mouse Cells and Cell Lines

Quantitative real-time RT-PCR was performed using previously published methods[2] and specific, intron-spanning primers (Invitrogen and Roche Applied Science, Indianapolis, IN):

Actb, forward: 5'-ATGGTGGGAATGGGTCAGAAGG-3'; reverse: 5'-GCTCATTGTAGAAGGTGTGGTGCC-3'; *Fam96a*: forward: 5'-CCTATTCTCTGCTCCCCTAAAC-3'; reverse: 5'-AGTATTGGGCTTTTCTGGGTC-3'. For detection of the *FAM96A aa1*-55 deletion construct in 2xSCS70 cells, the following primers were used: F1-55, forward: 5´-ATGCAGCGGATGTCCGGGCTG-3´; reverse: 5´-GCAAGTATTGGTCTTTTCTGGG-3´. For housekeeping gene expression, *GAPDH* and *HPRT* were analyzed: *GAPDH* forward: 5´-AATGGAAATCCCATCACCATCT-3´, reverse: 5´-CGCCCCACTTGATTTTGT-3´; *HPRT* forward: TGACACTGGCAAAACAATGCA-3´, reverse: 5´-GGTCCTTTTCACCAGCAAGCT-3´. The cDNA was amplified on a Bio-Rad CFX96 (Bio-Rad Life Science Research, Hercules, CA, USA) or a Roche LightCycler 480 (Roche Applied Science) real-time PCR detector using the SYBR GreenER qPCR SuperMix (Invitrogen). In the experiments utilizing the LightCycler 480 Relative Quantification Software (Roche Applied Science), the crossing point values for each amplification curve were calculated using the LightCycler 480 Abs Quant/2nd Derivative Max method.

Temperature-sensitive SV40 large T antigen was detected by Western immunoblotting using LI-COR technology and reagents (LI-COR Bioscience, Lincoln, NE) as described.[1] The mouse monoclonal anti-SV40 large T antigen antibody (PAb 101; IgG_{2a}; BD Bioscences, Franklin Lakes, NJ) was applied at a dilution of 1:1,000 and the rabbit anti-GAPDH antibody (Sigma-Aldrich) was applied at 1:38,000.

Supplementary Tables

Supplementary Table 1. Antibodies used for the isolation of ICCs, ICC-SCs and FLCs from day 10-20 BALB/c mouse gastric corpus+antrum *tunica muscularis*

Target	Supplier	Host/Source	Clone/ID	Isotype	Label	Final conc. or µg/10^6 cells
Kit[a]	eBioscience	Rat mc[b]	ACK2	IgG_{2b}, κ	APC[c]	5 µg/ml
Kit	eBioscience	Rat mc	2B8	IgG_{2b}, κ	APC	0.25 µg[d]
Pdgfra[e]	eBioscience	Rat mc	APA5	IgG_{2a}, κ	PE	0.25 µg[d]
Cd11b[f]	eBioscience	Rat mc	M1/70	IgG_{2b}, κ	PE[g]-Cy7[h]	0.0312 µg[d]
Cd45[i]	eBioscience	Rat mc	30-F11	IgG_{2b}, κ	PE-Cy7	0.0312 µg[d]
F4/80[j]	eBioscience	Rat mc	BM8	IgG_{2a}, κ	PE-Cy7	0.0625 µg[d]
Cd34[k]	eBioscience	Rat mc	RAM34	IgG_{2a}, κ	eFluor 450 or FITC[l]	0.2 µg[d]
Cd44[m]	BioLegend	Rat mc	IM7	IgG_{2b}, κ	APC-Cy7	0.0625 µg[d]

[a], Kit oncogene; [b], mc, monoclonal; [c], APC, allophycocyanin; [d], amount added to 100 µl of staining volume; [e], Pdgfra, Platelet derived growth factor, alpha polypeptide; [f], Cd11b, integrin alpha M; [g], PE, phycoerythrin; [h], Cy7, cyanine 7; [i], Cd45, protein tyrosine phosphatase, receptor type, C; [j], F4/80, EGF-like module containing, mucin-like, hormone receptor-like sequence 1; [k], Cd34 antigen; [l], fluorescein isothiocyanate; [m], Cd44 antigen. Suppliers: eBioscience, Inc., San Diego, CA, USA; BioLegend, San Diego, CA, USA.

Supplementary Table 2. Potential APAF1 interaction partners identified in a saturation screen by their ability to bind both CED4 and APAF1 in yeast-two-hybrid assays

Gene name	Function
FAM96A	MIP18 family protein
SF3B1	Splicing factor 3b, subunit 1
SF3B2	Splicing factor 3b, subunit 1
HERC2	E3 ubiquitin protein ligase

Supplementary Table 3. The top 10 tumor entities with the most frequent cases of *FAM96A* locus deletions, as extracted from the CGH database Progenetix (June 2014)

Tumor entity	**No. of cases with deletions involving the *FAM96A* locus/total no. of cases**	**% of cases with deletions involving the *FAM96A* locus**
GIST	165/334	49.4
Seminoma, NOS	12/29	41.4
Bronchoalveolar carcinoma	57/147	38.8
Small cell lung carcinoma	57/148	38.5
Large cell neuroendocrine carcinoma	30/80	37.5
Plasmacytoma, extramedullary	7/21	33.3
Myxopapillary ependymoma	7/22	31.8
Meningioma, malignant	19/60	31.7
Fibrosarcoma, NOS	18/59	30.5
Malignant peripheral nerve sheath tumor	37/122	30.3

Supplementary Table 4. Demographic and clinicopathological characteristics of the GIST cases in the Mayo Clinic series

Tissue	Age (yr)	Gender	Site	Histotype[a]	Neoadjuvant imatinib (mo)	Genotype[b]	FAM96A staining score[c]
GIST-1	71	M	Stomach	E+S	-	KIT exon 11 c.1669_1674del p.W557_K558del	0
GIST-2	58	M	Stomach	E	-	KIT exon 11 c.1735_1737del p.D579del	0
GIST-3	59	F	Small intestine	S	-	KIT exon 11 c.1670_1678del p.W557_V560delinsF	0
GIST-4	49	F	Small intestine	E	-	KIT exon 11 c.1669T>A p.W557R	0
GIST-5	70	F	Abdomen	S	4	KIT exon 11 c.1676T>A p.V559D	0
GIST-6	82	M	Stomach	S	-	KIT exon 11 c.1655_1666del p.M552_Q556delinsK	1
GIST-7	58	M	Rectum	S	5	KIT exon 9 c.1504_1509dup p.A502_Y503dup; negative for KIT exon 11 mutation	0
GIST-8	68	F	Abdomen	S	7	KIT exon 11 c.1669_1674del p.W557_K558del	1
GIST-9	45	M	Rectum	S	6	KIT exon 11 c.1669_1674del p.W557_K558del	1
GIST-10	81	M	Stomach (fundus)	E	24	KIT exon 11 c.1674_1676del p.K558_V559delinsN	0
GIST-11	74	M	Rectum	S	4	KIT exon 11 c.1679T>A p.V560D	1
GIST-12	82	M	Stomach	S	2	KIT exon 11 c.1654_1659del p.M552_Y553del	1
GIST-13	44	M	Abdomen	S+E	4	KIT exon 11 c.1669_1674del p.W557_K558del	0
GIST-14	71	M	Stomach	S	-	KIT exon 11 c.1727T>C p.L576P	1
GIST-15	45	F	Small intestine	S	-	Negative for KIT exon 9, 11, 13 & 17 and PDGFRA exon 12 & 18 mutations	1
GIST-16	45	F	Stomach	S	-	KIT exon 11 c.1679_1681del p.V560del; negative for KIT exon 9 mutation	0
GIST-17	61	F	Gastroesophageal junction	S	4	KIT exon 11 c.1739_1753dup p.H580_F584dup	0
GIST-18	55	M	Abdomen	S	8	KIT exon 11 c.1650_1670del p.P551_W557del	0
GIST-19	52	M	Stomach	S	-	Failed (no DNA)	1
GIST-20	80	M	Stomach	S	-	KIT exon 11 c.1713_1739dup p.D579_H580ins9; negative for KIT exon 9 mutation	0

[a], E, epithelioid; S, spindle-cell; [b], By PCR and sequencing; [c], Blind scores; scale: 0-3

Supplementary Figures

Supplementary Figure 1. Purification of ICCs, ICC-SCs and FLCs from day 10-20 BALB/c mouse corpus+antrum *tunica muscularis* by FACS. (*A*) Gating sequence. ICCs, ICC-SCs and FLCs were identified by flow cytometry in the hematopoietic marker-negative fraction of dissociated gastric corpus+antrum *tunica muscularis* as $Kit^{+}Cd44^{+}Cd34^{-}$, $Kit^{low}Cd44^{+}Cd34^{+}$ and $Kit^{-}Cd44^{-}Cd34^{-}Pdgfra^{+}$ cells, respectively.[1, 2, 14] (*B,C*) Representative projections illustrating key cell populations identified by numbers in *A*. Population boundaries were drawn along lines representing identical event distribution probabilities in contour plots. Numbers in the two-dimensional projections indicate the proportion of cells within the specified clusters as percentages of the total cell count in the projection. (*B*) Gating steps for ICC and ICC-SC purification. ⓪: Cells with light scatter properties characteristic of single live cells (LS^{+}). ①: Separation of cells of hematopoietic origin ($Cd45^{+}Cd11b^{+}F4/80^{+}$ leukocytes; HP^{+}) from other components of the gastric muscles (HP^{-}). ②: Identification of $Cd44^{+}$ cells in the HP^{-} population. ③: Kit^{-}, Kit^{low} and Kit^{+} cells in the $HP^{-}Cd44^{+}$ parent population. ④: $Cd34^{+}$ cells in the $HP^{-}Cd44^{+}Kit^{low}$ population. ⑤: $Cd34^{-}$ cells in the $HP^{-}Cd44^{+}Kit^{+}$ population. (*C*) Gating steps specific for FLC sorting. ⑥: $Cd34^{-}$ cells in the HP^{-} population. ⑦: $Cd44^{-}$ cells in the $HP^{-}Cd34^{-}$ population. ⑧: Kit^{-} cells in the $HP^{-}C34^{-}Cd44^{-}$ population. ⑨: $Pdgfra^{+}$ cells in the $HP^{-}C34^{-}Cd44^{-}Kit^{-}$ population.

Supplementary Figure 2. (*A*) Validation of the rabbit anti-mFAM96A antibody and demonstration of its cross-reactivity with hFAM96A. Cell lysates were prepared from untransfected HEK293T cells (wt) or HEK293T cells transfected either with *pcDNA3.1 hFAM96A or pcDNA3.1 Flag-hFAM96A*. Both the native and the Flag-tagged protein were readily detectable by Western immunoblotting using the rabbit antiserum raised against GST-mFAM96A. The authenticity of the immunodetection was verified with an anti-Flag antibody. (*B*) Validation of the anti-hFAM96A antibody. The anti-FAM96A antiserum raised against the hFAM96A-derived peptide YDLIRTIRDPEKPN (EMC Microcollections GmbH, Tübingen, Germany) was pre-incubated with the immunizing peptide at the ratios indicated prior to incubation with a nitrocellulose membrane loaded with lysates of Flag-hFAM96A-overexpressing HEK293T cells. The antiserum's binding to hFAM96A was readily blocked by the immunizing peptide at the lowest concentration tested thus verifying the specificity of the immunoreaction.

Supplementary Figure 3. Northern blot analysis of adult human and mouse tissues demonstrates ubiquitous FAM96A mRNA expression. RNA membranes with 20 µg of total

human RNA (upper panel) or 2 µg of polyA$^+$ murine mRNA (lower panel) were hybridized with a ^{32}[P]-labeled *mFAM96A* cDNA probe. Only a single transcript (1.2 kb for human *FAM96A*) was detected.

Supplementary Figure 4. FAM96A is required for UV-induced apoptosis in zebrafish embryos. Antisense morpholino oligonucleotides targeting *zFAM96A* (*zFAM96A* MO) or *tp53* (*tp53* MO; positive control) were injected into 1-2-cell zebrafish embryos (0-1 hours post fertilization; hpf). Embryos injected with a morpholino bearing 5 mismatch mutations (*zFAM96A* 5mm MO) and uninjected embryos served as negative controls. At 4 hpf, the sphere-stage embryos underwent UV irradiation (20 s; 50 mJ/cm^2). (*A*) Apoptotic cells detected by TUNEL staining (dark brown dots) at 8 hpf in representative whole embryos. (*B*) Apoptotic cells counted in 3 UV-irradiated zebrafish embryos. Data are means±SD. Inset: Verification of knock-down efficiency by Western immunoblotting. To this end, zebrafish embryos were injected with an *in vitro*-transcribed Myc-tagged *zFAM96A* mRNA alone or in combination with *zFAM96A* MO or *zFAM96A* 5mm MO. Embryonic protein lysates prepared at 6 hpf were analyzed using an anti-Myc antibody. Cadherin was used as loading control.

Supplementary Figure 5. KIT$^+$ mast cells of the gastric mucosa do not express FAM96A. (*A*) In the *tunica mucosa*, only glandular cells but not mast cells expressed FAM96A. (*B*) Preabsorption of the FAM96A antibody with 10-fold molar excess of the immunogen completely abolished the strong mucosal labeling thus verifying antibody specificity.

Supplementary Figure 6. Overexpression of FAM96A severely reduces tumorigenicity of RKO colon carcinoma cells. 4x10^6 RKO cells stably transduced either with *hFAM96A* or the empty lentiviral expression vector, *LeGoiG2*, were injected subcutaneously into the flank of NOD/SCID mice (n=7 mice/group). Successful engraftment was detected in 5 of 7 empty-vector controls and in 3 of 7 mice injected with *hFAM96A*-transduced cells (inset). Tumor growth was significantly reduced in mice injected with FAM96A-overexpressing cells at 20 and 36 days post-injection ($P \leq 0.05$).

Supplementary Figure 7. Re-expression of FAM96A in GIST882 cells does not influence the expression of proteins involved in iron metabolism. Western immunoblotting was performed in lysates prepared from GIST882 cells transduced with *LeGoiG2 hFAM96A* or the control lentiviral vector *LeGoiG2*. Membranes were probed with antibodies recognizing ferritin heavy chain (FTH), iron regulatory protein 1 (IRP1) and transferrin receptor (TfR; the antibody detects both the monomer and the dimer due to insufficient reduction of the disulfide bonds). β-Actin and Ponceau S staining are loading controls.

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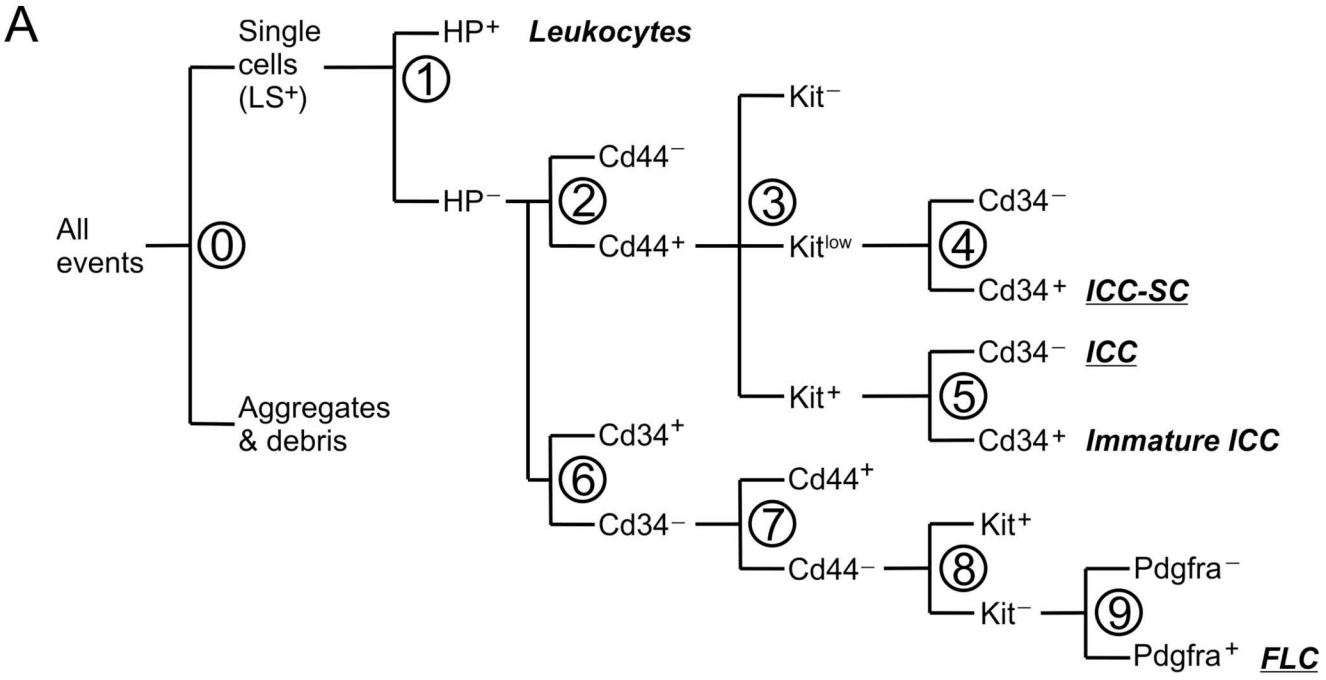

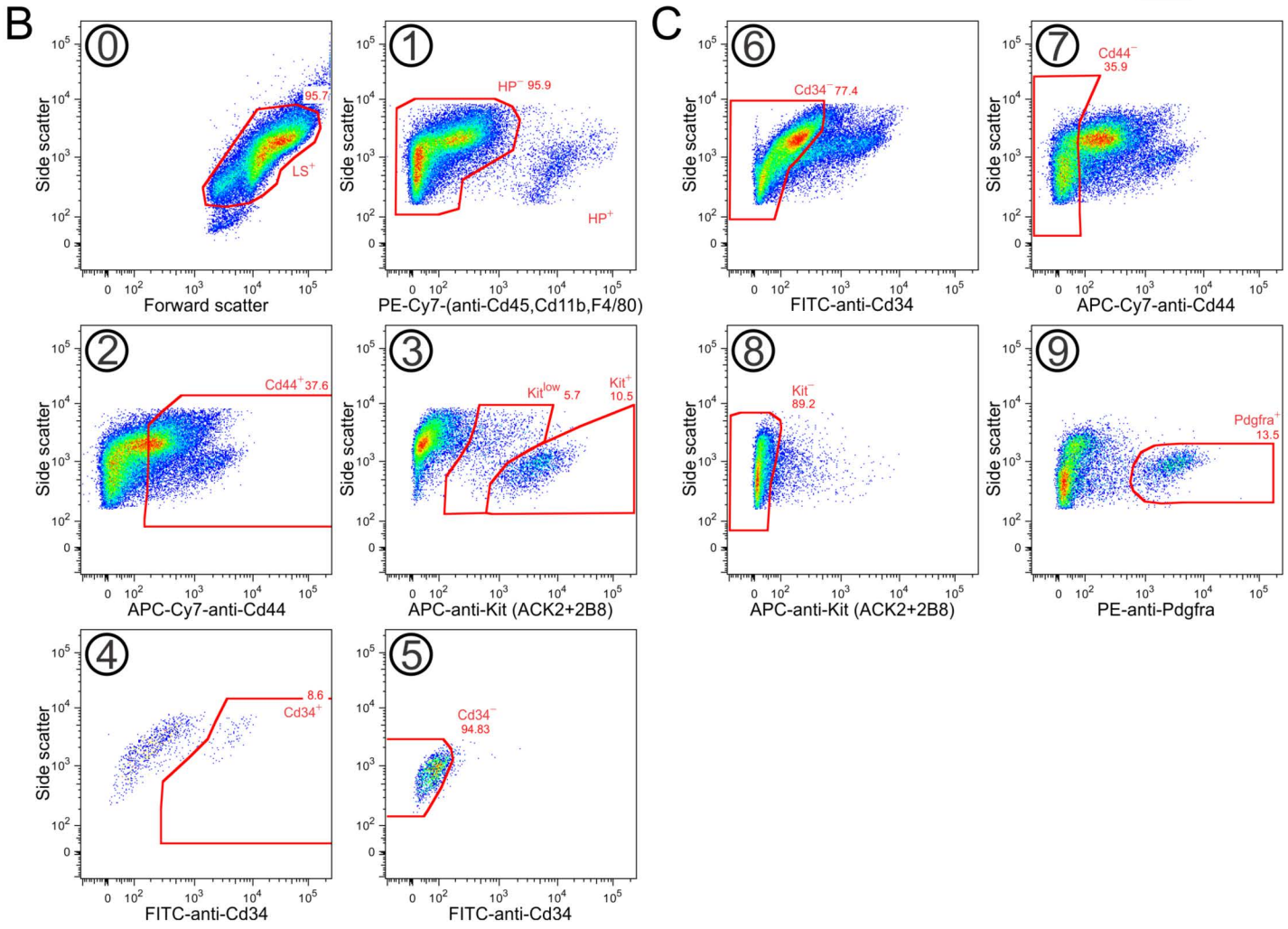

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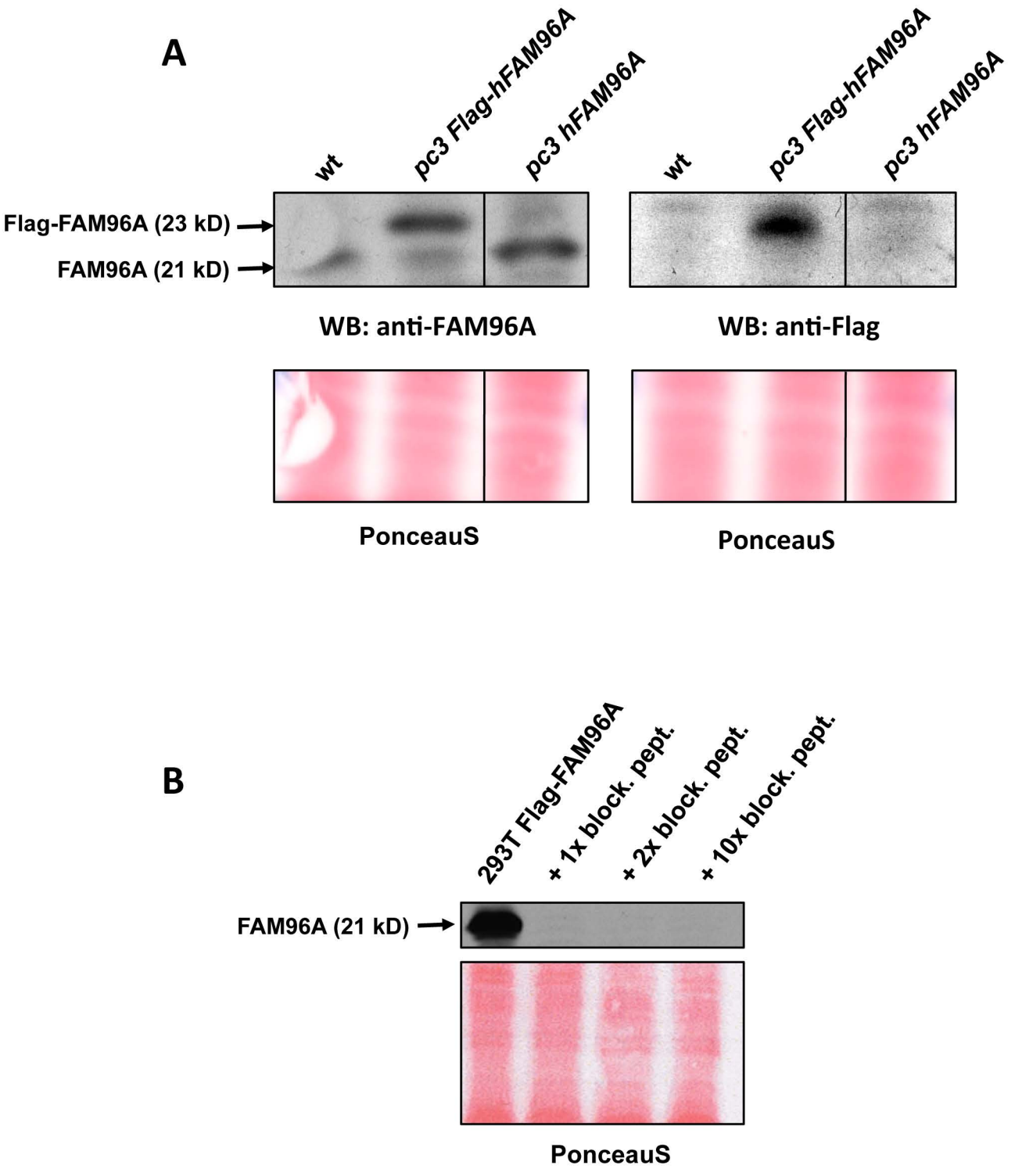

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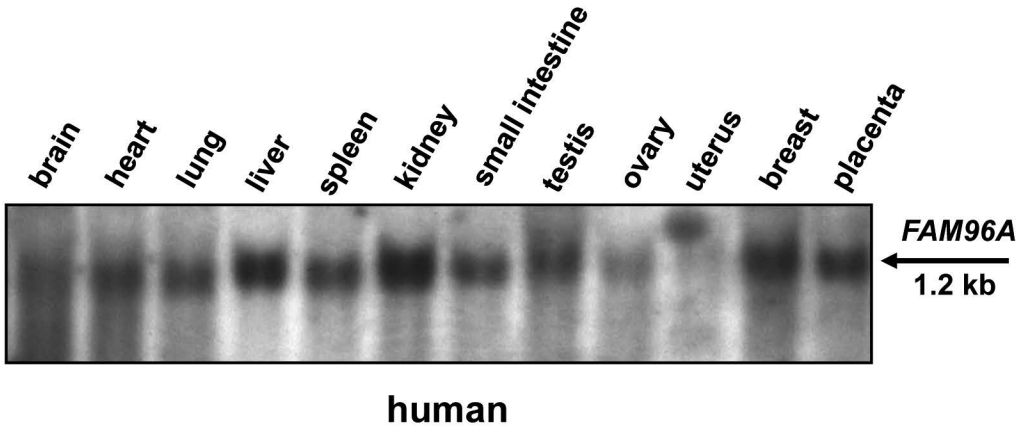

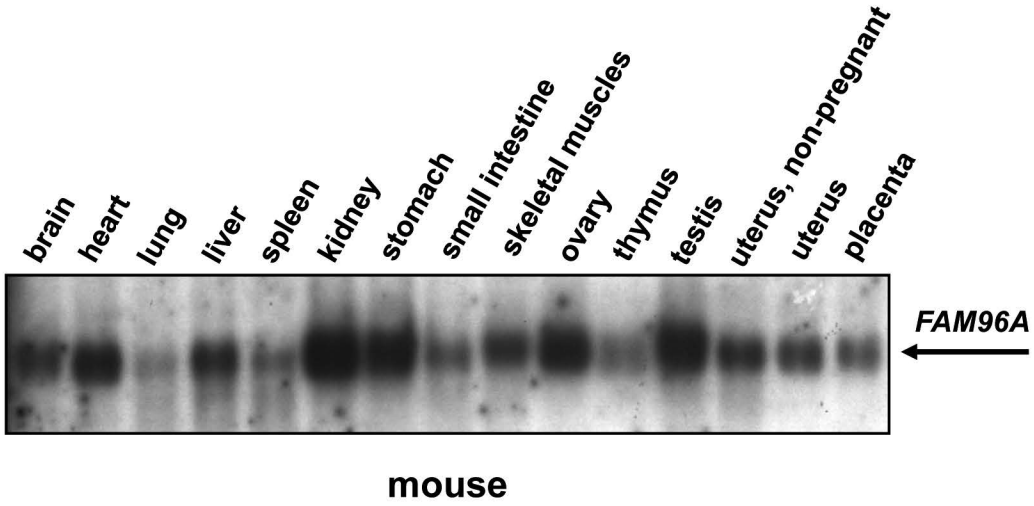

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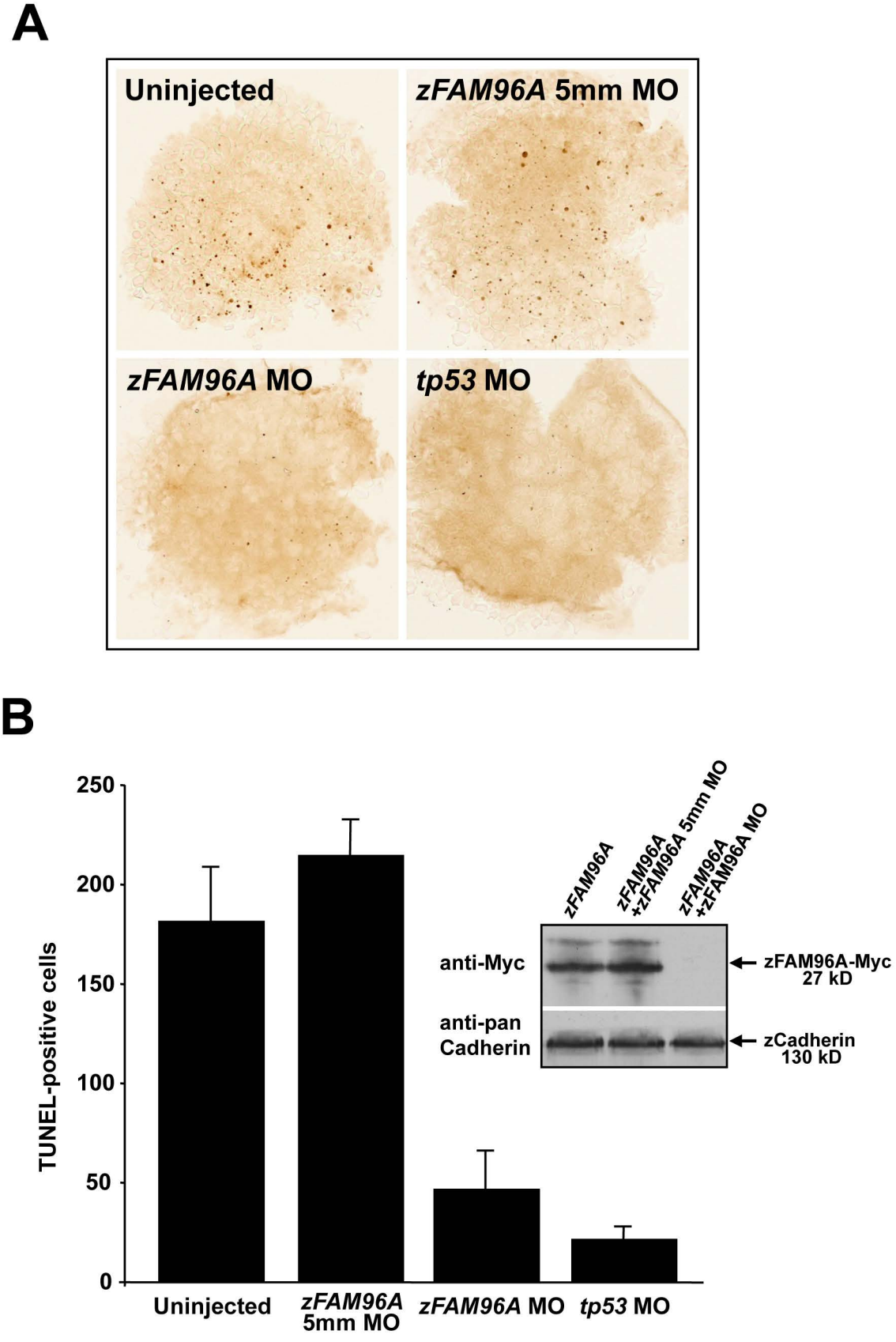

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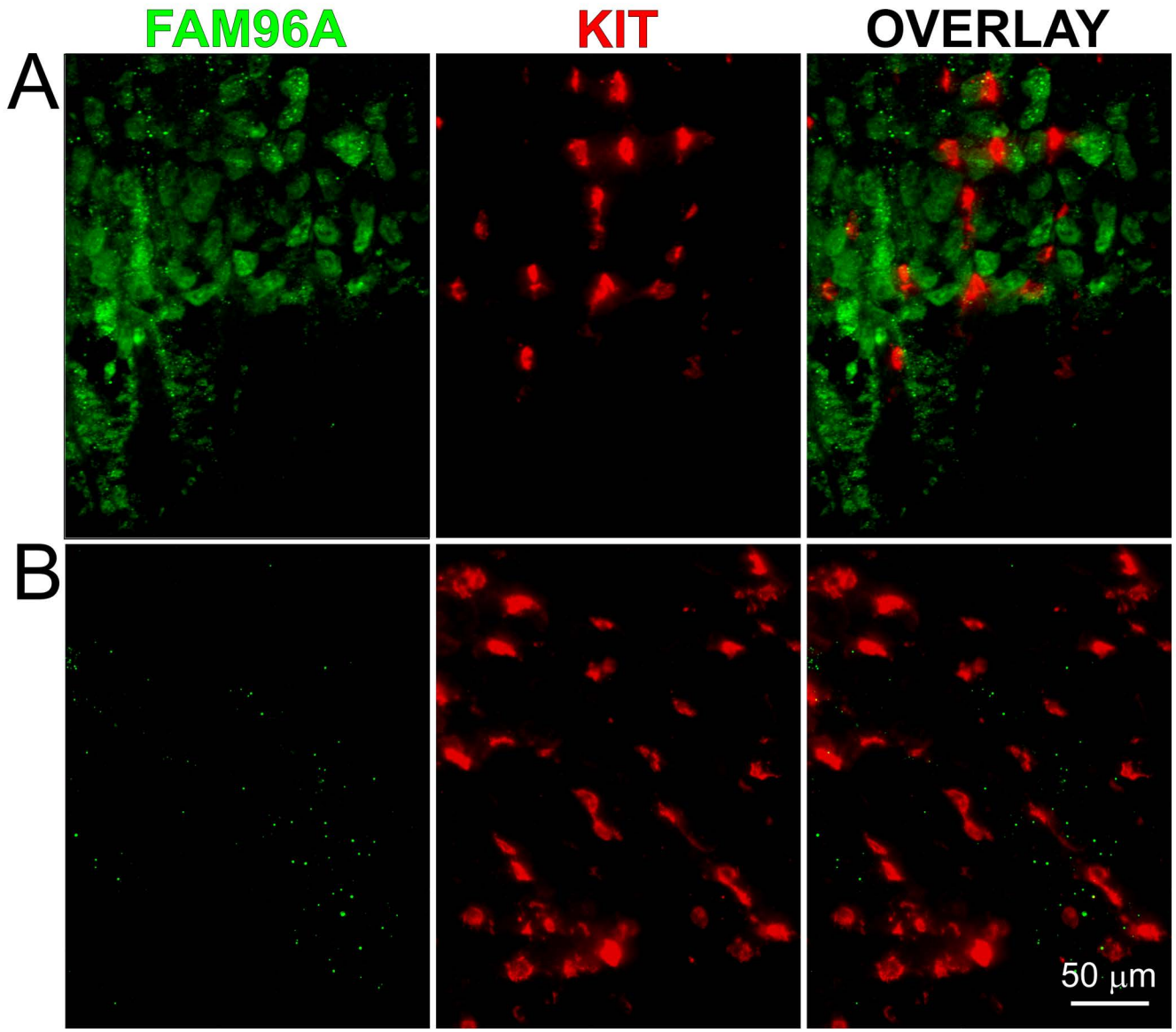

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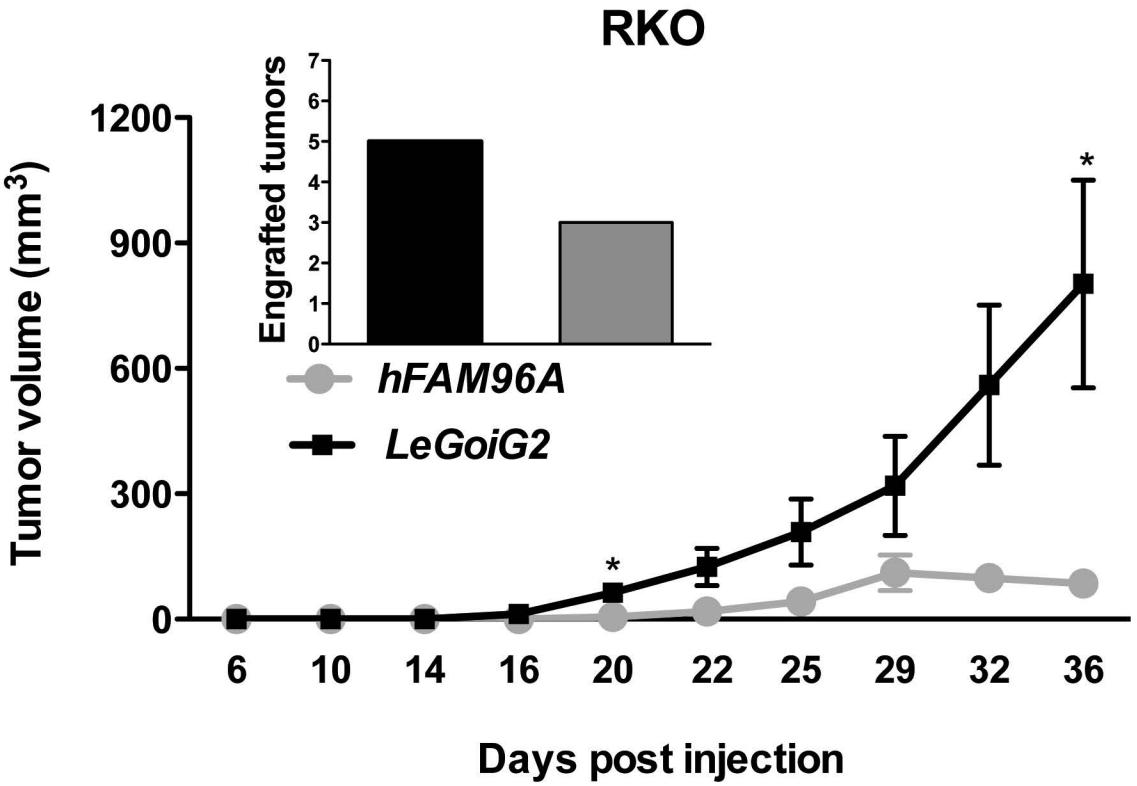

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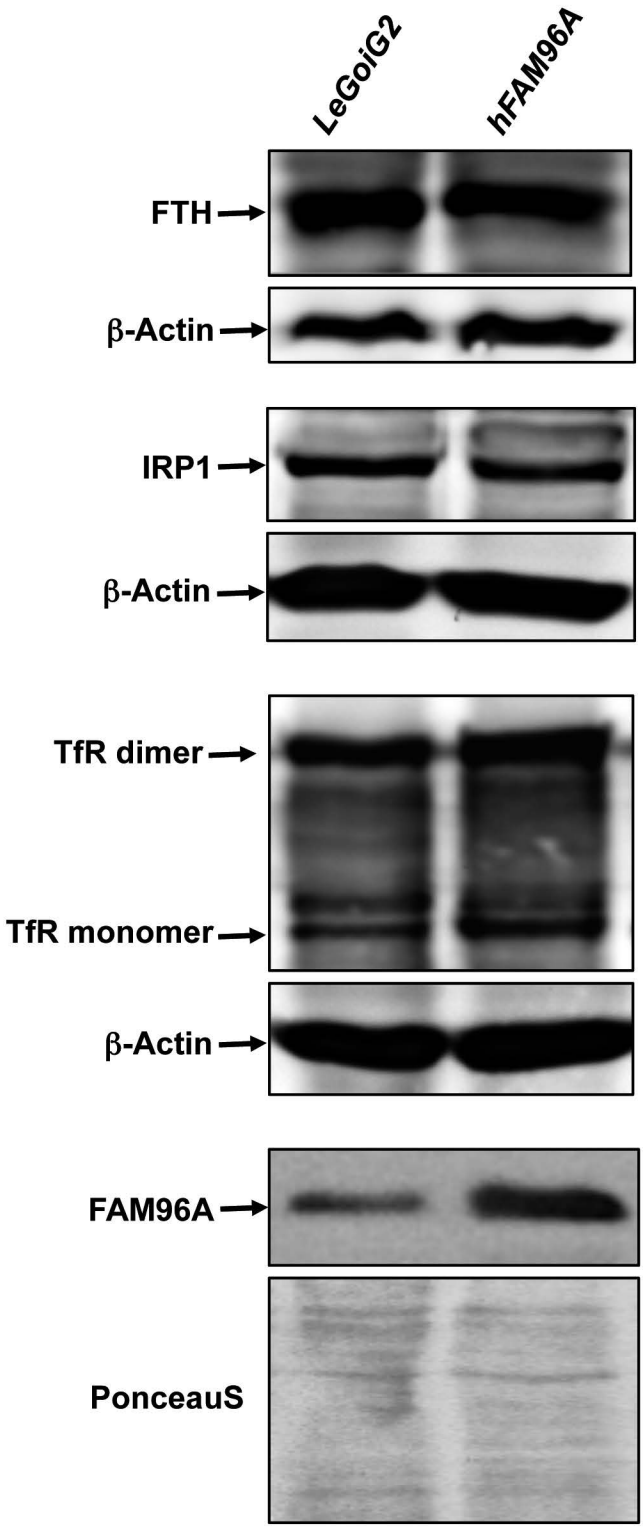

Supplementary Figure 7