Supplemental Methods

Reagents. N-formyl-methionyl-leucyl-phenylalanine (fMLP), LY294002 [2-(4-morholinyl)-8-phenyl-4H-1-benzopyran-4-one, Akt2 inhibitor (Akti XII) were purchased from Calbiochem. Wortmannin was obtained from Cell Signaling Technology. S-2302 (H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroaniline) was purchased from Diapharma. Probenecid, adenosine 5´-triphosphate (ATP), Ionomycin, N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), phorbol-12-myristate-13-acetate (PMA), gelatin and monoclonal anti-beta-actin antibody (A5441/AC-15) were purchased from Sigma. Purified mouse uPAR was purchased from My BioSource. Electrochemiluminescence western blotting detection reagents were purchased from ThermoFisher. CM5 chip, ethanolamine, EDC, NHS and HBS-P buffer were from GE Healthcare Life Sciences. Human FXII (1.26 mg/ml) was purchased from Haematologic Technologies Inc. Recombinant mouse FXII (0.51 mg/ml) was purchased from Innovative Research. Thioglycolate modified Brewer medium was from BD Biosciences. Sytox Green and Fluo-4-AM were purchased from Life Technologies. siRNA to hepatic FXII was generously provided by Alnylam Pharmaceuticals, Inc. PE-conjugated rat anti-mouse antibody to F4-80 (12-4801-80/BM8), rat anti-mouse to αM (12-0112-82/M1/70), PE rat IgG 2αk isotype control (12-432-42/eBR2a) were from eBioscience. PerCP-Cy 5.5-conjugated rat anti-mouse antibody against CD11b (550993/M1/70) and PerCP-Cy 5.5 rat IgG2b κ isotype control (550764/A95-1) were from BD Pharmingen. For immunostaining, purified rat anti-mouse antibody to CD11b (553308/M1/70) was purchased from BD Pharmingen; rat anti-mouse antibody to Ly6G (BE 0075-1/1A8) was from Bio X Cell; rabbit polyclonal antibody to Citrullinated Histone H3 (citrulline R2 + R8 + R17, ab1791) was purchased from Abcam. A23187 was also purchased from Abcam. Alexa Fluor 488-[donkey anti-rat (A21208), donkey anti-rabbit (A21206)] and 594-conjugated [donkey anti-rat

(A21209)] antibodies were obtained from ThermoFisher. Polyclonal antibody to total Akt (9272), phospho-Akt (Ser473; 9271) and phospho-Akt2 (Ser474; 8599) were purchased from Cell Signaling Technology. Primary polyclonal antibody against human FXII (GAFXII-AP) was from Affinity Biologicals. Mouse control IgG (2025), primary polyclonal antibody against mouse FXII (56750/G-20), CD31 (PECAM-1; 18916/MEC 13.3) and mouse monoclonal antibody to uPAR (376494/E-3) were purchased from Santa Cruz Biotechnology. 4'6'-diamidino-2-phenylindole, dilactate (DAPI) was purchased from Vector Laboratories. Signaling medium (serum-free without growth factors) was purchased from Cell Systems. DMEM/F12-10 medium was from ThermoFisher. Reduced growth factor matrigel was purchased from Corning. Pierce protein G agarose beads were purchased from ThermoFisher. CytoSelect 24-well cell migration kits (3 µm, fluorometric), and Cytoselect 48-well cell adhesion kits (ECM array, fluorometric), were purchased from Cell Biolabs, Inc. Mouse neutrophil and monocyte isolation kits were purchased from Miltenyi Biotec. LRG20 peptide was synthesized at the Department of Cellular and Molecular Medicine, Cleveland Clinic.

Skin wound assays. Full-thickness excisional wounds were made on the dorsal skin of mice under aseptic conditions. A fold of the dorsal skin was then picked up and punched with a 5-mm disposable sterile biopsy punch (Acu Punch). Two wounds were generated per mouse. External wound area was determined using an electronic caliper. Wounds were considered closed when their area relative to day 0 was less than 5%. Area was calculated with the formula: area= ¼ x length x width x 3.14. Wounds were harvested on Days 2 and 5. Skin sections from Day 2 or 5 wounds were embedded in optimal cutting temperature compound (OCT) (Tissue-Tek) and flash-frozen in liquid nitrogen. Ten µm thick sections were obtained by cryostat for immunofluorescence staining. The coverslips were mounted with DAPI. Photomicrographs were viewed on a Nikon

Eclipse TE2000-S microscope with a QImaging Retiga 2000R, Fast 1394 camera at final magnification as indicated in each figure. For all immunofluorescence and immunohistochemistry experiments, parallel sections were stained with only primary or secondary antibody as a control. ImageJ (NIH) analysis was used to determine CD11b, Ly6G, or H3Cit stained cells per high power field (HPF) or CD31 (PECAM) stained area/HPF.

Day 5 skin wounds also were harvested, fixed in 4% paraformaldehyde, embedded in paraffin and stained with hematoxylin and eosin (H&E). Photomicrographs of the sections were obtained on a Leica SCN 400 Slide Scanner equipped with a Hamamatsu line sensor color camera and a 40X/0.65 objective. Re-epithelialization of the wounds was quantitated in a standardized manner allowing for small differences in original wound size between mice that occur due to experimental variability. The wound gap, defined as the distance between the two epithelial tongues (i.e. the keratinocytes migrating into the wound bed) was measured and subtracted from the total length of the original wound size (i.e. the distance between the normal skin/wounded skin border on each side of the lesion) to provide the total re-epithelialization length. This number was then divided by the original length of the wound to supply the percent re-epithelialization. Therefore, each wound was internally controlled by taking into consideration the original wound size.

WT or *Bdkrb2*$^{-/-}$ mice were wounded as described above and wounds were harvested for immunofluorescence studies, on Days 2 and 5. Another group of *Bdkrb2*$^{-/-}$ mice had an osmotic pump placed (ALZET Model 1002**,** ALZET Corp., Cupertino, CA) such that saline or 1 mg/kg/day of bradykinin 1 receptor antagonist (R715) (Tocris Bioscience) was administered for 14 days. Wounds were created eight days after osmotic pump placement and were similarly harvested on Days 2 and 5 after wounding, for immunofluorescence studies.

For immunohistochemistry, Day 2 skin wounds were stained with antibodies against NE or double stained with antibodies against NE and pAktS^{473} (4060L, Cell Signaling). Antigen retrieval was performed with citrate buffer (Vector Laboratories) using a pressure cooker (Dako). First, slides were blocked with peroxidase, washed with PBS and subsequently blocked in normal goat serum (1:20, S-1000, Vector Laboratories) for 1 h at room temperature. Tissue was stained with a primary antibody against pAktS^{473} (1:50) overnight at 4°C. The following day, tissue was incubated with horse-radish peroxidase-conjugated anti-rabbit secondary antibody (K4010, Dako) for 1 h at room temperature and visualized by DAB (Dako) oxidation reaction. Denaturing Solution (DNS001L, Biocare Medical) was used to remove any remaining first primary antibody. Slides were washed with PBS. NE (1:100), was applied to the tissue using the same procedure and visualized by Fast Red (Enzo Life Sciences) or Vina Green (Biocare Medical). Tissue sections were counterstained with hematoxylin, dehydrated with ethanol, and mounted with Permount (ThermoFisher).

Sterile peritonitis assays. Peritonitis was induced by intraperitoneal injection of 1 ml of 5% (wt/vol) Brewer Thioglycolate (TG) Medium. At 4 h or 72 h, peritoneal cells were harvested by injection of 5 ml PBS into the peritoneum. Loaded mice were briefly massaged and 4 ml of this lavage fluid was collected with a 25 g needle, followed by a second aspiration step with a Pasteur pipette to collect any remaining fluid from the peritoneal cavity. Peritoneal lavage fluid was dispensed into a 50 ml conical centrifuge tube on ice and centrifuged for 10 min at 400 g. The supernatant was discarded and cells were resuspended in 200 µl cold DMEM/F12-10 solution. Peritoneal exudative cells were counted on a hemacytometer with trypan blue. PEC were also transferred on a glass slide and stained with Wright-Giemsa stain for manual differential counting.

siRNA studies. Lipid nanoparticle-formulated *F12* siRNA (0.47 mg/ml) (Alnylam Pharmaceuticals) and luciferase siRNA (0.38 mg/ml) were diluted in 1X PBS prior to each use.

Formulations were administered by i.v. tail vein injection at a 10 µl/g volume. WT mice received *F12* siRNA and luciferase siRNA at a dose of 0.1 mg/kg. Mice were sacrificed at 8 h, 12 h, 24 h, daily from Day 2 to 7, on Day 10. Liver was harvested for real-time PCR and blood was collected by IVC venipuncture for coagulation assays and immunoblotting. A different group of WT mice were treated with *F12* siRNA and luciferase siRNA and were wounded 24 h later. Wounds were harvested on Days 2 and 5 for immunofluorescence studies. *F12*- and luciferase-siRNA-treated mice were also subjected to TG-induced peritonitis. Peritoneal exudate fluid was harvested 4 h after thioglycolate instillation and the # of PECs was determined as above.

Flow cytometry. Peritoneal lavage fluid was collected 4 h after TG instillation and centrifuged for 10 min at 400 g. Cells were resuspended in 1 ml PBS/10% fetal bovine serum (FBS) to a final concentration of 1 x 10^6 cells/ml and incubated for 30 min with 1 µg/ml PerCP-Cy 5.5 anti-CD11b and PE-conjugated anti-F4/80 antibodies. Cells were fixed and flow cytometry was performed on a BD FACS LSR II equipment. Neutrophils were detected by CD-11b positive, F4-80 negative staining. Antibody specificity was verified using an appropriate isotype-labeled antibody (anti-rat IgG2b, κ), 1 µg/ml. Similarly, peripheral neutrophils (2 x 10^6 cells/ml) were resuspended in DMEM/F12/10, stimulated with or without 30 µM fMLP or 62 nM FXII/10 µM Zn^{2+} for 5 min and incubated with 10 µg/ml of PE-conjugated control IgG or anti-αM antibodies for 15 min. Cells were fixed and analyzed on a BD FACS LSR II equipment.

FXII reconstitution experiments. Purified human FXII or recombinant mouse FXII were administered by tail vein injection to *F12*$^{-/-}$ mice to make the plasma concentration of FXII 450 and 650 nM, respectively. Mice were immediately subjected to TG-induced peritonitis. Peritoneal exudate fluid was harvested at 4 h and the number of PEC was determined. At the time of sacrifice, blood was collected by IVC venipuncture for coagulation assays.

Preparation of mouse neutrophils and monocytes from bone marrow. Bone marrow was harvested from both femurs and tibias of donor mice and collected in PBS. The cell suspension was centrifuged at 400 g for 10 min and resuspended in DMEM/F12-10. Cells were then applied on a three-layer Percoll gradient of 78%, 69%, and 52% Percoll (Cosmo Bio USA), respectively, diluted in DMEM/F12-10, and centrifuged at 1500 g for 30 min. We obtained $6 \pm 0.6 \times 10^6$ cells per mouse, and 93 ± 2% of them were morphologically mature neutrophils.

Bone marrow-derived monocytes were isolated with magnetic bead separation system (Miltenyi Biotec) according to the manufacturer's instructions. The eluted cells were used for cDNA preparation and FXII sequencing, described below.

cDNA preparation, FXII sequencing and mRNA studies. Total mRNA was isolated from homogenized mouse livers or bone marrow-derived neutrophils and monocytes using the TRIZOL chloroform method and first strand cDNA was synthesized with Superscript III reverse transcriptase (ThermoFisher). All samples were processed in duplicate. To control for genomic contamination in samples, the reaction was also carried out in the absence of reverse transcriptase. PCR premixtures were prepared from TaqMan Universal Mastermix, water, and solutions of primers. PCR was performed with 1 µl of cDNA samples or water (control) using 3 pairs of sequential, overlapping primers to the coding *F12* region. The cycling conditions were: 2 min at 50º C, 5 min at 95º C, 35 cycles with 1 min at 95º C and 45 s at 54º C. PCR products were sequenced by the Genomics Core Facility at CWRU using the same primers. Nucleotide sequence results were uploaded in the Basic Local Alignment Search Tool (BLAST) and identified murine FXII cDNA in BM-derived neutrophils. Real-time PCR was performed on iCycler IQ5 with sybergreen (BioRad). Relative *F12* expression, normalized to control gene (18S), was determined by:

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Where $\Delta\Delta C_t = (C_{t\ F12} - C_{t\ 18S}) - \text{average}\ (C_{t\ F12} - C_{t\ 18S})$, as previously described (31).

Creation of FXII variants. FXII cDNA was introduced into pcDNA3.1+ vector and expressed in HEK293 cells under serum-free conditions. In addition to wild type FXII (rFXII WT), variants were made with proline substituting for arginine at 353 (FXII Locarno, FXII-353P) or a double variant (FXII-D) with combined R353P and alanine replacing the active site serine (S544A). The supernatant was collected 48 h after transfection, concentrated with Amicon Ultra centrifugal filters (30K, EMD Millipore), resolved on SDS-PAGE, and the recombinant FXII variants were detected with anti-FXII antibody under reducing conditions. Conditioned media from non-transfected (henceforth called "mock") and transfected cells (labeled as "media") were also collected, handled as above, and used in coagulation assays and signaling experiments.

In vitro functional assays. In order to obtain purified, resting, non-stimulated neutrophils for immunoblotting and *in vitro* functional assays, whole blood (500 µl) was subjected to red cell lysis with double distilled H_2O (9 parts) and 10X PBS (1 part), the reaction was stopped immediately by the addition of serum-free media and the mixture was centrifuged at 300 g for 10 min at room temperature. Cells were filtered through a sterile MACS 30-µm pre-separation filter to remove cell clumps, and neutrophils were separated by negative selection using the MACS magnetic bead separation system (Miltenyi Biotec) according to the manufacturer's instructions. The filtered cells were incubated with a cocktail of biotin-conjugated monoclonal antibodies against antigens that are not expressed on neutrophils in PBS containing 1% BSA for 10 min at 4º C, followed by anti-IgG microbeads for 15 min at 4º C. Cells were then loaded onto MS columns connected to the MACS magnet. The eluted cells were resuspended in 1 ml of DMEM/F12-10 medium containing 0.5% BSA, 2 mM $CaCl_2$ and 2mM $MgCl_2$ for *in vitro* functional assays; and in serum-free medium

without growth factors for signaling and immunoblotting. Neutrophils were stimulated for 5 min with 10 µM fMLP and 62 nM FXII/10 µM Zn^{2+}, unless otherwise stated.

Static neutrophil adhesion assays. WT and *F12*$^{-/-}$ neutrophils, 1 x 10^6/ml, in DMEM/F12-10 containing 0.5% BSA, 2 mM $CaCl_2$ and 2mM $MgCl_2$, were incubated with or without fMLP in BSA-coated and fibrinogen pre-coated plates. After 60 min incubation at 37ºC, media was carefully discarded from each well and cells were gently washed 3 times with PBS. Lysis buffer (1X)/CyQuant dye solution were added to each well containing cells in 1:300 ratio and allowed to incubate for 20 min at room temperature with shaking. The mixture containing cells, was transferred to a 96-well plate and fluorescence was determined in a NOVOstar plate reader (BMG-Labtech) with excitation set at 480 nm and emission at 520 nm.

Microfluidic channel chemotaxis. The microfluidic devices were fabricated by means of a lamination based technique (89). Briefly, a rectangular polymethylmethacrylate (PMMA) top piece was attached onto a glass microscope slide by using a biocompatible double-sided adhesive (DSA), which is sandwiched in between the two parts. Prior to lamination, two DSA films (50 µm) were attached on the bottom and top surfaces of a PMMA sheet (200 µm) to obtain a separation gap of 300 µm between the PMMA and glass slide. Following adhesive preparation, the 300 µm thick film was fixed on a glass slide while the upper part was still covered with a liner and a 10 µl of matrigel stock solution was perfused over the slide on ice so that it covered the surface confined by the DSA film. Two µl each of chemoattractant solution (media, 2.5 µM fMLP, or 62 nM FXII containing 10 µM Zn^{2+}) was applied into a pocket created in each channel with fluorescent beads to clearly mark the interphase. After a short period of incubation at room temperature, the top liner of the DSA film was removed and a 3.175 mm thick PMMA top piece was fixed onto the surface-functionalized glass slides by forming three separate channels each with an inlet and outlet hole.

Once the microfluidic devices were prepared, they were placed on a fully motorized inverted microscope (Olympus, model: IX83). A total number of 1 x 10^6 neutrophils obtained were loaded into the channels and allowed to adhere to the matrigel surface for 10 min. Thereafter, the bright field images in the vicinity of matrigel/chemoattractant interphases were recorded at 5 minute intervals for each channel up to 120 min at 20X magnification.

Mouse peripheral blood neutrophil and monocyte isolation. Murine peripheral blood was drawn by IVC venipuncture into sodium citrate tubes (ratio: 1 part anticoagulant to 9 parts whole blood). Peripheral monocytes were isolated with a magnetic bead separation system (Miltenyi Biotec) according to the manufacturer's instructions. The eluted cells were resuspended in serum-free medium without growth factors for immunoblotting. Neutrophils were isolated with Polymorphprep gradient (3, 4). For immunofluorescence studies, 1 x 10^6/ml neutrophils were resuspended in serum-free DMEM/F12-10 containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, plated in 35 mm glass-bottom dishes No 1.5 (MatTek Corporation) and incubated with media or fMLP (10 µM, unless otherwise stated) for 2 h. Cells were subsequently fixed with 4% formalin for 4 min, washed in PBS, permeabilized with Triton X-100 0.3% in 0.3% BSA for 3 min. Next, cells were blocked with 3% BSA for 1 h and incubated with primary antibody against a peptide from the C-terminus of FXII of mouse origin (8 µg/ml) at 4 ºC overnight. Cells were then incubated with a donkey anti-goat antibody (1:750 dilution) conjugated with Alexa Fluor 488 for 1 h at room temperature. Coverslips were mounted with DAPI. Fluorescent images were obtained using a Nikon TE2000-S microscope at magnifications indicated in each figure legend. In some experiments, cells were fixed but not permeabilized before they were stained with the antibodies.

Human neutrophil isolation and immunofluorescence studies. Blood was drawn from three healthy individuals and a FXII deficient individual after their written informed consent. Neutrophils were

isolated as murine neutrophils using Polymorphprep gradient. For immunofluorescence studies, 1 x 10^6/ml neutrophils were similarly processed but stimulated with 1 µM fMLP and incubated with primary antibody against human FXII (1 µg/ml) at 4ºC overnight. Cells were then incubated with a donkey anti-goat antibody (1:750 dilution) conjugated with Alexa Fluor 488 for 1 h at room temperature. Coverslips were mounted with DAPI. Images were acquired on a Zeiss 510 confocal microscope at various magnifications as indicated in each figure. In some experiments, cells were fixed but not permeabilized before they were stained with the antibodies.

Murine CBC. Mouse complete blood counts were measured in 50 µl of whole blood on a HEMAVET analyzer according to the manufacturer's instructions.

Coagulation assays. The activated partial thromboplastin time (aPTT) was performed by mixing 50 µl thawed citrated plasma with 50 µl pre-warmed aPTT reagent (Helena Laboratories) in a glass tube and incubated for 5 min at 37ºC. The reaction was initiated by adding 50 µl of 35.3 mM $CaCl_2$. The endpoint clotting time was determined visually by constantly tilting the cuvette in a 37ºC water bath. The prothrombin time (PT) was similarly performed by the addition of 100 µl of pre-warmed PT reagent (Thromboplastin Reagent, Helena) to 50 µl thawed citrated in a glass tube. Factor XII assays were performed using FXII-deficient plasma as substrate in an aPTT-based coagulant assay. FXII deficient plasma was obtained from George King.

Chromogenic assay. Neutrophils were seeded at 1×10^6 cells per well in a gelatin-coated 96-well plate in DMEM/F12-10 containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, incubated in the absence or presence of 10 µM fMLP, 62 nM FXII/10 µM Zn^{2+}, or increasing concentrations of FXIIa (0.62 nM, 6.2 nM, 62 nM) and 10 µM Zn^{2+}. In each well, 200 mM S-2302 was added and optical density (OD) at 405 nm was continuously monitored over 180 min on a NOVOstar microplate reader.

Surface plasmon resonance studies. Real-time biomolecular interaction analysis was performed using a BiaCore 3000 instrument (GE Healthcare, Piscataway, New Jersey). Purified mouse uPAR was covalently linked at pH 5.0 in 10 mM sodium acetate buffer to a CM5 Chip using EDC/NHS amine coupling of the primary amine of the protein to a carboxyl group of a chip linked carboxymethylated dextran using HBS-P running buffer (10 mM HEPES, 0.15 M NaCl, 0.005% polysorbate 20, pH 7.4)at 25°C. In order to prevent FXII binding onto the chip's dextran surface, 0.05% gelatin solution was injected over coated chip. Ethanolamine was used to block unwanted carboxy groups after linkage (flow rate was 30 µl/min). An ethanolamine blocked Fc1 was used as blank for background subtraction of any non-specific response to the derived dextran chip. In kinetic determinations, rising concentrations of FXII (0 nM, 1 nM, 100 nM, 400 nM), in the absence or presence of 10 µM Zn^{2+} were produced from a stock solution using the BiaCore software. Kinject was used for injection. Flow rate was 30 µl/min. The running buffer used was BiaCore HBS-P (10 mM HEPES, 0.15 M NaCl, 0.005% polysorbate 20, pH = 7.4). After each analyte injection, the chip surface was regenerated with ethanolamine injection. For affinity constant determinations, merging of triplicate injections of each concentration was performed to obtain a single sensorgram line at each concentration. A Lang-muir binding model with local fit (stoichiometry of 1:1) was used to analyse *kon* (association rate constant), *koff* (dissociation rate constant) and KD (equilibrium dissociation constant).

Neutrophil signaling studies

Cell-supernatant studies. WT neutrophils (PMNs) were washed in serum-free media without growth factors and either processed immediately (untreated; UT) or stimulated with 2.5 µM fMLP for 1, 2, and 5 min. Cells were centrifuged at 2,000g for 5 min. The cell pellet and supernatant were separated and mixed with 2X Laemmli sample buffer which was reduced with 5% β-

mercaptoethanol. Cell lysates and supernatant were loaded, resolved, and transferred onto nitrocellulose. The membranes were incubated with purified primary polyclonal FXII antibody (1:1000 dilution) for 1 h. The primary antibody was detected with a horseradish peroxidase–conjugated anti–goat immunoglobulin G (1:5000) for 1 h. The membranes were developed using the Odyssey Infrared Imaging System.

FXII immunoblotting. Wild type neutrophils (PMNs) were incubated in the absence (UT) or presence of 62 nM FXII and 10 μM Zn^{2+} for 1, 2 and 5 min. The reaction was stopped by the addition of 1 mM p-A-phenylmethylsulfonyl fluoride (APMSF) and protease inhibitor cocktail (Roche). Western blot analysis for FXII was performed under reduced and non-reduced conditions using polyclonal anti-FXII antibody. Full-length FXII was a single band at ~ 78 kDa under reducing conditions. When cleaved to FXIIa, the heavy chain was 52 kDa under reduced conditions.

Immunoblotting. For $AktS^{473}$ and $AktS^{474}$ immunoblotting, neutrophils were washed in serum-free media without growth factors and unless stated otherwise, some aliquots were pretreated with 50 nM Wortmannin, 100 μM LY294002, 10 μM Akti-XII, 10 μM TPEN, or 300 μM LRG20. Then, cells were incubated with 62 nM FXII in the absence or presence of 10 μM Zn^{2+}, 10 μM fMLP or vehicle for 5 to 7 min. Following stimulation, cells were treated at 4℃ with RIPA lysis buffer containing protease inhibitor mixture (Roche) supplemented with NaVO3 (1 mM), leupeptin (25 μM), pepstatin (25 μM), aprotinin (25 μM), NaF (25 mM), levamisole (1 mM), PMSF (1 mM), and 1% (wt/vol) NP-40. In all cases, cells lysates were mixed with 2X Laemmli sample buffer which was reduced with 5% β-mercaptoethanol. Equal amounts of protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then electroblotting onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% (wt/vol) nonfat

dried milk in TBST and incubated overnight at 4°C with antibodies to phospho-Akt (Ser^{473}), phospho-Akt2 (Ser^{474}) or anti-Akt (1:1000 dilution in 3% BSA in TBST). Primary antibodies were detected with a horseradish peroxidase–conjugated anti–rabbit immunoglobulin G (1:5000) for 1 hour at room temperature. The blots were scanned using Scion Image (v4.0) software. The band density of untreated (UT) samples was considered zero percent; band density of fMLP-treated samples subtracted from UT cell band density was set at 100%. Test sample or lane density (% Relative Densitometry Units or % RDU) was determined as follows:

(Test sample RDU- UT RDU) / (fMLP RDU – UT RDU) x 100 = % RDU.

For H3-C immunoblotting, cells were placed on ice after activation with 10 μM fMLP, 1μM PMA, or 62 nM FXII/10 μM Zn^{2+} for 2 hours and similarly treated with RIPA lysis buffer. Cells were then sonicated three times, 10 seconds each, using an aquasonic sonicator (Fisher Scientific Ultrasonic Bath 5.7L at the highest power setting) and centrifuged at 20,000 g for 30 min to remove insoluble particles. The samples were then incubated with an equal volume of 2X Laemmli sample buffer which was reduced with 5% β-mercaptoethanol. The transferred blots were blocked with 5% (wt/vol) nonfat dried milk in TBST for 1 h at room temperature. The antibodies used were anti-H3-C (1mg/ml, 1:10000 dilution) and anti-beta-actin (2.6 mg/ml, 1:10000 dilution). Blots were scanned using Scion Image (v4.0) software. The band density of untreated samples was used to calculate the fold increase in H3-C among treated samples on the same immunoblot.

Neutrophil chemotaxis migration assays

Boyden chamber chemotaxis. Wild type and *F12*$^{-/-}$ neutrophils, 1 x 10^6/ml, resuspended in DMEM/F12-10 containing 0.5% BSA, 2 mM $CaCl_2$ and 2mM $MgCl_2$, were applied to the top insert of a Boyden chamber (3 μm pore size). Media containing fMLP was added to the lower well and the chambers were incubated for 1 h. Next, media was aspirated from the top and the insert itself

was transferred to a clean well containing cell detachment solution and incubated for 30 min at 37ºC. Medium containing migratory cells was mixed well and transferred to a 96-well plate. Lysis buffer (4X)/CyQuant GR dye solution were added to each well containing cells in 1:75 ratio and allowed to incubate for 20 min at room temperature. Fluorescence was determined in a NOVOstar plate reader at 480/520 nm. Cells (1 x 10^6/ml) plated directly at the bottom well served as positive control (100% neutrophil migration); an empty bottom well containing 4X lysis buffer/CyQuant GR dye only, served as negative control (0% neutrophil migration). Fluorescence intensity was subtracted from that of negative control, normalized to the intensity of positive control and expressed as % neutrophil migration.

Fluo-4–based assay of cytosolic [Ca2+]. Wild type neutrophils, resuspended in DMEM/F12-10 containing 0.5% BSA, 2 mM $CaCl_2$ and 2mM $MgCl_2$ were briefly washed with PBS prior to the addition of 1 mM Fluo-4-AM, Pluronic F-127 (premixed with fluo-4-AM in 1:1 proportion by volume), and 2.5 mM probenecid. After incubation at 37°C for 45 min, cells (1 x 10^6/ml) were plated in a 24-well plate. The plate was placed into the Synergy HT reader preheated to 37°C. Baseline fluorescence (485 nm excitation → 528 nm emission at 30-s intervals) was recorded for 5 min. Cells were then stimulated with 5 mM ATP, 3 µM ionomycin, 10 µM fMLP, or FXII (62 nM or 100 nM) and 10 µM Zn^{2+}. Changes in 485ex→528em fluorescence were recorded at 30-s intervals. Assays were terminated by permeabilization of cells with 1% Triton X-100 to quantify the maximum Ca^{2+}-dependent fluorescence (*Fmax*) of the Fluo-4 indicator dye. The wells were then supplemented with 15 mM EGTA/50 mM Tris to chelate Ca^{2+} and quantify the minimum Ca^{2+}-independent fluorescence of Fluo-4 (*Fmin*). The *Fmax* and *Fmin* values were used to calculate the cytosolic [Ca2+] corresponding to changes in 485ex→528em fluorescence of Fluo-4 within intact cells as described by Tsien and colleagues (5).

NETosis plate reader assay. Neutrophils were stimulated with 4 µM A23187, 1 µM phorbol-12-myristate-13-acetate (PMA), 100 nM fMLP, or 62 nM FXII and 10 µM Zn^{2+} or 10 µM Zn^{2+}. Where indicated, neutrophils were pre-incubated with 5 µM Akti XII or 300 µM LRG20 for 30 min, before stimulation with FXII/Zn^{2+}. Cells were seeded at 3×10^4 cells per well in a 96-well plate in DMEM/F12-10 containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, in the presence of 5 µM Sytox Green cell-impermeable nucleic acid stain. The fluorescence was measured using NOVOstar microplate reader at specific time intervals for up to 400 min after the activation of cells. To calculate the NETotic index, fluorescence readout obtained from cells lysed with 0.5% Triton X-100 was considered as 100% DNA release, and the index was calculated as the percentage of total value at each time point. The NETotic rate was calculated from the slope of the linear regression curves of percent total DNA relative to time.

Bone marrow transplantation. Bone marrow cells of WT and *F12*$^{-/-}$ mice (6-8 weeks old) were harvested from both femurs and tibias of donor mice and collected in PBS with 2% fetal bovine serum under sterile conditions. WT and *F12*$^{-/-}$ recipient mice underwent lethal irradiation (11 Gy) before receiving 12 x 10^6 bone marrow cells in 400 µl PBS by tail vein injection. Recipient mice received water containing enrofloxacin (Baytril, Bayer) for 2 weeks. Six weeks after transplantation, marrow reconstitution was confirmed by complete blood count analysis of the transplanted mice.

Supplemental Figures

Supplemental Figure 1. *Characterization of F12$^{-/-}$ mice.* (**A**) Representative separate agarose gels from polymerase chain reaction showing the genotype of wild type (*F12*$^{+/+}$), heterozygous (*F12*$^{+/-}$) and FXII deficient (*F12*$^{-/-}$) mice run simultaneously. *F12*: 152 base pairs (bp), single NEO band 128 bp denotes *F12*$^{-/-}$ genotype. (**B**) aPTT was determined in WT and *F12*$^{-/-}$ plasma (n=5-8). (**C**) Plasma FXII coagulant activity from WT and *F12*$^{-/-}$ mice (n=5-8). (**D**) Immunoblot for FXII antigen in murine and human WT and *F12*$^{-/-}$ plasmas. This image represents non-contiguous lanes of the same blot. Each image is representative of n=5 experiments.

Supplemental Figure 2. *Reduced bradykinin signaling does not influence leukocyte migration into skin wounds.* (**A**) Number of neutrophils (Ly6G positive cells) per HPF in Day 2 skin wounds of WT, bradykinin 2 receptor knock-out (Bdkrb2$^{-/-}$), and Bdkrb2$^{-/-}$ mice treated with the bradykinin 1 receptor antagonist R715 are shown. Data represent mean ± SD (n = 6-8 mice per group). p=0.74 vs. WT control mice by one-way ANOVA with Bonferroni correction. (**B**) Number of neutrophils in Day 5 skin wounds of WT and bradykinin 2 receptor knock-out (Bdkrb2$^{-/-}$) mice. Data represent mean ± SD (n = 6 mice per group). p=0.18 vs. WT control mice by Student's t-test. Fluorescent images were obtained using a Nikon TE2000-S microscope, 20X magnification. The number of Ly6G positive cells in all groups of animals was compared by morphometric analysis using the NIH ImageJ software. These data indicate that the reduction in plasma bradykinin levels or absence of its receptors does not account for the reduced neutrophil migration seen in wounds of *F12*$^{-/-}$ mice.

Supplemental Figure 3. *Leukocyte migration in thioglycolate-induced peritonitis.* WT and *F12*$^{-/-}$ mice were injected intraperitoneally with thioglycolate (TG) solution. At 72 h, mice were subjected to peritoneal lavage and the peritoneal exudate cell (PEC) number was determined. WT: n=10, F12$^{-/-}$: n=10 (mean ± SEM. *p=0.13 vs. WT control mice by Student's t-test). Since the majority

of peritoneal exudate cells at this time point are macrophages, these data show that *F12*$^{-/-}$ mice have normal macrophage infiltration at sites of sterile inflammation at 72 h.

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Supplemental Video. Neutrophil chemotaxis in a microfluidic channel.

Supplemental Tables

Supplemental Table 1. *Hematologic parameters in WT and F12$^{-/-}$ mice*. Hematologic parameters in WT and *F12$^{-/-}$* mice. Blood cells from WT and *F12$^{-/-}$* mice were counted using an automated HEMAVET 950 analyzer (Drew Scientific). Data represent the mean ± SD (n = 6 mice per group). The prothrombin time was performed as indicated in the Methods.

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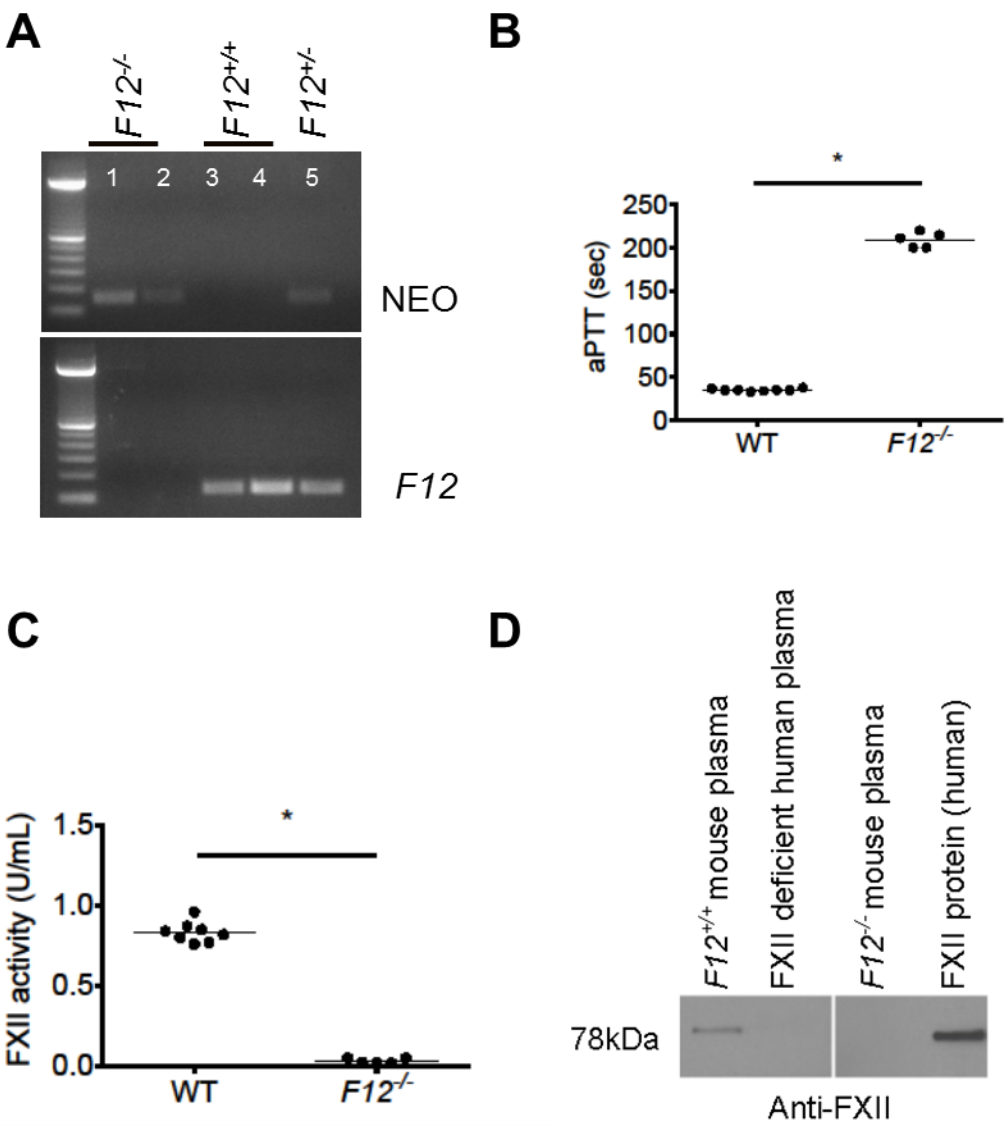
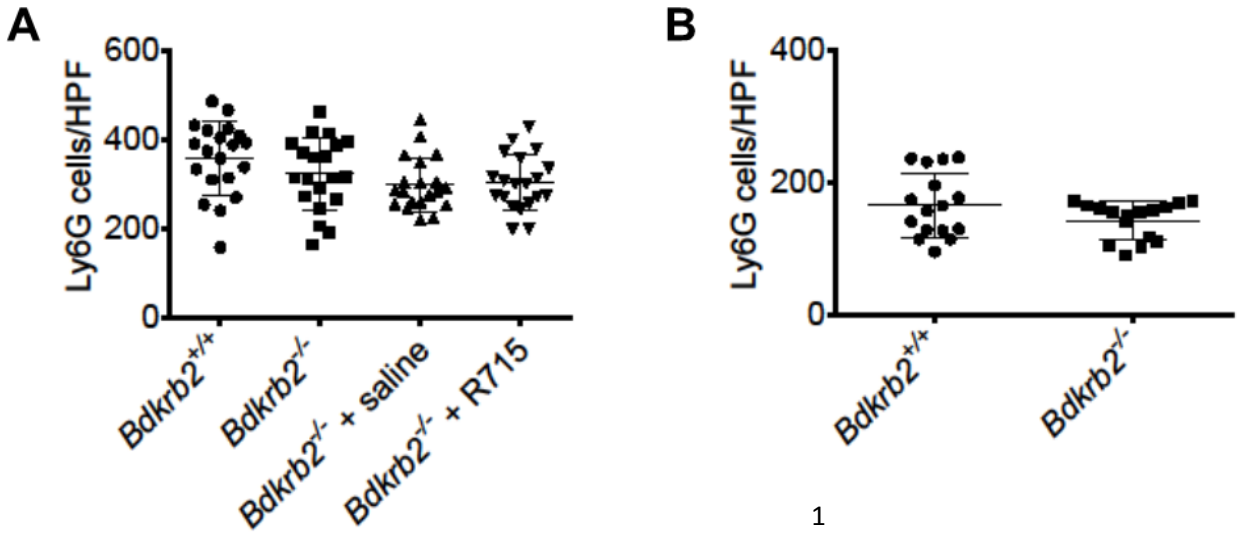
Supplemental Table 3. *CBC parameters 6 weeks after adoptive bone marrow transfers*. Data represent the mean ± SD (n = 4 mice per group).



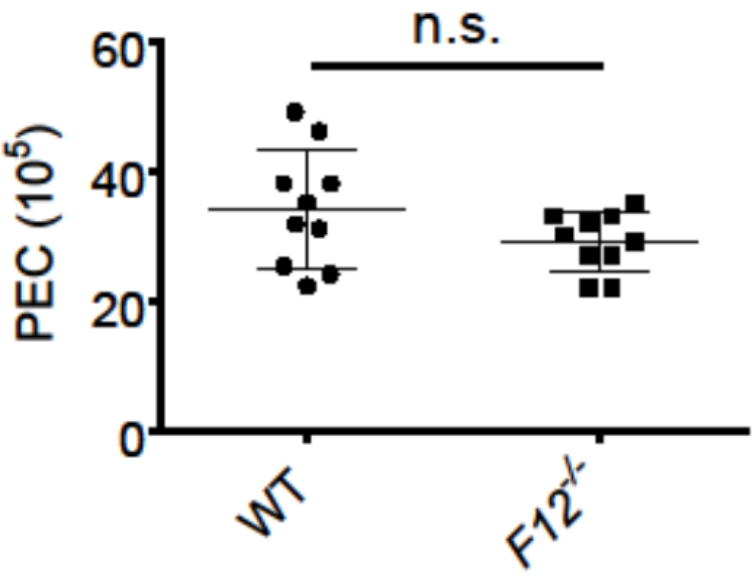
Supplemental Figure 1

Supplemental Figure 1. *Characterization of F12$^{-/-}$ mice*. (**A**) Representative separate agarose gels from polymerase chain reaction showing the genotype of wild type (*F12*$^{+/+}$), heterozygous (*F12* $^{+/-}$) and FXII deficient (*F12*$^{-/-}$) mice run simultaneously. *F12*: 152 base pairs (bp), single NEO band 128 bp denotes *F12*$^{-/-}$ genotype. (**B**) aPTT was determined in WT and *F12*$^{-/-}$ plasma (n=5-8). (**C**) Plasma FXII coagulant activity from WT and *F12*$^{-/-}$ mice (n=5-8). (**D**) Immunoblot for FXII antigen in murine and human WT and *F12*$^{-/-}$ plasmas. This image represents non-contiguous lanes of the same blot. Each image is representative of n=5 experiments.



Supplemental Figure 2

Supplemental Figure 2. *Reduced bradykinin signaling does not influence leukocyte migration into skin wounds.* (**A**) Number of neutrophils (Ly6G positive cells) per HPF in Day 2 skin wounds of WT, bradykinin 2 receptor knock-out (Bdkrb2$^{-/-}$), and Bdkrb2$^{-/-}$ mice treated with the bradykinin 1 receptor antagonist R715 are shown. Data represent mean ± SD (n = 6-8 mice per group). p=0.74 vs. WT control mice by one-way ANOVA with Bonferroni correction. (**B**) Number of neutrophils in Day 5 skin wounds of WT and bradykinin 2 receptor knock-out (Bdkrb2$^{-/-}$) mice. Data represent mean ± SD (n = 6 mice per group). p=0.18 vs. WT control mice by Student's t-test. Fluorescent images were obtained using a Nikon TE2000-S microscope, 20X magnification. The number of Ly6G positive cells in all groups of animals was compared by morphometric analysis using the NIH ImageJ software. These data indicate that the reduction in plasma bradykinin levels or absence of its receptors does not account for the reduced neutrophil migration seen in wounds of *F12*$^{-/-}$ mice.



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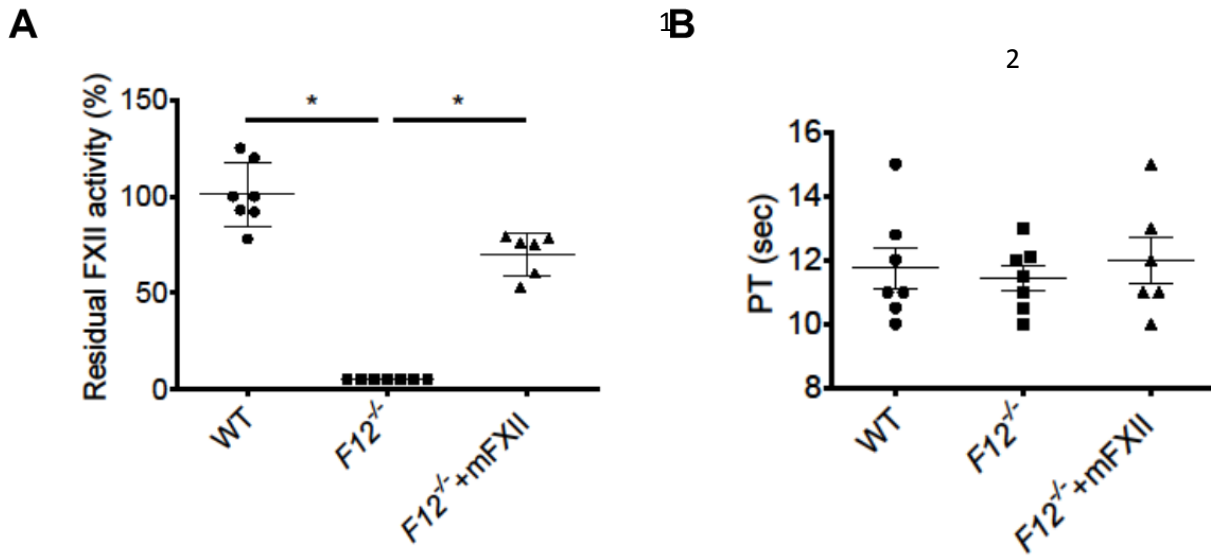

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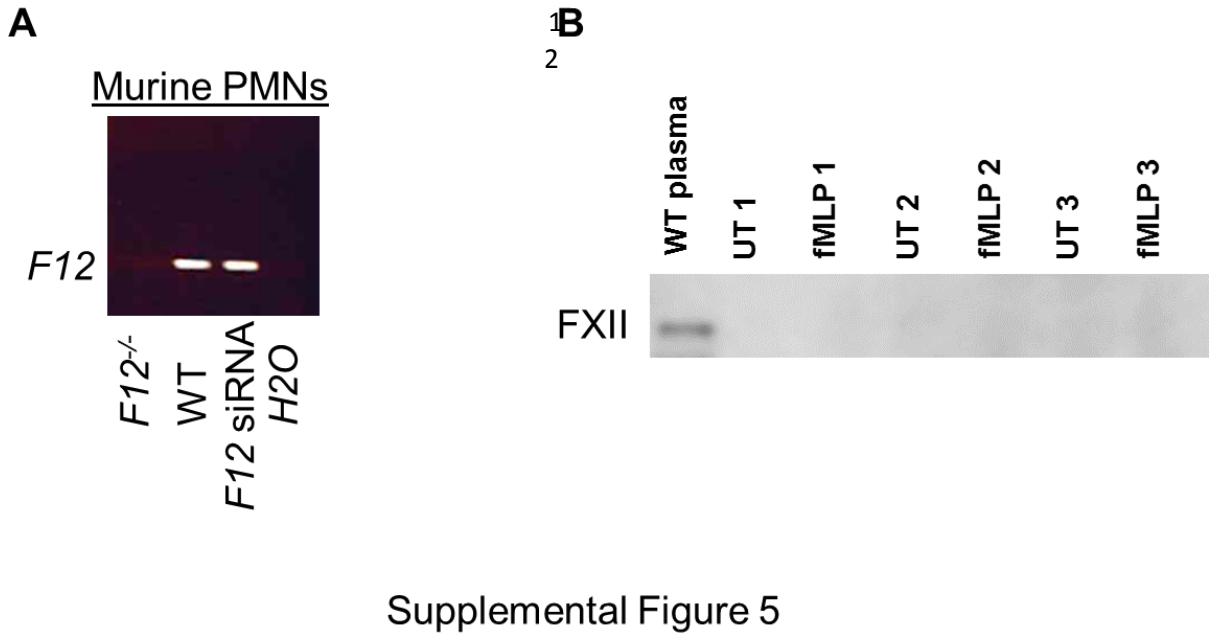

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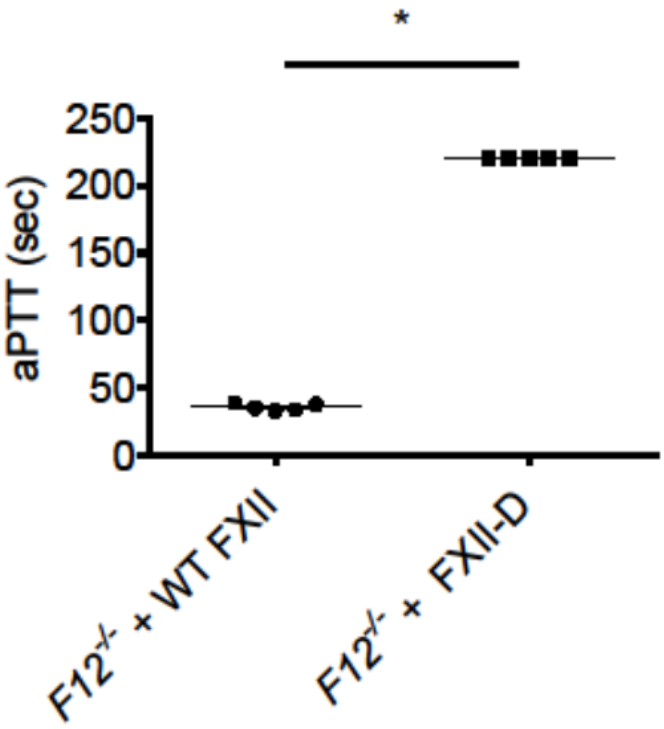

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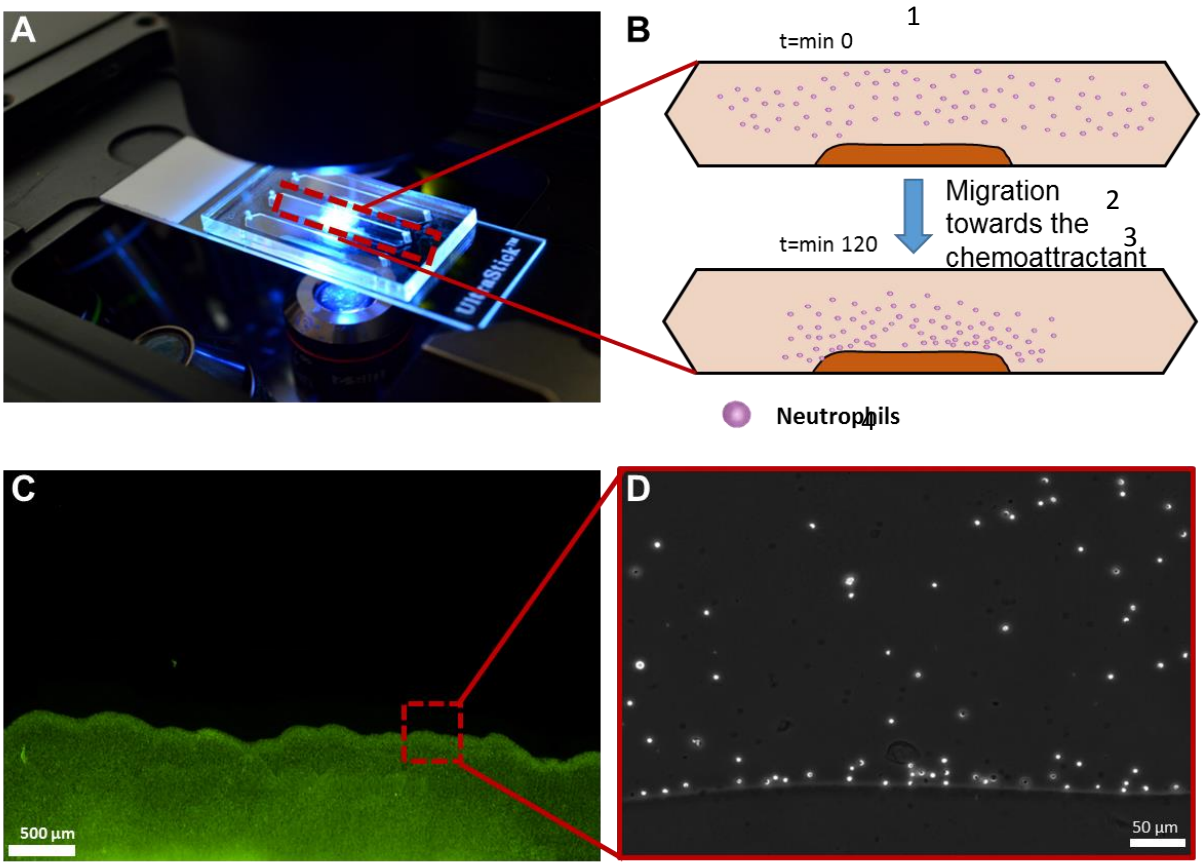

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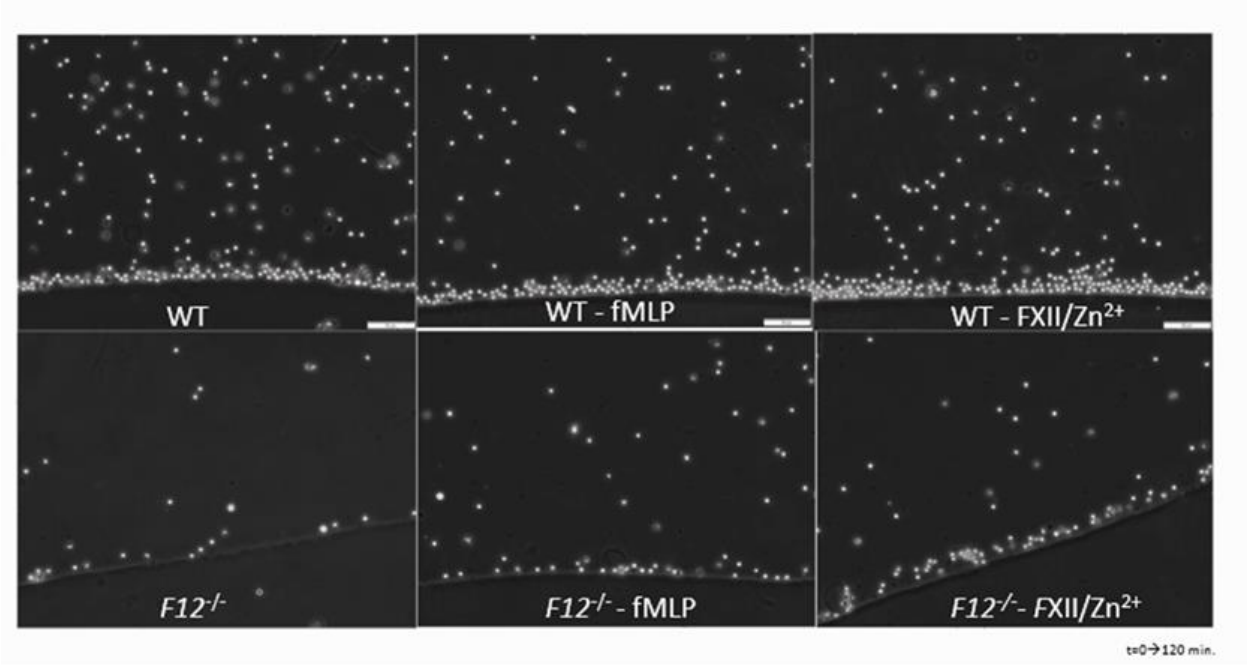
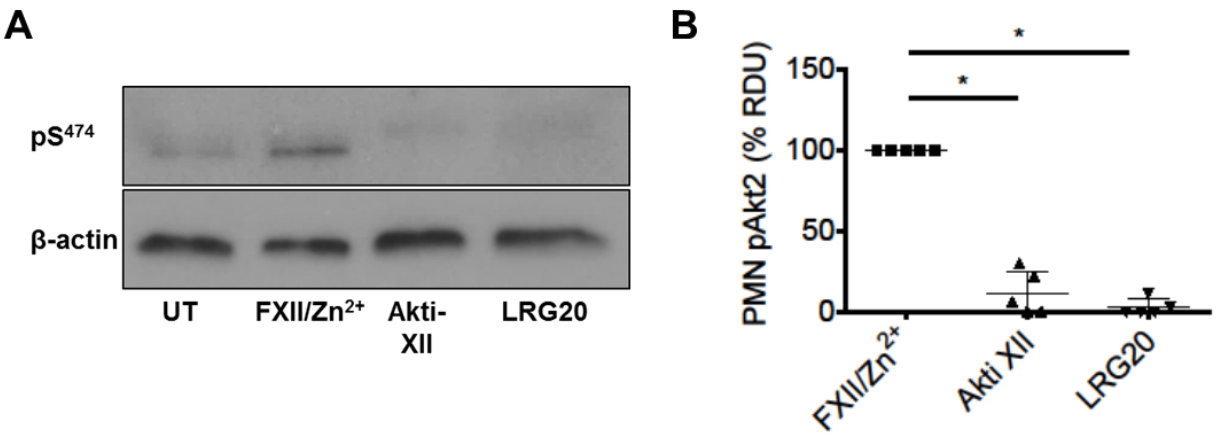


Supplemental Figure 8

Supplemental Figure 8. *Neutrophil chemotaxis in a microfluidic channel assay.* Pictures are representative images acquired at t=2 h.



Supplemental Figure 9

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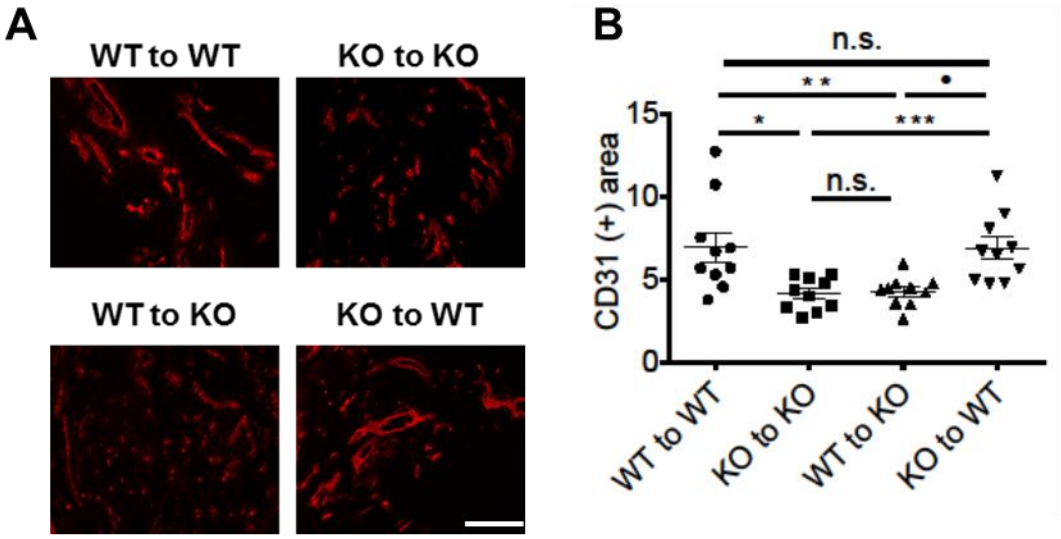

Supplemental Figure 10

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Supplemental Table 1

	WBC (10^3/μL)	Neutrophils (10^3/μL)	Lymphocytes (10^3/μL)	Monocytes (10^3/μL)	Hgb (g/dL)	Platelets (10^3/μL)	Mean PT (sec)
WT	5.0 ± 1.3	1.06 ± 0.5	3.76 ± 1.0	0.17 ± 0.0	12.1 ± 1.1	722 ± 166	12.1
***F12*$^{-/-}$**	4.18 ± 1.4	1.27 ± 0.3	2.6 ± 1.0	0.31 ± 0.1	12.2 ± 1.3	692 ± 137	11.7

Supplemental Table 1. *Hematologic parameters in WT and F12$^{-/-}$ mice*. Hematologic parameters in WT and *F12*$^{-/-}$ mice. Blood cells from WT and *F12*$^{-/-}$ mice were counted using an automated HEMAVET 950 analyzer (Drew Scientific). Data represent the mean ± SD (n = 6 mice per group). The prothrombin time was performed as indicated in the Methods.

Supplemental Table 2

k_{on}	k_{off}	K_D
10^5 M^{-1} s^{-1}	10^{-3} s^{-1}	nM
2.03 ± 0.85	5.02 ± 2.82	37.1 ± 29.4

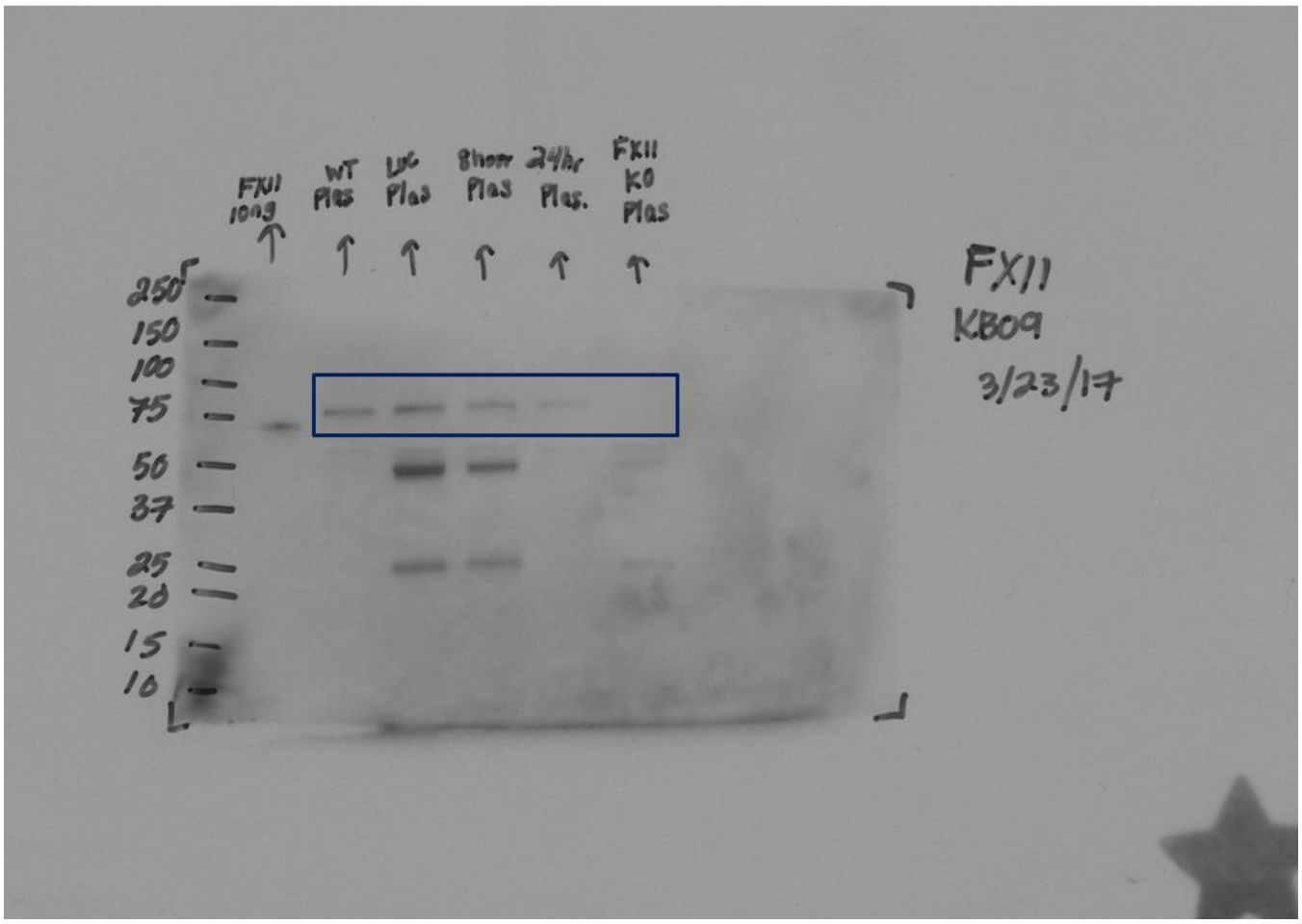
Supplemental Table 2. *Binding constants of FXII to immobilized uPAR.* Data reflect binding kinetics in the presence of 10 µM zinc. Mean ± SD of binding constants from n=3 sensorgrams using BiaCore Langmuir binding model.

Supplemental Table 3

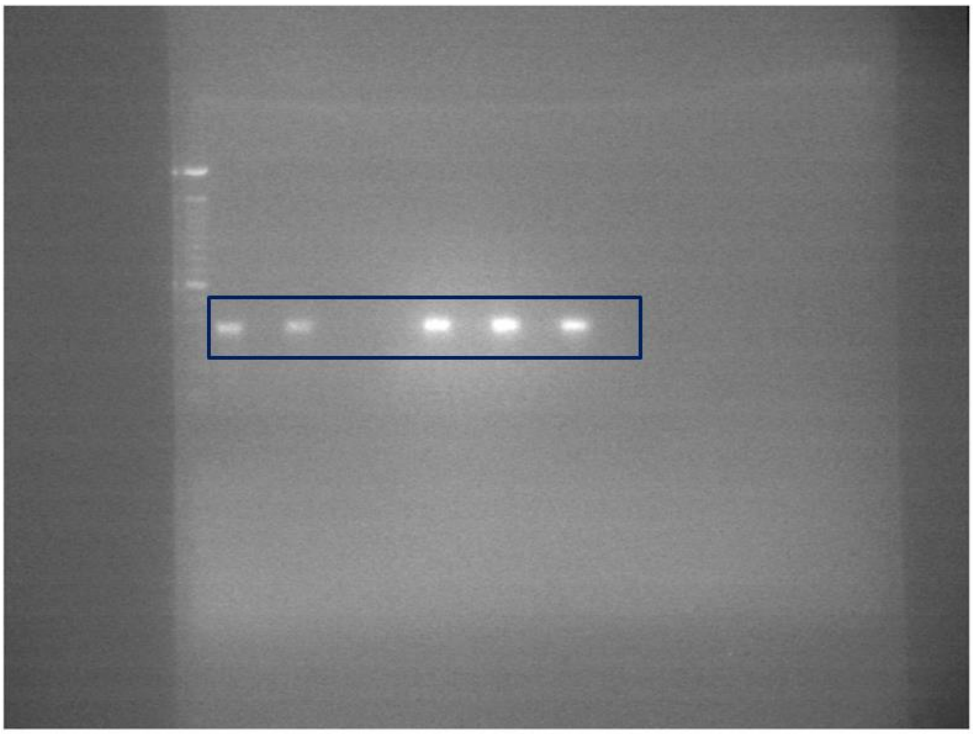
	WT-WT	KO-KO	WT-KO	KO-WT
WBC (10^3/μL)	4.04 ± 1.3	3.42 ± 1.0	3.96 ± 1.0	4.78 ± 1.4
ANC (10^3/μL)	1.94 ± 0.4	1.54 ± 0.3	1.47 ± 0.3	1.73 ± 0.2
Hgb (g/dL)	13.3 ± 1.4	12.4 ± 1.03	13.1 ± 1.9	12.9 ± 1.2
PLT (10^3/μL)	753 ± 111	742 ± 130	692 ± 122	707 ± 117

Supplemental Table 3. *CBC parameters 6 weeks after adoptive bone marrow transfers*. Data represent the mean ± SD (n = 4 mice per group).

Full Unedited Gel for Figure 4B: FXII

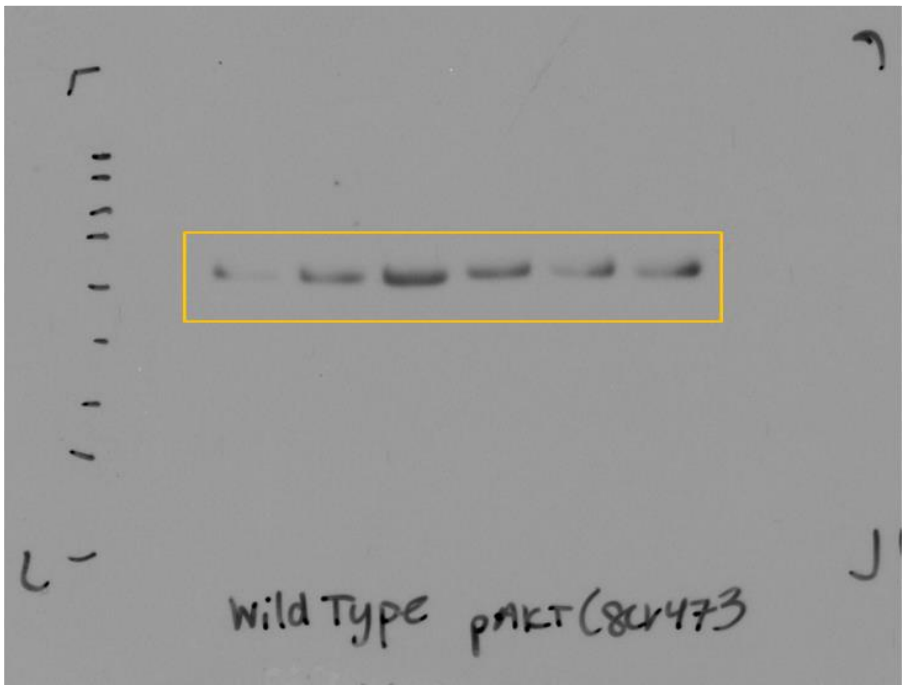


Full Unedited Gel for Figure 5A: *F12* PCR

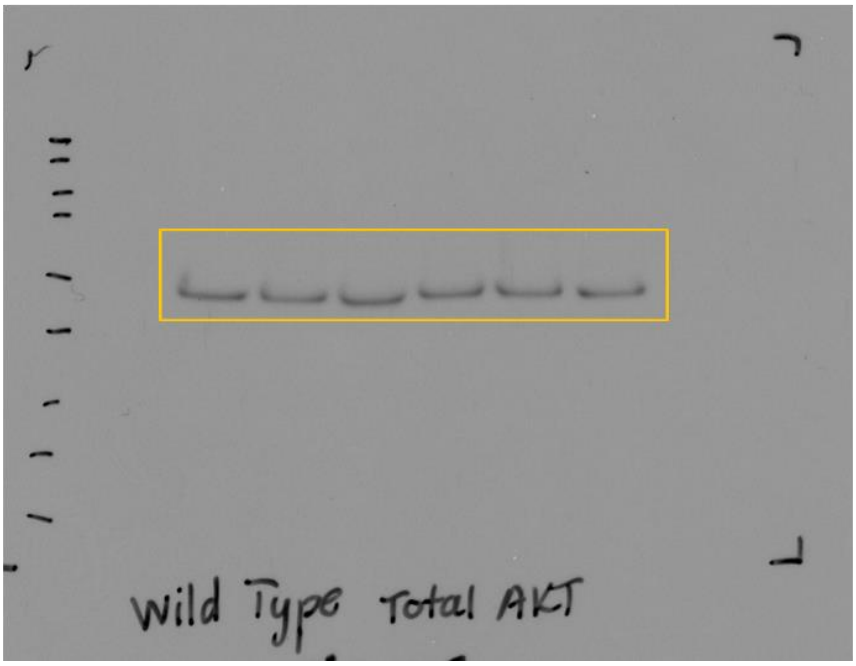


Full Unedited Gel for Figure 6A: FXII switched to Odyssey Infrared Imaging System for blot development

Full Unedited Gel for Figure 6C:
pAkt Ser^{473} (TOP)



Full Unedited Gel for Figure 6C: total Akt (BOTTOM)



Full Unedited Gel for Figure 6D:
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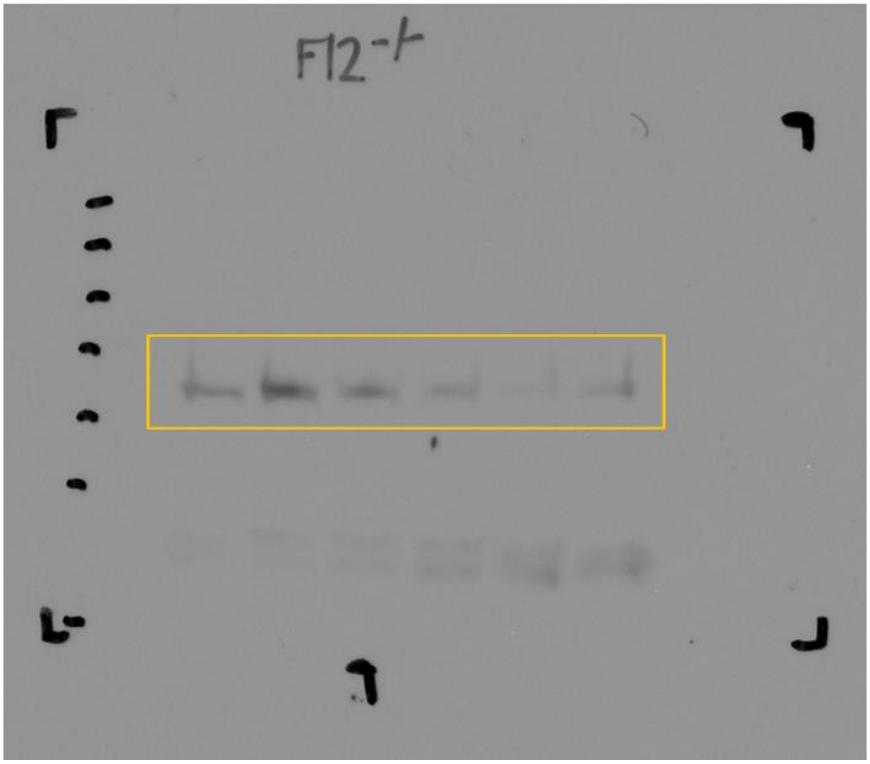

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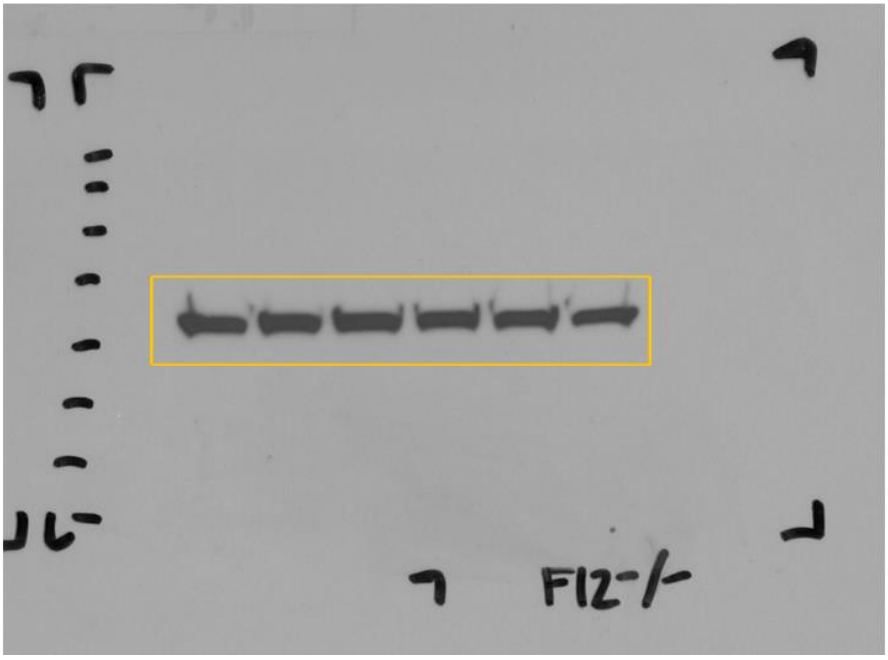

Full Unedited Gel for Figure 6E:
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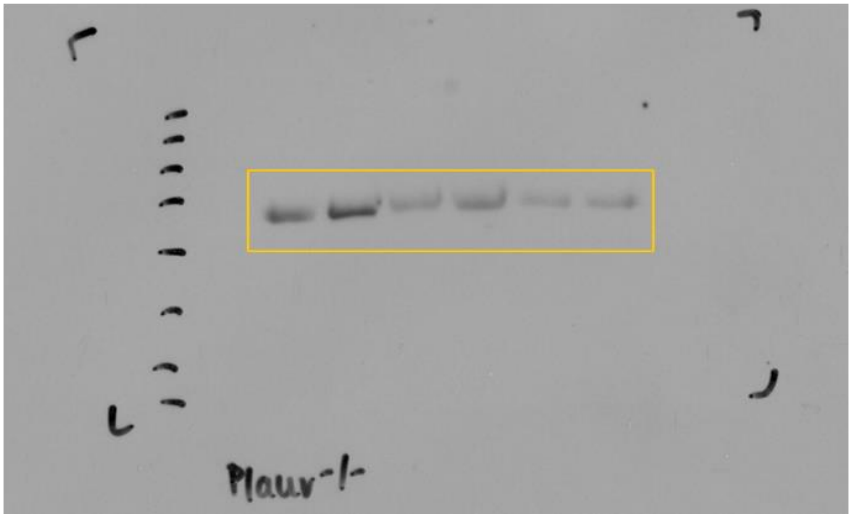

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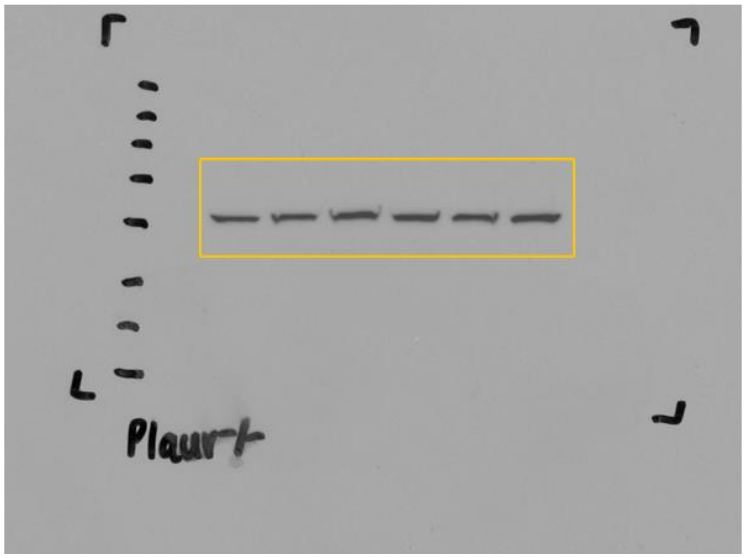
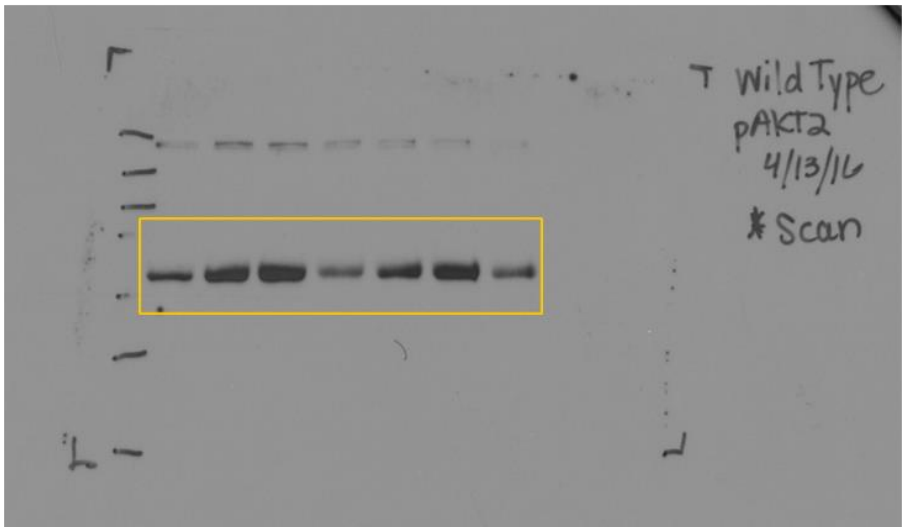


Full Unedited Gel for Supplemental Figure 6F: pAktS^{474} (TOP)



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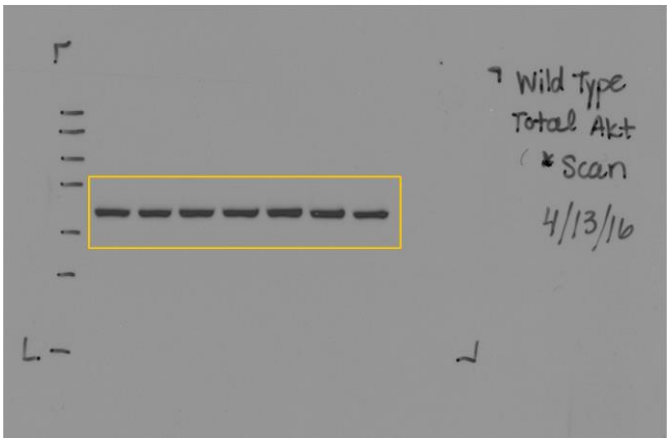
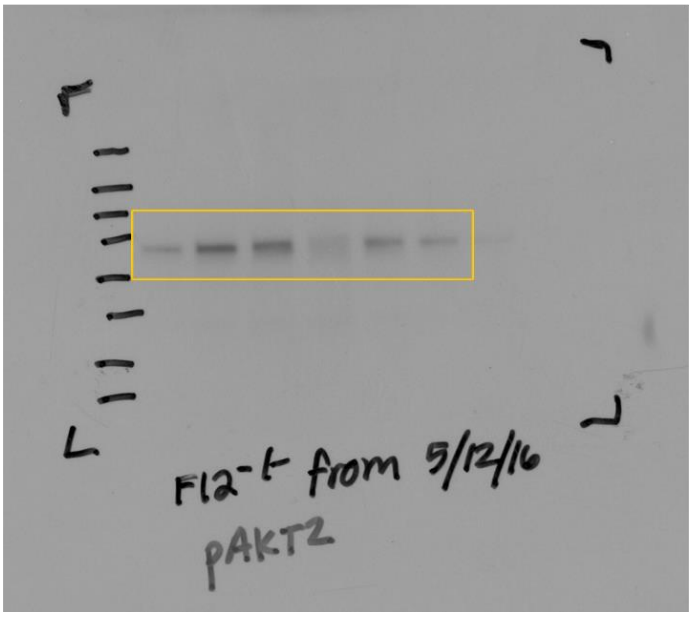


Full Unedited Gel for Supplemental Figure 6G: pAktS^{474} (TOP)



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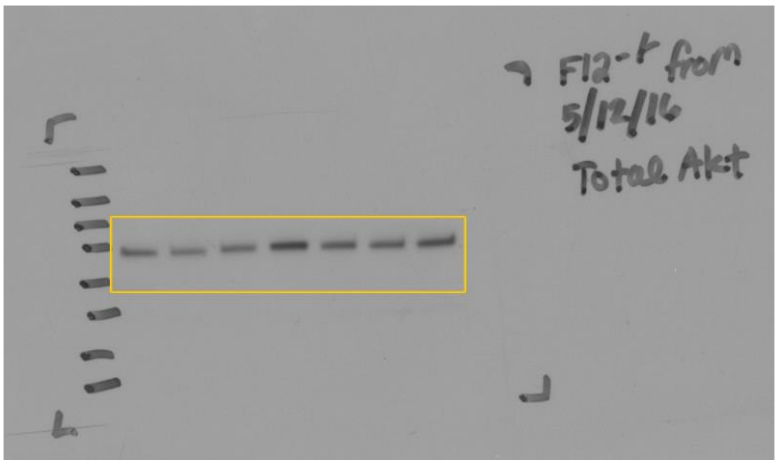

Full Unedited Gel for Supplemental Figure 6H:
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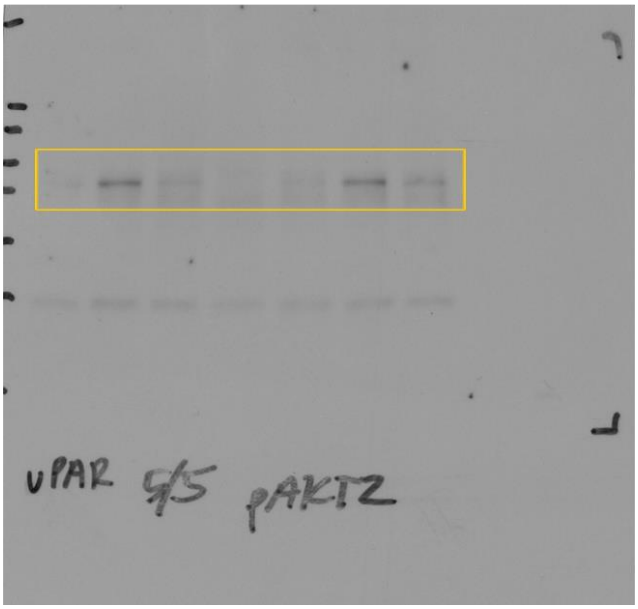
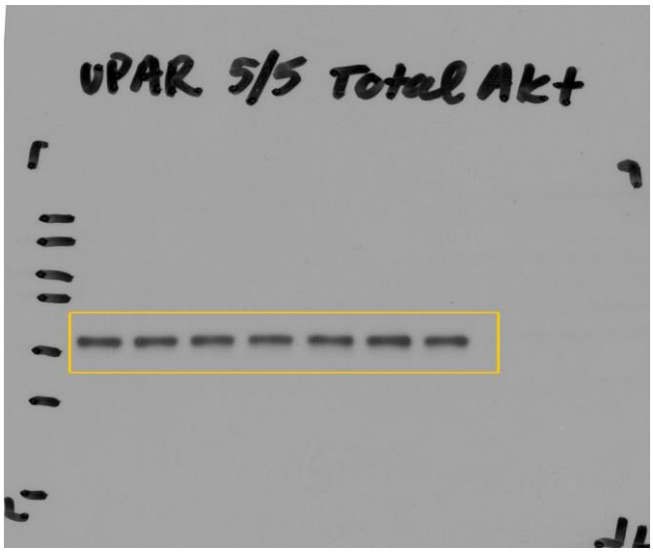
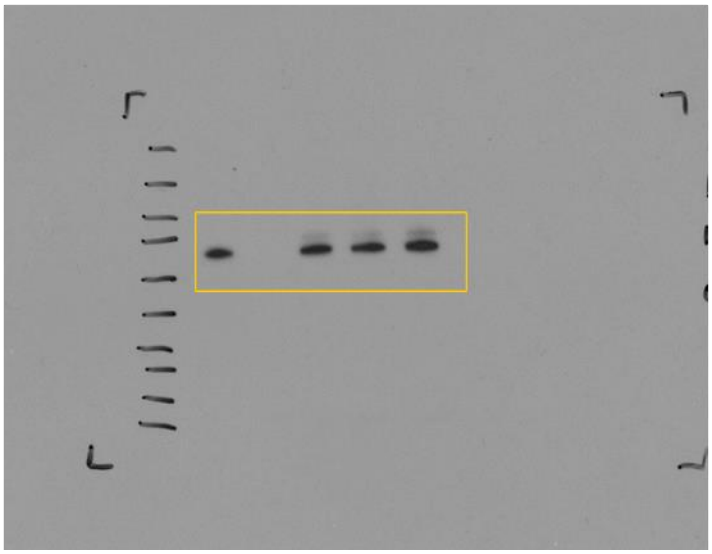


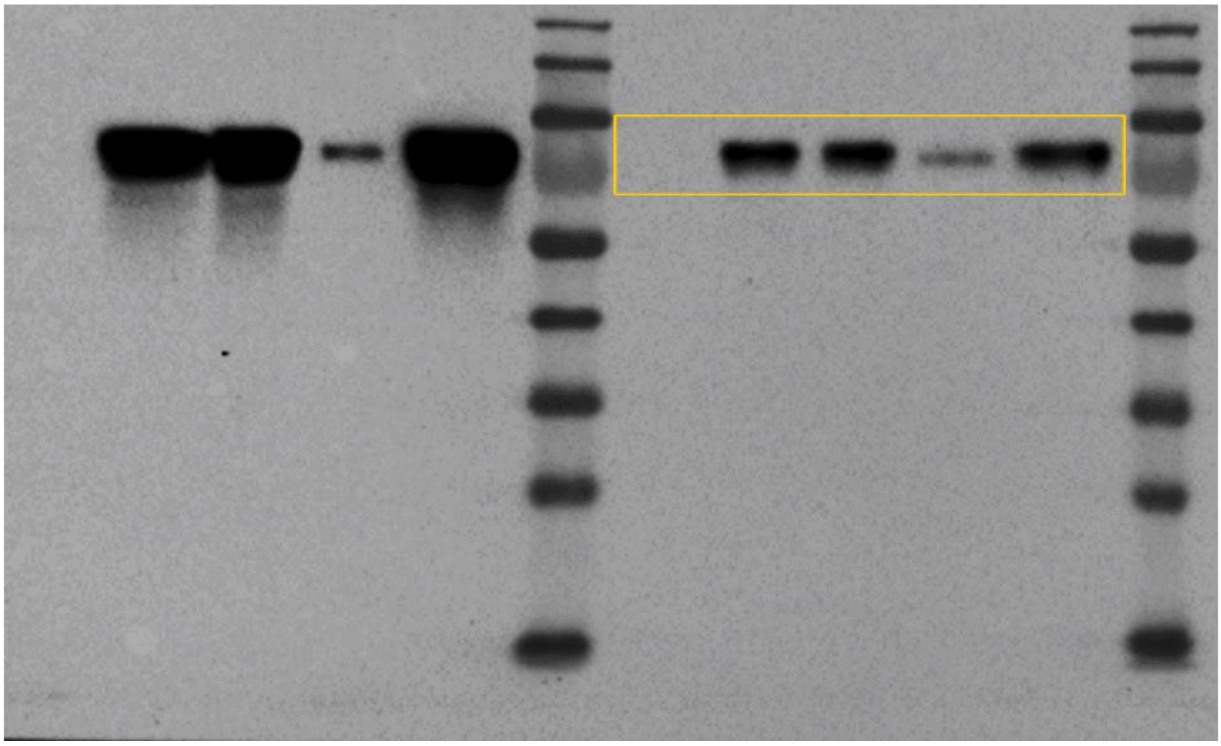
Full Unedited Gel for Supplemental Figure 6H:
total Akt (BOTTOM)



Full Unedited Gel for Figure 7A



Full Unedited Gel for Figure 7D:
FXII immunoblotting of FXII variants



Full Unedited Gel for Figure 7E:
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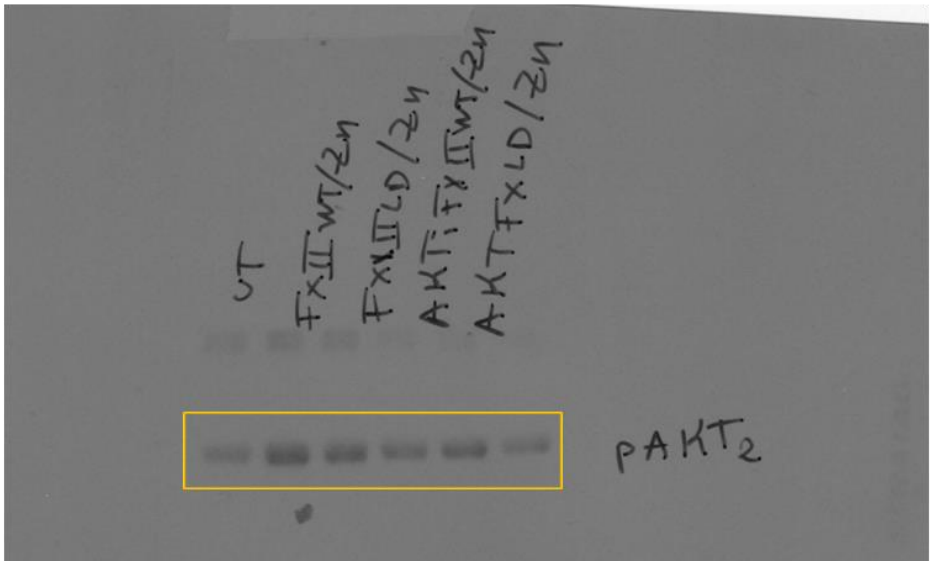

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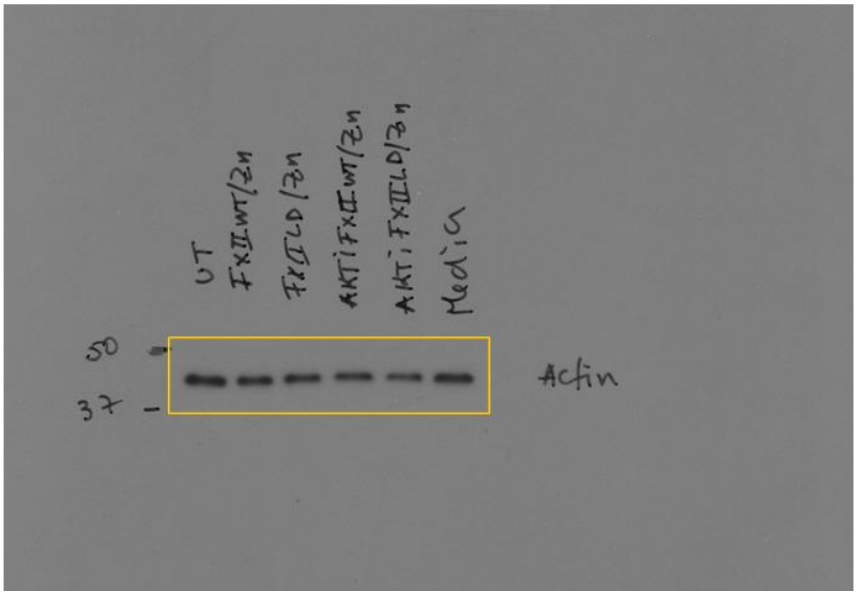
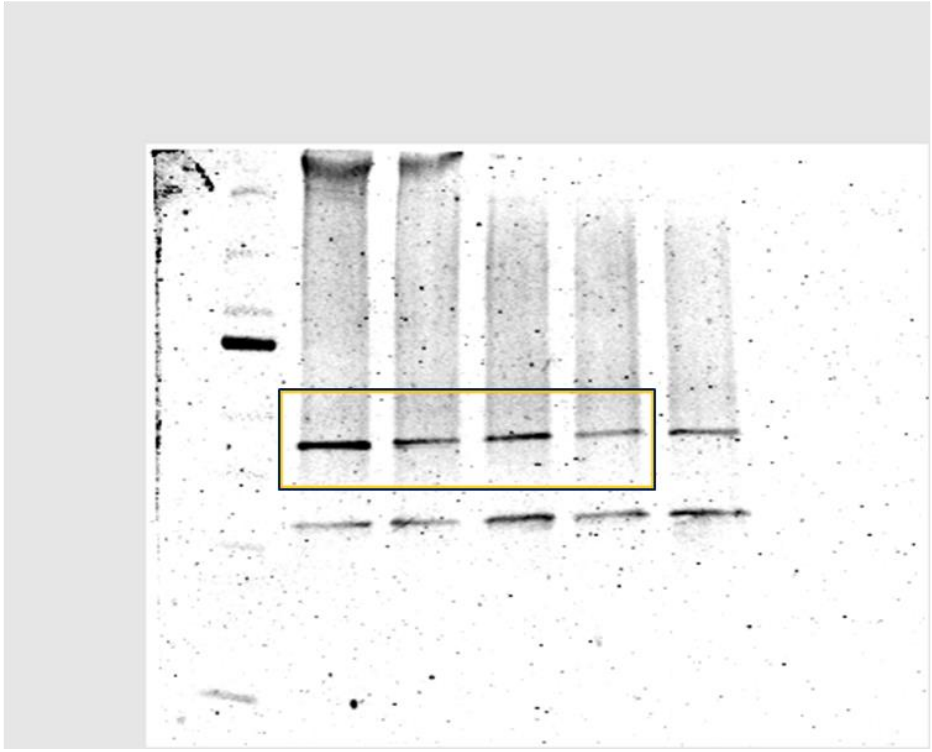
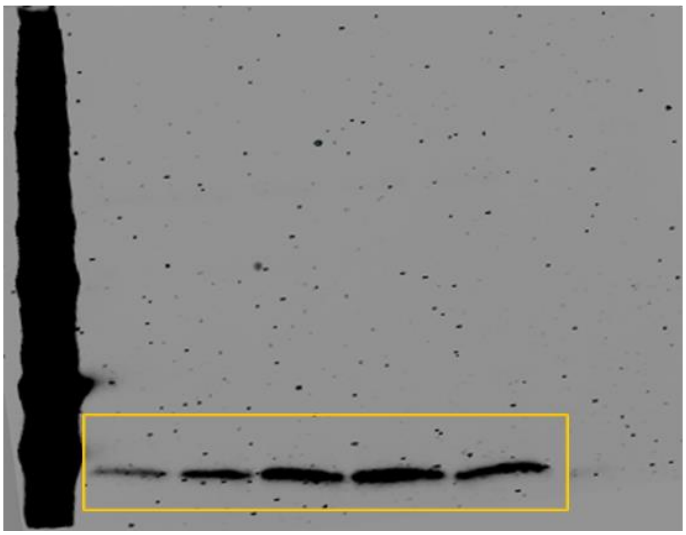


Full Unedited Gel for Figure 9E: H3-C (TOP)

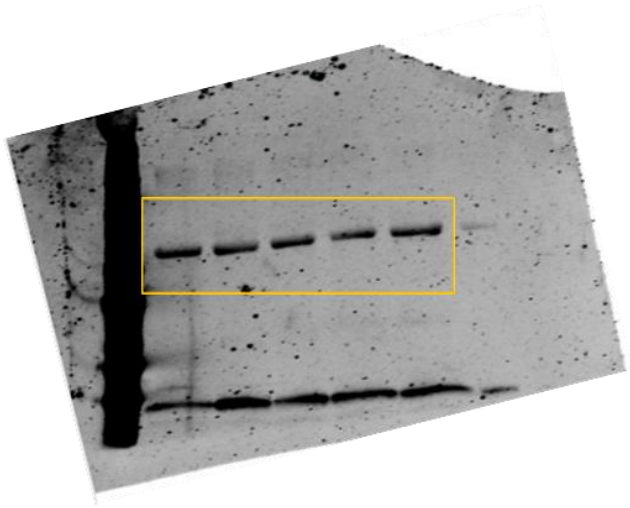
Full Unedited Gel for Figure 9E: β-actin (BOTTOM)



Full Unedited Gel for Figure 9G: H3-C (TOP)



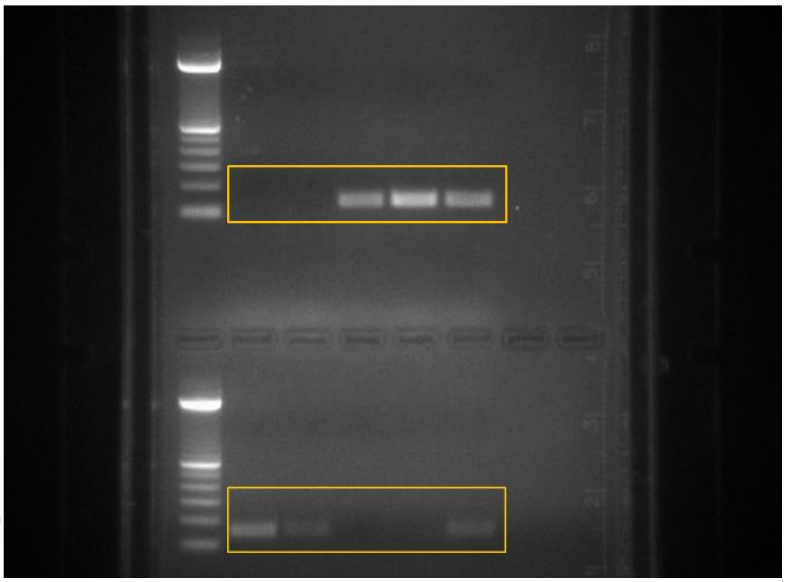
Full Unedited Gel for Figure 9G: β-actin (BOTTOM)



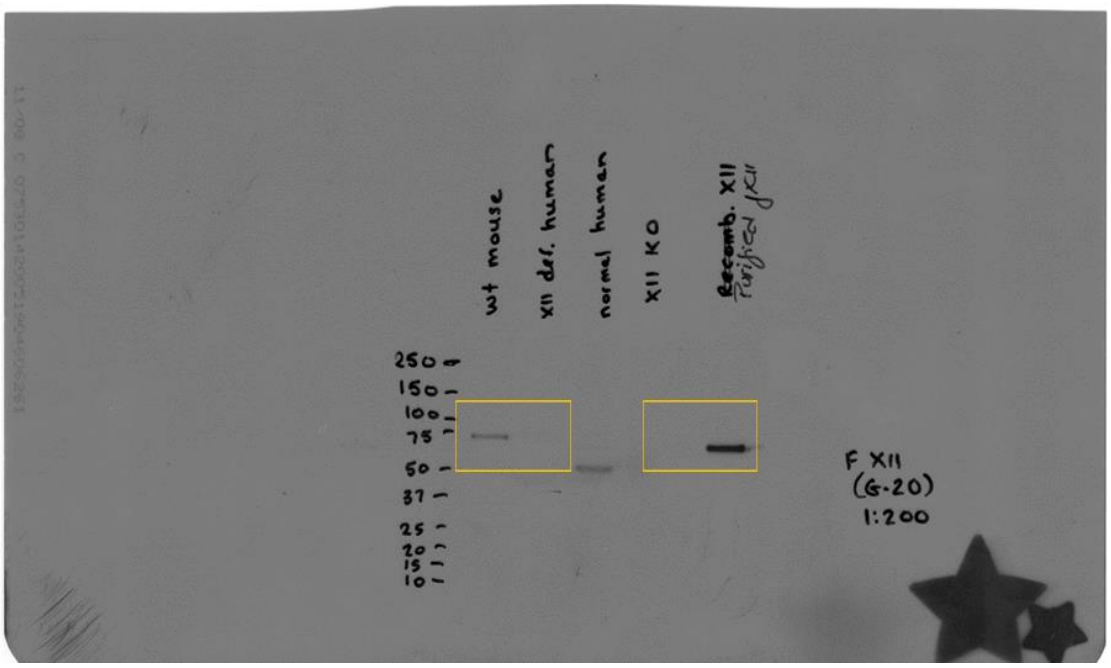
Full Unedited Gel for Supplemental Figure 1A

F12
(BOTTOM band)

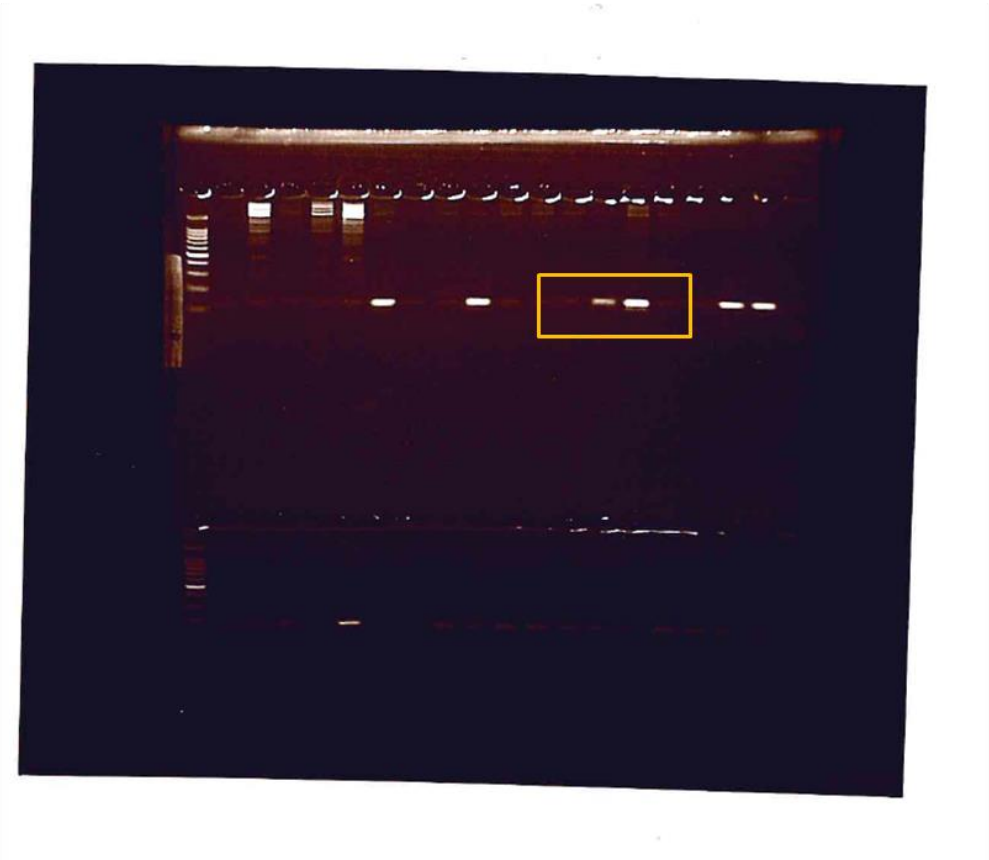
NEO
(TOP band in manuscript)



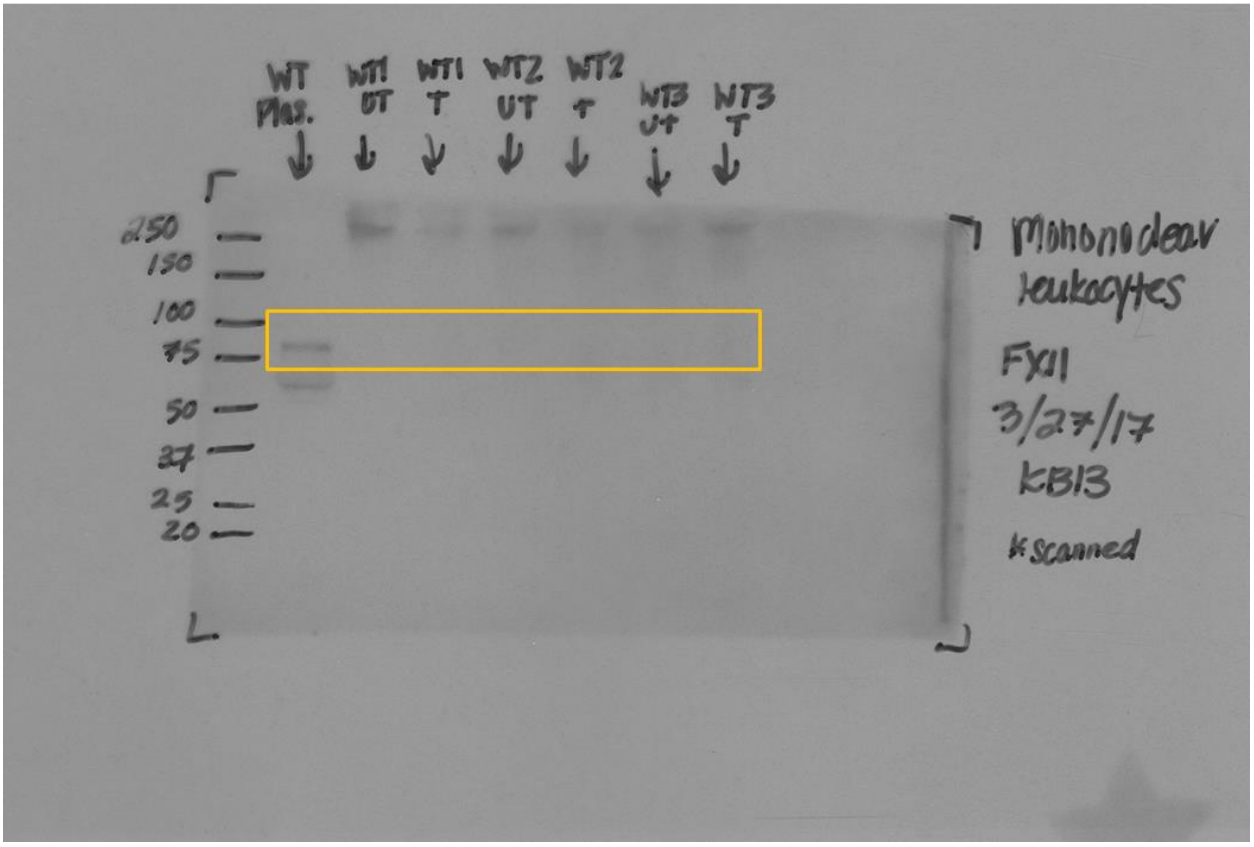
Full Unedited Gel for Supplemental Figure 1D



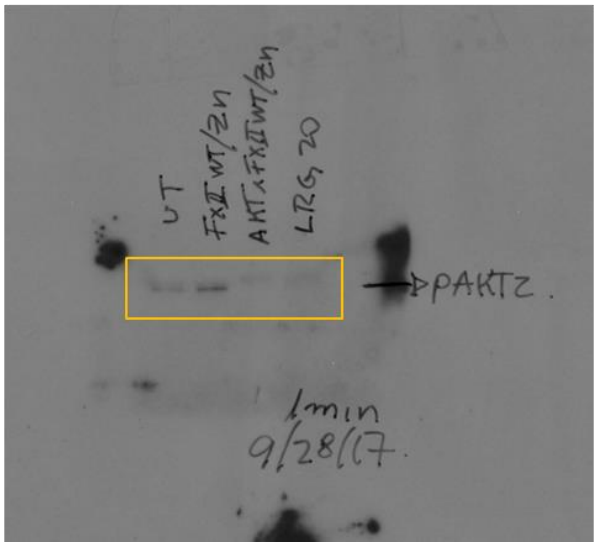
Full Unedited Gel for Supplemental Figure 5A: *F12* PCR



Full Unedited Gel for Supplemental Figure 5B: FXII



Full Unedited Gel for Supplemental Figure 9A: pAkt2S^{474} (TOP)



Full Unedited Gel for Supplemental Figure 9A: β-actin (BOTTOM)