PNAS

Supplementary Information for

Selective Targeting of Metastatic Ovarian Cancer Using an Engineered Anthrax Prodrug Activated by Membrane Anchored Serine Proteases

Nadire Duru[1#], Nisha R. Pawar[1#], Erik W. Martin[1], Marguerite S. Buzza[1,2], Gregory D. Conway[1], Rena G. Lapidus[2], Shihui Liu[3], Jocelyn Reader[2,4], Gautam G. Rao[2,4], Dana M. Roque[2,4], Stephen H. Leppla[3], Toni M. Antalis[1,2,5*]

[*]**Corresponding Author:** Toni M. Antalis Ph.D., Department of Physiology and the Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine, 800 West Baltimore Street, Baltimore MD 21201. Ph: 410-706-8222; Fax: 410-706-8121
Email: tantalis@som.umaryland.edu

This PDF file includes:

Supporting Materials and Methods
Figures S1 to S7

Supporting Materials and Methods

Cell culture

ES-2 (high grade ovarian serous carcinoma), OVCAR3 (high grade ovarian serous adenocarcinoma derived from ascites fluid), CAOV3 (primary ovarian carcinoma) and COV362 (high grade serous adenocarcinoma derived from pleural effusion) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and NCI/ADR-Res (multi-drug resistant adenocarcinoma derived from OVCAR8) cells from the National Cancer Institute Division of Cancer Treatment and Diagnosis (NCI-DCTD, Frederick, MD, USA) repository. IOSE397 (immortalized ovarian surface epithelial cells) were obtained from the Canadian Ovarian Tissue Bank (University of British Columbia, Vancouver, Canada). Cell lines were cultured and maintained at 37°C in a 5% CO_2/95% air environment. ES-2, CAOV3, and COV362 were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma) and 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. NCI/ADR-Res cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. OVCAR3, cells were cultured in RPMI 1640 supplemented with 20% FBS and 0.01mg/mL insulin. Cells obtained from patient ascites and IOSE cells were cultured in Medium 199:MCDB 105 (1:1) supplemented with 15% FBS, 1% non-essential amino acids and 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. All cells were routinely tested and confirmed to *Mycoplasma* negative using the MycoAlert Mycoplasma Detection Kit assay (Lonza).

Reagents

Restriction enzymes for DNA cloning were purchased from New England Biolabs Inc. Human matriptase was provided by Dr. Richard Leduc (Université de Sherbrooke, Québec, Canada) (1). Human furin was provided by Dr. Iris Lindberg (University of Maryland School of Medicine, Baltimore, MD) (2). Recombinant human hepsin, mouse testisin, human prostasin, and human MMP-2/-9 were purchased from R&D Systems Inc. Recombinant human uPA was purchased from American Diagnostica. cDNA expression plasmids encoding wild-type testisin, S238A-testisin catalytic triad mutant (S238A-Test), R41A "zymogen-locked" testisin mutant (R41A-Test) and cDNA plasmids encoding human hepsin are described previously (3). Human matriptase cDNA (4) was cloned into pEF1a-IRES-AcGFP1 vector (ClonTech Laboratories). pcDNA3.1-HAI-1 (5), and

pcDNA3.1-HAI-2 (6) were provided by Dr. Chen-Yong Lin (Georgetown University, Washington DC). pMSCV-Luciferase PGK-hygro luciferase was provided by Dr. Stuart Martin (University of Maryland School of Medicine, Baltimore, MD). pCDH-EF1-MCS-IRES-Puro was purchased from Systems Biosciences. PA-U7 is from Kerafast, Inc.

Expression plasmid mutagenesis

Two-step overlap PCR mutagenesis (7) was used to replace the P4-P4' furin cleavage site in the PA expression plasmid pYS5-PA33 with the sequences listed in Fig. 1B. pYS5-PA33 served as the template for the first round of PCR using the primers denoted 'A' (below). The resulting PCR reaction products were digested with DpnI and the mutant plasmids cloned by standard techniques. The cloned plasmids were used as the templates for the second round of PCR using primers denoted 'B' (below). The resulting PCR reaction products were digested with DpnI and the final mutant plasmids cloned and verified by DNA sequencing. For UAS, only one round of PCR was required. The product from the first round of PCR resulting from the use of the PAS primer set 'A' produced the template for PCR using the UAS 'B' primers.

Primers:

PAS 'A': F:5'GAGCTGCTAGAATCACGGGTGGAGGACCTACGGTTCC3'
PAS 'A': R:5'GGAACCGTAGGTCCTCCACCCGTGATTCTAGCAGCTC3'
PAS 'B': F:5'CTTCGAATTCACCACAGGCTAGAATCACGGGTGGA3'
PAS 'B': R:5'TCCACCCGTGATTCTAGCCTGTGGTGAATTCGAAG3'
TAS 'A': F:5'CGAATTCAAGAGCTTCTAGAATCGTGGGTGGAGGACC3'
TAS 'A': R:5'GGTCCTCCACCCACGATTCTAGAAGCTCTTGAATTCG3'
TAS 'B': F:5'CTTCGAATTCAATACCTTCTAGAATCGTGGGTGG3'
TAS 'B': R:3'CCACCCACGATTCTAGAAGGTATTGAATTCGAAG3'
UAS 'B': F:5'CTTCGAATTCACCACGTTTTAGAATCACGGGTGG3'
UAS 'B': R:3'CCACCCGTGATTCTAAAACGTGGTGAATTCGAAG3'

Purification of ZMTs and cleavage assays

Recombinant ZMTs and LF proteins were expressed using *B. anthracis* strain BH480, an avirulent, sporulation-defective, protease deficient *B. anthracis* strain, and purified as described previously (8). For cleavage assays, ZMTs (1μM) were incubated with recombinant proteases (50nM) for indicated intervals. BH480LF and FP59 used herein have the native N-terminal sequence of AGG. For PA protein cleavage assays, the

recombinant PA proteins (1 μM) were incubated with recombinant proteases (50 nM) for 2.5h, or indicated intervals, at 30˚C, in 50 mM HEPES (pH 7.3), 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% (v/v) Brij-35. Reactions were stopped by addition of Laemmli sample buffer containing 10% β-mercaptoethanol to the samples. PA cleavage was analyzed by SDS-PAGE followed by immunoblotting using a rabbit anti-PA pAb (9).

Cell lysis and immunoblotting

Cells were lysed in RIPA buffer (Sigma) with Complete Mini Protease Inhibitor Cocktail & PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Protein concentrations were determined by Bradford assay utilizing Protein Assay Dye (Bio-Rad) and samples containing equal protein were prepared with 6X LDS sample buffer containing 5% β-mercaptoethanol and heated at 70°C for 10 min. Samples were separated on SDS-PAGE gels (NuPAGE™ 4-12% Bis-Tris Protein Gels, Invitrogen) according to standard methods, were transferred by immunoblot to 0.45μm PVDF membrane (EMD Millipore) and blocked in 5% milk in PBST. Membranes were probed using the following antibodies: anti-PA Rabbit pAb (no. 5308, Stephen Leppla, NIH), rabbit anti-human p44/42 MAPK (total ERK1/2) pAb (Cell Signaling 9102), rabbit anti-human phospho-p44/42 MAPK (phospho-ERK1/2) (Thr 202/Tyr204) mAb (Cell Signaling 20G11), rabbit anti-human β-tubulin pAb (Santa Cruz H-235), goat anti-mouse-HRP and mouse anti-rabbit-HRP secondary antibodies (Jackson Immunoresearch Laboratories). HRP activity was detected by SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher). Densitometry analysis of immunoblots was performed using ImageJ software and signals were normalized to β-tubulin.

Transient transfections

HEK293T cell transient transfections were performed using Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions. Matriptase and hepsin expression plasmids were transiently co-transfected with expression plasmids encoding HAI-1 and HAI-2 respectively. The HAI proteins function as co-factors or chaperones that enhance MASP cell surface expression and protein stability. MASP expression was confirmed by qPCR at 72h post transfection. Cell surface serine protease activity was determined by fluorogenic peptide cleavage assay using Boc-QAR-AMC at 48h post transfection. For cytotoxicity assays, cells were treated at 48h post transfection with PAS:FP59 and viability assessed at 96h post transfection by MTT assay.

Real-time Quantitative PCR (qPCR)

RNA was prepared from cultured tumor cells using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. For RT-qPCR, cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). RT-qPCR analysis was performed with Taqman Fast Advanced Master Mix and Taqman primers using QuantStudio 3 Real Time-PCR System (ThermoFisher). RT-qPCR was performed using primers for human testisin (Hs00199035_m1), hepsin (Hs01056332_m1), matriptase (Hs00222707_m1), HAI-1 (Hs00173678_m1), HAI-2 (Hs01070442_m1), ANTXR1 (Hs00216777_m1), ANTXR2 (Hs00292467_m1), GAPDH (Hs02758991_g1) and β-actin (Hs99999903_m1). The TissueScan Ovarian Cancer cDNA Array II (Origene) (HORT102) containing 40 samples covering four disease stages (8 stage I, 9 stage II, 17 stage III, and 6 stage IV) of varying OvCa histopathologies, and 8 normal tissues, was screened for hepsin, testisin and matriptase mRNA gene expression using the primers listed above.

MTT cytotoxicity assays

Cells (~8,000 cells per well) were seeded onto 96-well plates and incubated overnight. Cells were treated with PAS:LF (0ng/mL-1000ng/mL) or PAS:FP59 (0ng/mL-1000ng/mL) at equal concentrations (molar ratios: PAS:LF 1:0.92; PAS:FP59 1:1.4) in 100µL growth media for 48h. For sequential treatments, cells were seeded at 5,000 cells per well and treated with PAS:LF (1000ng/mL) every 48h for a total of three treatments over a period of 7 days. For AEBSF inhibition experiments, cells were pretreated with AEBSF (100nM) for 30 min, treated with PAS:FP59 (100ng/mL) or PAS:LF (250ng/mL) for 4h, replaced with fresh media, and viability measured after 48h. To assess viability, 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen) in 1X PBS was added to each well (10µl) and incubated for 90 min at 37°C. Cells were solubilized with 0.5% (w/v) SDS, 2.5% 1M HCl (v/v), in 90% (v/v) isopropanol. Absorbance was measured using a FlexStation 3® spectrophotometer (ThermoFisher-Molecular Devices) at 570nm and 600nm (reference wavelength). Absorbance values of cells treated with PAS toxin or LF or FP59 alone were normalized to absorbance values of untreated cells. Dose-response data from replicate experiments were combined and logarithmic-transformed to determine a logarithmic trendline equation using Microsoft Excel and calculate EC_{50} values.

Spheroid formation and viability

Ovarian tumor cell lines were seeded at 2-3x10^4 cells/well on 0.75% agarose in 96-well plates and allowed to aggregate overnight. Each spheroid was treated with PAS:LF or PAS:FP59 (1000ng/mL) in 100µL growth media once for 48h or every 48h for a total of three treatments over a period of 7 days. The spheroids were imaged just before treatment, at 48h, and at 144h using the EVOS FL Auto Cell Imaging System (Life Technologies). Spheroid viability was assessed by comparing the circumference of the treated and untreated spheroids at the end of each treatment using ImageJ, and/or by measuring viability at the experimental endpoint with PrestoBlue® (Thermo Fisher; colorimetric) according to the manufacturer's instructions, comparing treated spheroids to untreated control spheroids.

Fluorogenic peptide cleavage assay

Cells were grown to ~40% confluence in 96-well, black-walled, clear-bottom plates, washed in Opti-MEM I, and incubated with 100 µM Boc-QAR-AMC peptide (R&D Systems) in Opti-MEM I, in the absence or presence of the serine protease inhibitor AEBSF (100 µM). Cleavage of the peptide and release of the AMC group was monitored at excitation 380 nm and emission 460 nm, using a FlexStation 3® microplate reader, at times indicated in the figures. AEBSF-insensitive values were subtracted from the total relative fluorescence in the absence of AEBSF at each time point. Relative fluorescence was normalized to background values in the absence of cells and then to average cell number.

Luciferase expressing OvCa cell lines for *in vivo* imaging

The ES-2-Luc cell line used for *in vivo* studies and imaging was described previously (10, 11). Luciferase was introduced into NCI/ADR-Res cells by transfection with retroviral particles expressing pMSCV-Luciferase PGK-hygro). Cleared retroviral supernatants mixed with 6 µg/mL Polybrene (AmericanBio, Natick, MA) were applied to NCI/ADR-Res cells and pools of stably transduced cells selected by hygromycin. The NCI/ADR-Res-Luc pools were subcloned in 96-well plates to isolate single cell clones. A high expressing single cell-derived colony was selected after assay for luciferase activity levels by adding 250 µg/mL D-luciferin and detecting luminescence with a Berthold Technologies Centro LB-960 plate reader. Signals were normalized for cell number.

Animal Experiments

All animal experiments were conducted in compliance with PHS guidelines for animal research and approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee (IACUC).

Orthotopic xenograft models of OvCa metastasis. Female athymic nude (Nu/Nu) mice (6-8 weeks old) or ovariectomized mice for minimal residual disease experiments were purchased from Envigo (Frederick, MD). Ovariectomized mice were shipped one-week post-surgery and allowed to acclimate for 5-7 days upon arrival. ES-2-Luc xenograft model was described previously (10). The metastatic model of NCI/ADR-Res tumor growth was established by injecting the NCI/ADR-Res-Luc ovarian tumor cell line i.p. into female athymic nude (Nu/Nu) mice (**Supplementary Figure S3**). ES-2-Luc or NCI/ADR-Res-Luc ($5x10^6$ cells in 400 µL) were administered intraperitoneally (i.p.) into mice. Tumor burden was monitored longitudinally using the *in vivo* imaging system (IVIS-Xenogen, Alameda, CA, USA). Briefly the mice were injected with 150 mg/kg of D-Luciferin Firefly (Caliper) and luminescence assessed in anesthetized mice by monitoring photon intensity every 5 min, up to 30 min using the Xenogen IVIS Spectrum instrument. Peak total photon flux (photons/seconds) was determined and corrected for tissue depth by spectral imaging using Living Image software (Xenogen). Images shown represent the peak luciferase activity levels. As per the indicated treatment strategy, mice with similar mean photon intensity representing equal average tumor burden were sorted into cohorts of 5 mice/group. Mice were injected i.p. with the indicated doses of PAS:LF in 400 µL PBS according to the treatment schedules indicated in the Figures. Tumor burden was assessed by IVIS on a weekly or biweekly schedule. After the final day of imaging, mice were euthanized, and necropsies performed. The peritoneal cavity of each mouse was opened and peritoneal organs including the diaphragm and mesenteric arteries were photographed. Organs were collected and fixed in 4% paraformaldehyde (PFA) for histological analysis.

For survival studies and humane endpoints, mice were euthanized when they exhibited substantial weight gain (> 10%), were moribund, or exhibited other signs of significant malaise and/or distress due to tumor burden. An increase in body weight of > 10% was the primary endpoint in the absence of health conditions caused by tumor burden.

Patient-derived xenograft (PDX) model of OvCa. Female *NOD.Cg-Rag1<tm1Mom> Il2rg<tm1Wjl>/SzJ* (NRG) mice used for these studies were purchased from The Jackson Laboratory and bred at UMB veterinary resources. The human OvCa PDX tumor line NCI:572874-153-T was obtained from the NCI Patient-Derived Models Repository (PDMR). The PDX tumors were maintained in NRG mice and excised, dissociated into single cell suspensions, and mixed with 33% Matrigel for subcutaneous implantation into cohorts of 6-8 week old female NRG mice. Tumor volumes were measured in two dimensions using electronic calipers and volume calculated by [length × width^2 × 0.52]. When tumors reached at least 100 mm^3, mice were randomized and sorted into groups (n = 3-5/group) so that the mean tumor volume between groups was similar. Mice were observed daily, weighed 2 times per week, and tumor volume was measured once per week. Mice were injected with the indicated doses in a volume of 50 µL (intratumoral) or 400 µL (i.p) according to the treatment schedules indicated in the Figures. Mice were euthanized when tumors reached 1 cm^3, showed necrosis or if mice reached humane endpoints. At termination, tumors were excised, weighed, and then fixed in 4% PFA for histological analysis. Liver and kidney tissues from i.p. treated mice were also collected and fixed in 4% PFA for histopathological analyses.

Histopathological analyses

Fixed tissues were embedded in paraffin and 5 µm-thick sections were cut, deparaffinized, and stained with hematoxylin and eosin (H&E) using standard procedures. For immunohistochemical analyses, sections were deparaffinized and heat induced antigen retrieval was performed using citrate buffer pH 6.0. Sections were then washed twice in TBS, blocked for 1h at room temperature with 10% goat serum+1% BSA in TBS and incubated overnight at 4°C with the primary antibody diluted in 1% BSA in TBS. Hydrogen peroxidase blocking with 0.3% H_2O_2 in TBS was performed after primary antibody incubation. Sections were washed twice and incubated with biotinylated secondary antibody for 1h at room temperature. Detection of specific antigens was performed by development with the Vectastain Elite ABC Kit (Vector Laboratories). Sections were incubated with diaminobenzidine (DAB) substrate-chromogen solution for 4.5 min. Slides were counterstained with hematoxylin prior to being dehydrated and mounted with PermaMount. Images of slides were obtained by the EVOS FL Auto Cell Imaging System (Life Technologies). All tissues were completed in a single run for consistency.

PAS Toxicity analysis

Female CD1 outbred mice (n=4/group; Envigo, Frederick, MD) were challenged with vehicle (PBS) or 2 treatment doses of PAS:LF (15μg PAS:5μg LF and 45μg PAS:15μg LF) three times a week for 2 weeks by i.p. injection. At endpoint, mice were euthanized and whole blood was collected for blood counts and chemical analyses (VRL Laboratories, Gaithersburg, MD). Major organs were removed, weighed, and colon lengths measured. Colons were emptied, washed and prepared using the Swiss roll technique (12). Tissues were fixed in 4% paraformaldehyde, paraffin-embedded, and H&E stained (Histoserv, Inc., Germantown, MD). Spleen weights are expressed as % body weight and were recorded as an indicator of inflammation. 20X and 40X images were captured using EVOS Imaging System.

Isolation of human OvCa cells from ascites fluid

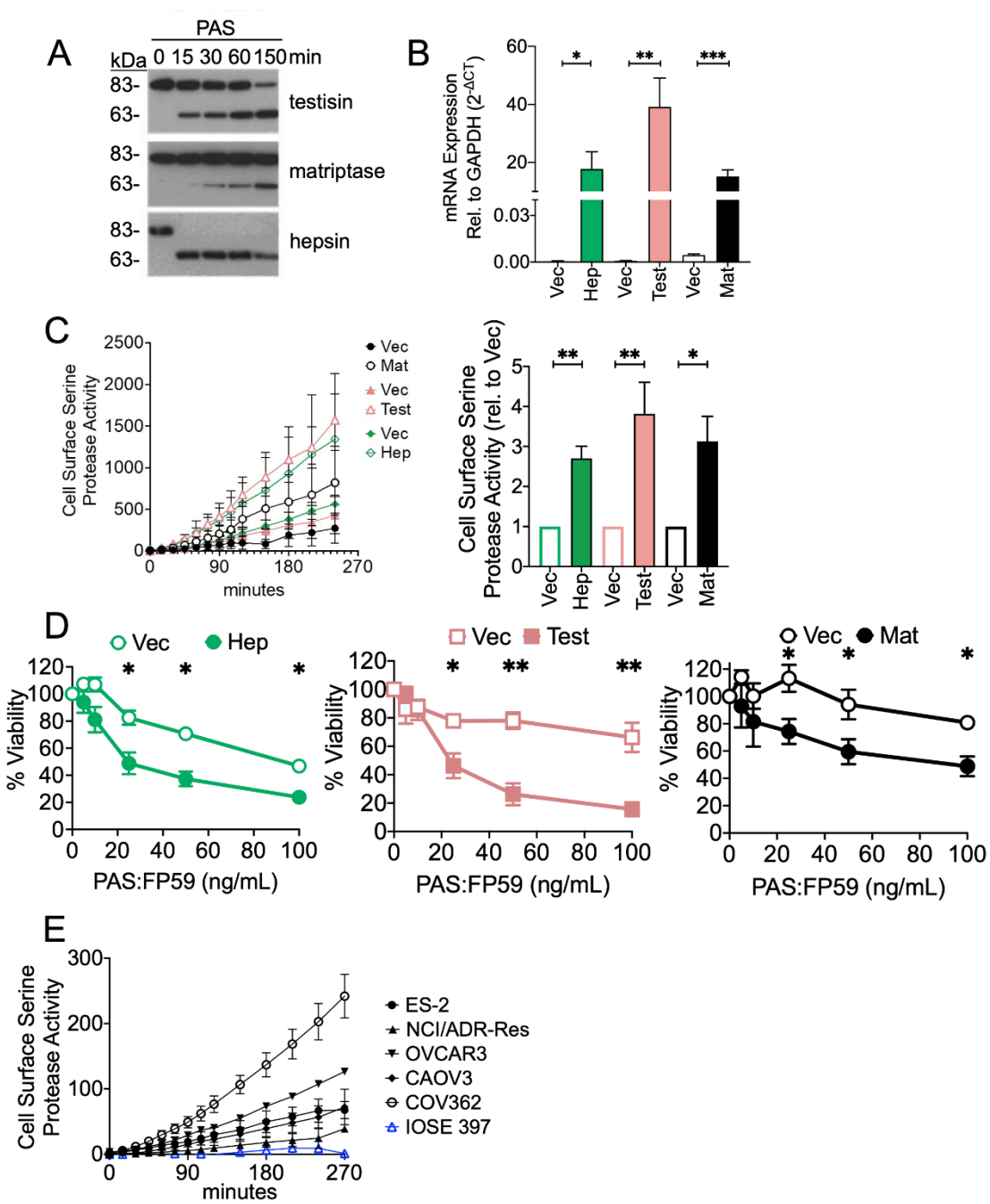
De-identified patient tumor cells and ascites fluids were used in this study. Tissues were recovered at the time of surgery in excess of pathology requirements with informed consent under protocol GCC1488. This study was approved by the Institutional Review Board of the University of Maryland, Baltimore. Ascites fluids were centrifuged at 1,000 x g for 10 min at room temperature and supernatant fluid was preserved. Cells were resuspended in ACK lysis buffer for 10 min to remove red blood cells. Cytospins from the cell suspension were prepared and were stained with Kwik-Diff™ and immunostained for key ovarian tumor cell markers CA125 (MUC16) and PAX8 (ProteinTech) to confirm tumor cell purity and percentage of tumor cells in the preparations. Stained cytospins were imaged using EVOS and fluorescent signals quantified using ImageJ (NIH). Cells isolated from ascites fluid were visualized and counted with a hemacytometer and adjusted to a concentration of $\sim 5 \times 10^5$ cells per mL for seeding on agarose-coated 96 well plates for 24h to generate organotypic multicellular spheroids. The spheroids were subjected to PAS toxin treatment and viability assessed as described above.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software Inc. San Diego, CA). Student's unpaired t-test was used to evaluate significant differences in *in vitro* studies and tumor burden in *in vivo* studies. The Kaplan-Meier plot was generated using GraphPad Prism software and significance was tested by Log-rank (Mantel-Cox) Test.

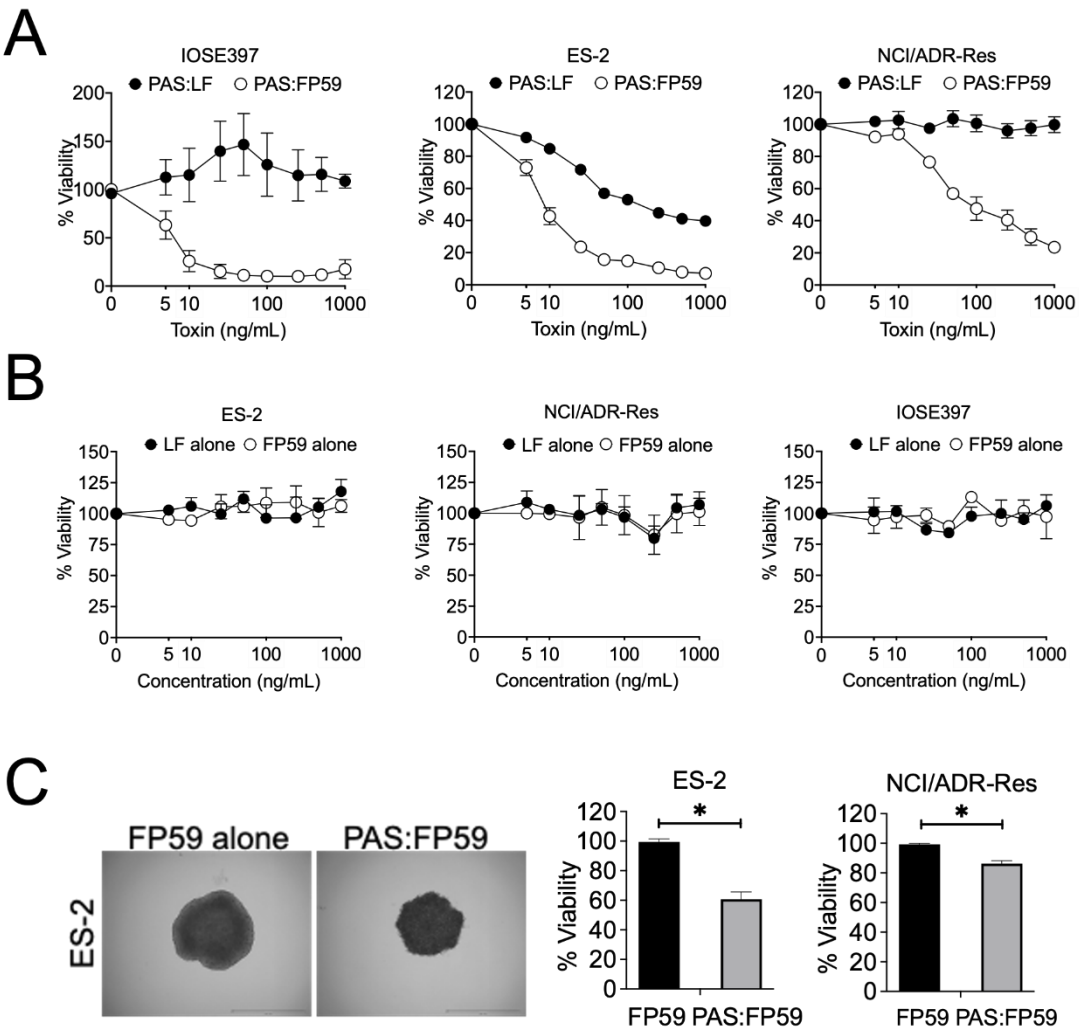
REFERENCES

1. A. Desilets, J. M. Longpre, M. E. Beaulieu, R. Leduc, Inhibition of human matriptase by eglin c variants. *FEBS Lett* **580**, 2227-2232 (2006).
2. M. M. Kacprzak *et al.*, Inhibition of furin by polyarginine-containing peptides: nanomolar inhibition by nona-D-arginine. *J Biol Chem* **279**, 36788-36794 (2004).
3. E. W. Martin *et al.*, Targeting the membrane-anchored serine protease testisin with a novel engineered anthrax toxin prodrug to kill tumor cells and reduce tumor burden. *Oncotarget* **6**, 33534-33553 (2015).
4. M. S. Buzza *et al.*, Prostasin is required for matriptase activation in intestinal epithelial cells to regulate closure of the paracellular pathway. *J Biol Chem* **288**, 10328-10337 (2013).
5. M. Oberst *et al.*, Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo. *Am J Pathol* **158**, 1301-1311 (2001).
6. C. H. Tsai *et al.*, HAI-2 suppresses the invasive growth and metastasis of prostate cancer through regulation of matriptase. *Oncogene* **33**, 4643-4652 (2014).
7. E. W. Martin (2015) Engineered Membrane-anchored Serine Protease-Activated Anthrax Toxins as a Potential Treatment Approach for Ovarian Cancer. in *Physiology* (University of Maryland, Baltimore), p 206.
8. A. P. Pomerantsev *et al.*, A Bacillus anthracis strain deleted for six proteases serves as an effective host for production of recombinant proteins. *Protein Expr Purif* **80**, 80-90 (2011).
9. S. Liu, T. H. Bugge, S. H. Leppla, Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. *J Biol Chem* **276**, 17976-17984 (2001).
10. G. D. Conway *et al.*, PRSS21/testisin inhibits ovarian tumor metastasis and antagonizes proangiogenic angiopoietins ANG2 and ANGPTL4. *J Mol Med (Berl)* 10.1007/s00109-019-01763-3 (2019).
11. E. J. Devor, J.R. Lapierre, D.P. Bender, ES-2 ovarian cancer cells present a genomic profile inconsistent with their reported history. *Obstet Gynecol Res* **4**, 233-238 (2021).
12. C. Moolenbeek, E. J. Ruitenberg, The "Swiss roll": a simple technique for histological studies of the rodent intestine. *Lab Anim* **15**, 57-59 (1981).

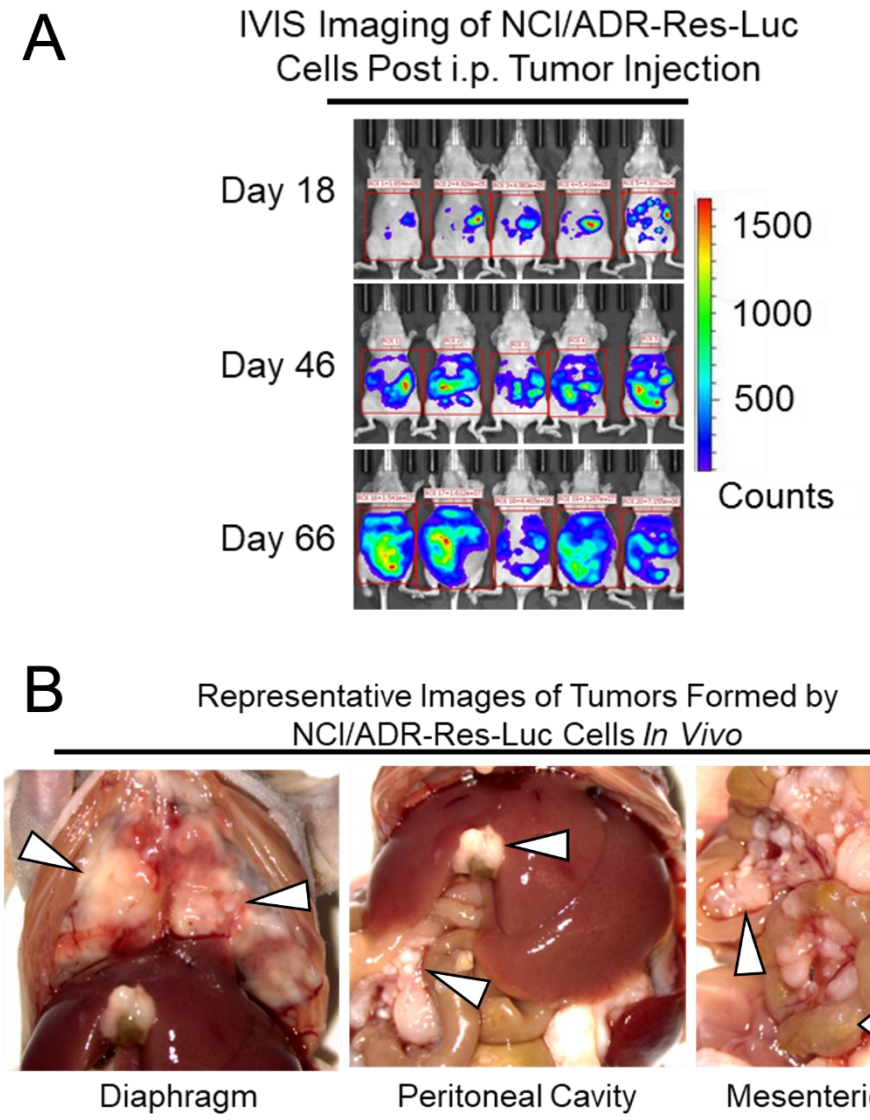


Supplementary Fig. S1. PAS activation and cytotoxicity of cells expressing the MASPs, hepsin, testisin and matriptase. (A) Time course of PAS incubation with the soluble serine protease domains of the indicated MASPs (50nM) for up to 2.5h (reactions were stopped at 15, 30, 60, 150 min). **(B)** HEK293T cells were transiently transfected with plasmids encoding MASPs (pcDNA3.1/Hepsin and pcDNA3.1-HAI-2, pDisplay/Testisin, pIRES/Matriptase and pcDNA3.1-HAI-1) or corresponding vector control plasmids. HAI-1

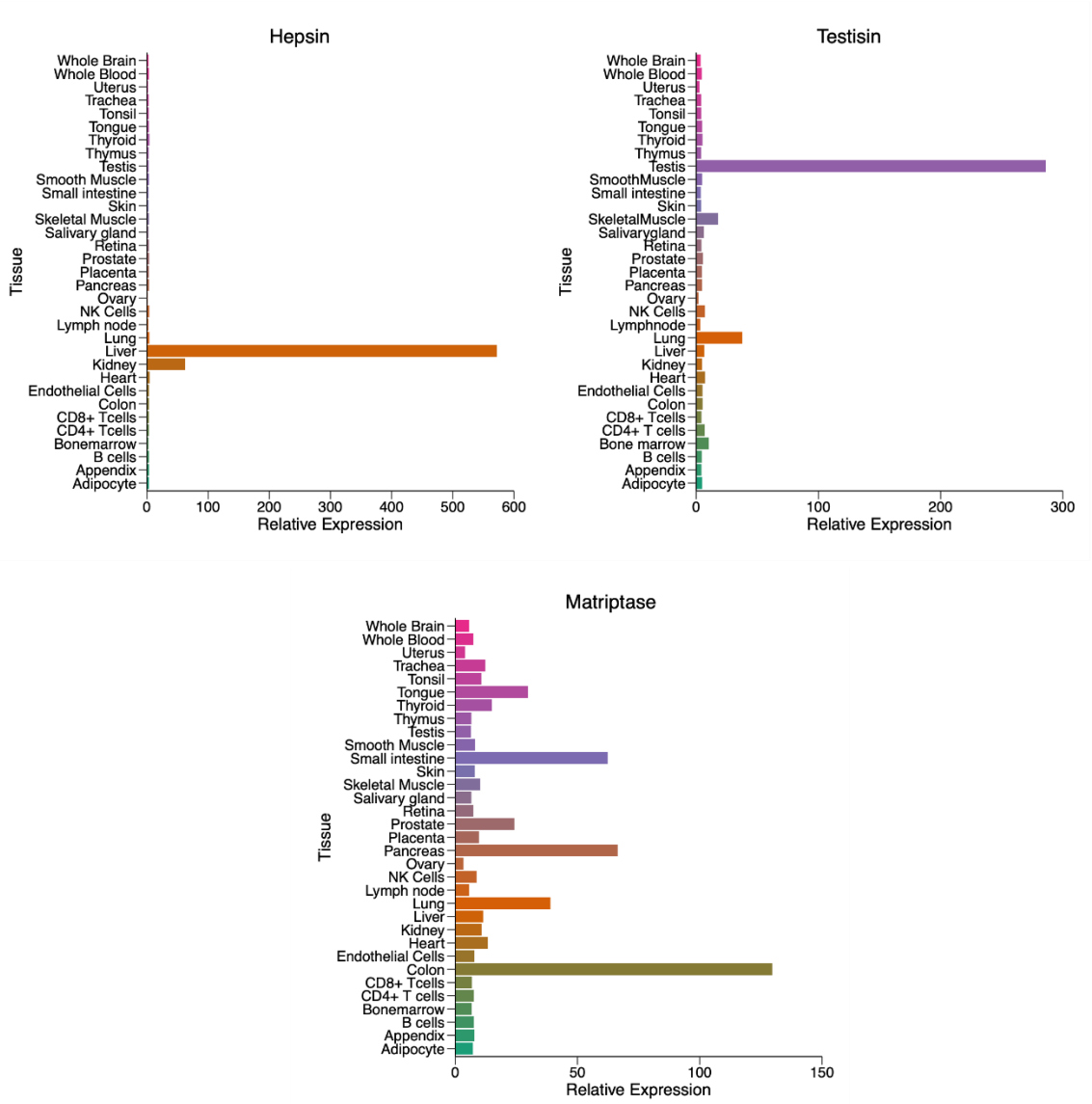
and HAI-2 are established chaperones for matriptase and hepsin, respectively, and are required for protein expression. mRNA expression of MASP expressing HEK293T cells relative to GAPDH ($2^{-\Delta CT}$) was determined by qPCR at 72h post transfection and is represented as an average ± SEM of 3-4 independent experiments performed in triplicate (*p<0.05, **p<0.01, ***p<0.005). **(C)** At 48h post transfection, AEBSF-sensitive cell surface serine protease activity in MASP expressing cells was measured over time using the fluorogenic peptide substrate (Boc-QAR-AMC) (*left panel*). Activities are normalized to cell number and data is represented as average activity ± SEM over time. MASP expressing cells show a 3-4-fold increase in cell surface AEBSF-sensitive serine protease activity compared to vector control cells Data is represented as fold change relative to respective vector controls after 240 min of incubation with Boc-QAR-AMC. Data is the average of 3 independent experiments ± SEM performed in triplicate (*p<0.05, **p<0.01) (*right panel*). **(D)** Expression of active MASPs in HEK293T cells increases PAS toxin induced tumor cell killing. The indicated MASP expressing cells were incubated with PAS:FP59 (0-100ng/mL) at 48h post transfection and cell viability was assessed 48h later by MTT assay. Data represents average percent viability relative to untreated controls ± SEM of 2-4 independent experiments performed in triplicate (*p<0.05, **p<0.01). **(E)** Comparison of cell surface serine protease activity in the panel of ovarian tumor cell lines measured by fluorogenic peptide cleavage assay. Cell lines were incubated with Boc-QAR-AMC (100μM) without or with AEBSF for 270 min. Data represent the average of triplicate values ± SEM from 2-3 independent experiments.



Supplementary Fig. S2. Cytotoxicity of PAS toxin in OvCa cell lines and spheroids. **(A)** IOSE397, ES-2, and NCI/ADR-Res cells were treated with various concentrations of PAS:LF or PAS:FP59 for 48h and viability measured by MTT assay. Data represents average viability ± SEM from 3 experiments performed with 3 or more replicates. **(B)** Cells were treated with various concentrations of LF or FP59 alone and viability measured by MTT after 48h. Data represents average viability ± SEM from 3 independent experiments performed in triplicate. **(C)** ES-2 and NCI/ADR-Res spheroids were formed on agarose-coated 96 well plates overnight, and then treated with 1µg/mL FP59 alone or PAS:FP59 for 48h. Representative images of ES-2 spheroids taken at 10X magnification are shown. Viability was measured by ImageJ quantification of the percent decrease in spheroid area compared to untreated spheroids. Data represents average viability ± SEM from 3 independent experiments performed in triplicate (*$p<0.05$).



Supplementary Fig. S3. Establishment of an Orthotopic NCI/ADR-Res-Luc Xenograft Model of Ovarian Cancer Metastasis. (A) A cohort of 5 female nude mice were injected with $5x10^6$ NCI/ADR-Res-Luc cells i.p. and were monitored at days 18, 46 and 66 by IVIS imaging to determine *in vivo* growth characteristics of the cell line. Images represent the peak luciferase activity levels in the individual mice. **(B)** Representative photographs taken at necropsy showing multiple widespread tumor nodules attached to major sites of metastasis, including the diaphragm, mesenteric arteries, and liver surrounding the gall bladder and throughout the peritoneal cavity (white arrowheads). As with the previous ES-2-Luc models, NCI/ADR-Res-Luc tumors appear as opalescent white plaques covering the organs or as white tumor foci in the peritoneal cavity. Necropsy images are representative of the tumor burden in each of the respective mice in the cohort.

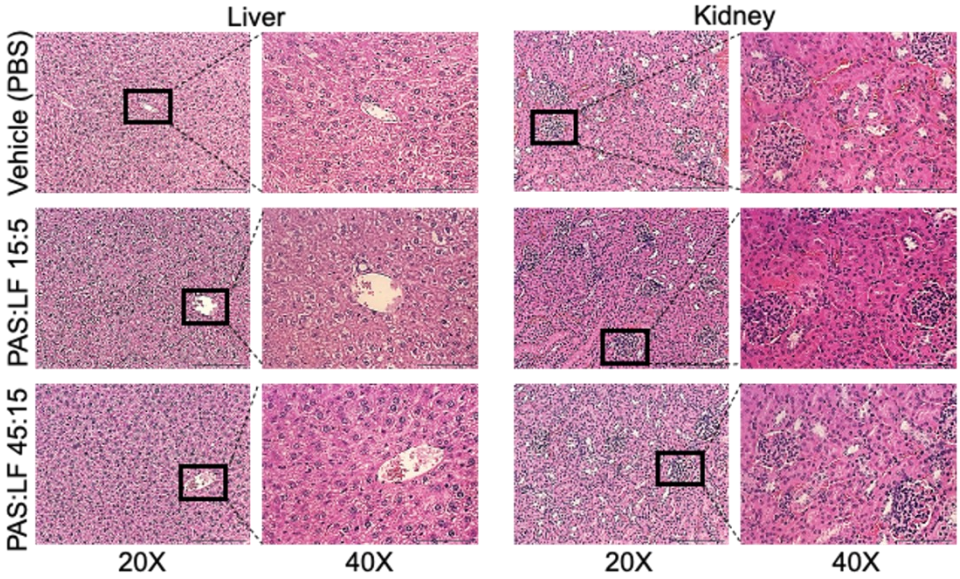


Supplementary Fig. S4. Hepsin, testisin, and matriptase have limited expression in normal human tissues. mRNA expression of MASPs hepsin, testisin, and matriptase in a range of healthy human tissues was determined using publicly available data generated from high-density oligonucleotide arrays (Human U133A/GNF1H Gene Atlas, BioGPS, The Scripps Research Institute). Data was analyzed and presented using RStudio.

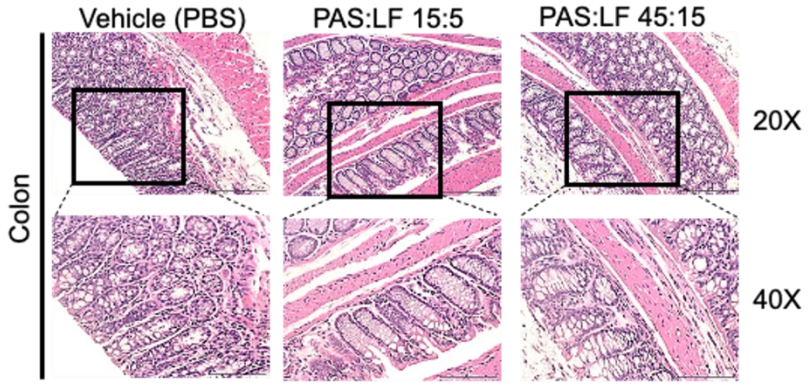
A

			Treatment Groups		
			Vehicle (n=4)	PAS:LF 15:5 (n=4)	PAS:LF 45:15 (n=4)
Bloodwork Parameter			Median		
Complete Blood Count	Absolute White Blood Cell Count (WBC	K/µL	6.91	8.66	8.16
	Absolute neutrophil Count	K/µL	8.82	7.00	6.22
	Absolute Lymphocyte Count	K/µL	14.35	12.65	8.80
	Absolute Monocyte Count	K/µL	53.55	39.90	40.80
	Absolute Eosinophil Count	K/µL	58.85	55.40	60.05
	Absolute Basophil Count	K/µL	16.20	17.05	20.90
	Absolute Red Blood Cell Count (RBC)	M/µL	785.00	669.50	652.50
	Hemoglobin (Hmg)	g/dL	0.71	1.12	1.25
	Hematocrit (Hct) %	%	5.01	5.38	3.49
	Mean Corpuscular Volume (MCV)	fL	0.16	0.10	0.10
	Mean Corpuscular Hemoglobin (MCH)	pg	0.70	0.81	1.22
	Absolute Platelet Count	K/µL	0.07	0.13	0.16
Chemistry	Albumin	g/dL	3.1	3.05	3.1
	Alanine Aminotransferase (ALT)	U/L	27.5	19	22
	Alkaline Phosphatase (ALP)	U/L	46	57	15
	Aspartate Aminotransferase (AST)	U/L	146	95	133
	Total Bilirubin	U/L	14.3	9.55	8.4
	Bicarbonate	mEq/L	0.85	0.3	0.3
	Creatinine	mg/dL	0.195	0.17	0.23
	Total Protein	g/dL	5.35	5.45	5.0

B

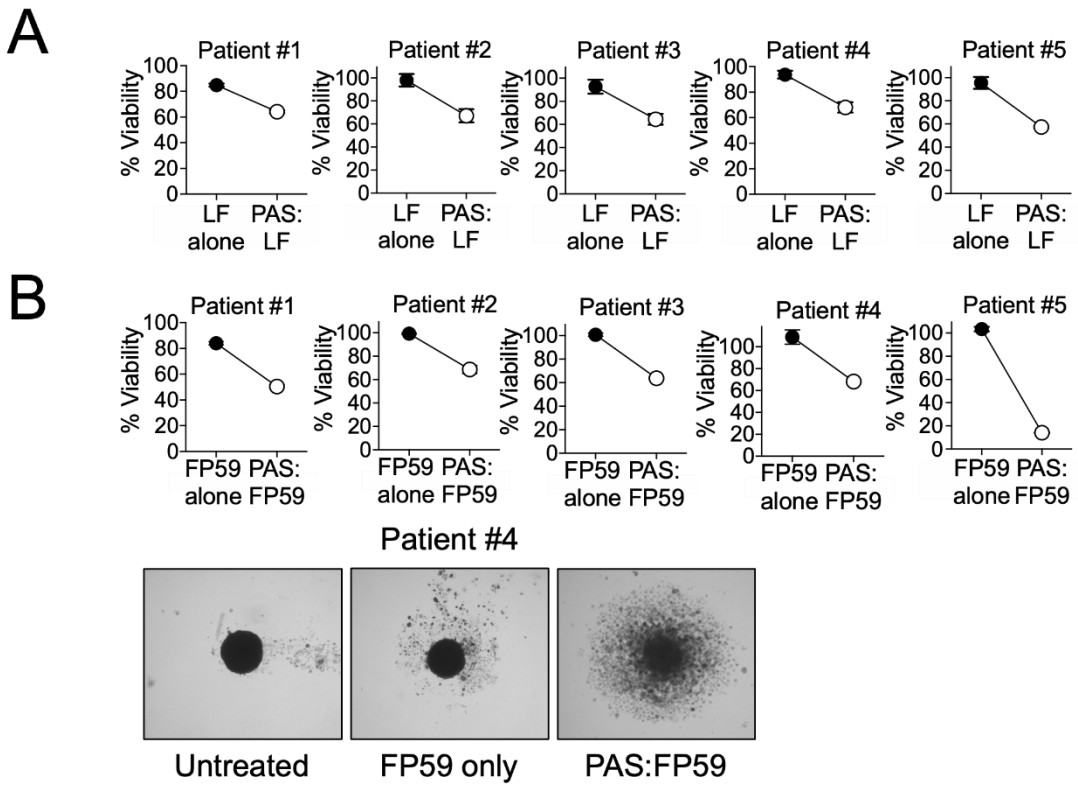


C

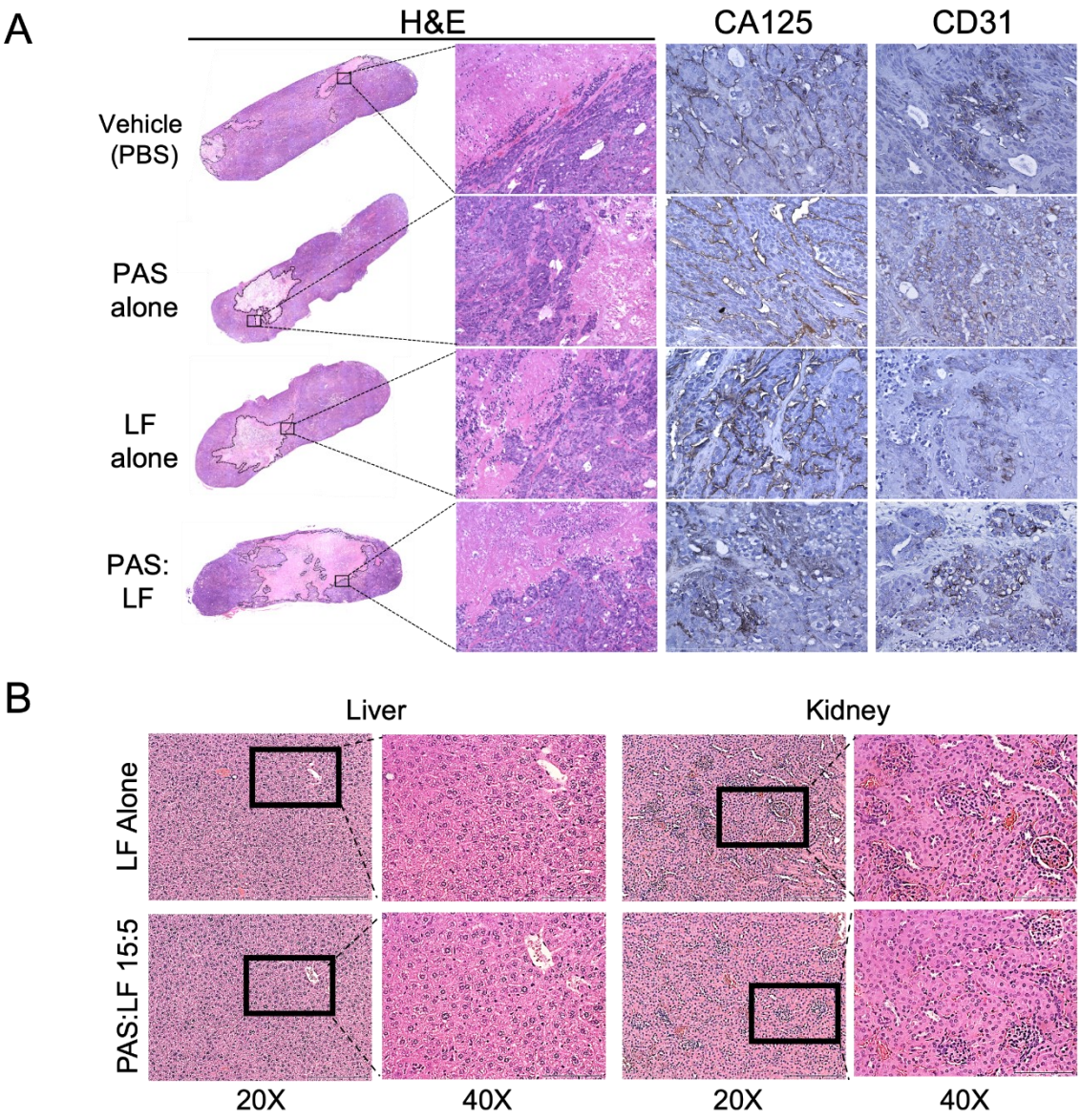


Supplementary Fig. S5. PAS:LF Toxin is well-tolerated in mice. Female CD1 mice (n=4/group) were treated with vehicle (PBS) or two different doses of PAS:LF (15:5 or 45:15) three times a week for 2 weeks. **(A)** Upon necropsy, whole blood was collected

and subjected to complete blood count and chemical analysis (VRL Laboratories). **(B)** Whole livers and kidneys were harvested, paraffin-embedded, sectioned and H&E stained. Representative images of 20X and 40X magnified areas show no gross morphological changes in PAS toxin treated organs compared to vehicle control. **(C)** Colons were prepared using standard Swiss roll technique, paraffin-embedded, sectioned and H&E stained. Representative images of 20X and 40X magnified areas show no gross morphological changes in PAS:LF toxin treated colons compared to vehicle control.



Supplementary Fig. S6. Cytotoxicity of PAS:LF toxin in patient ascites-derived OvCa spheroids. Recovered human ascites tumor cells were seeded on agarose-coated plates overnight and formed spheroids were treated twice with **(A)** LF alone or PAS:LF, **(B)** FP59 alone or PAS:FP59 (Patients 1-2: 2μg/mL, Patients 3-5: 6μg/mL) for a total of 120h. Spheroid viability relative to untreated controls was quantified via PrestoBlue™ or ImageJ and is shown as an average of 3-6 replicates ± SEM for each independent patient sample. Representative images of spheroids from Patient #4 after 120h of PAS:FP59 treatment are shown at 4X magnification (bottom panel).



Supplementary Fig. S7. PAS:LF toxin treatment of PDX models. (A) Antitumor effects of intratumoral PAS:LF treatment occur predominantly through necrosis. Ovarian PDX tumor cells were implanted and serially expanded in female NRG mice. Once tumors reached ~100mm^3, mice were treated with intratumoral injections of vehicle (PBS), PAS alone (15μg), LF alone (5μg), or PAS:LF (15μg:5μg) for a total of 7 injections. Tumors were measured over time and, at endpoint, were paraffin-embedded, sectioned, and H&E stained. Immunohistochemistry staining of the tumors were performed using CA125 and CD31 markers. Representative images of 10X whole scan and 20X magnified area of H&E stain showing the necrotic area and CA125 and CD31 staining in tumors. **(B)** Histopathological analyses of liver and kidney tissues after intraperitoneal PAS:LF

treatment. Representative images (20X and 40X magnification) of H&E-stained liver and kidney tissue of mice treated with intraperitoneal injections of PAS:LF (15μg:5μg) show no gross morphological changes.