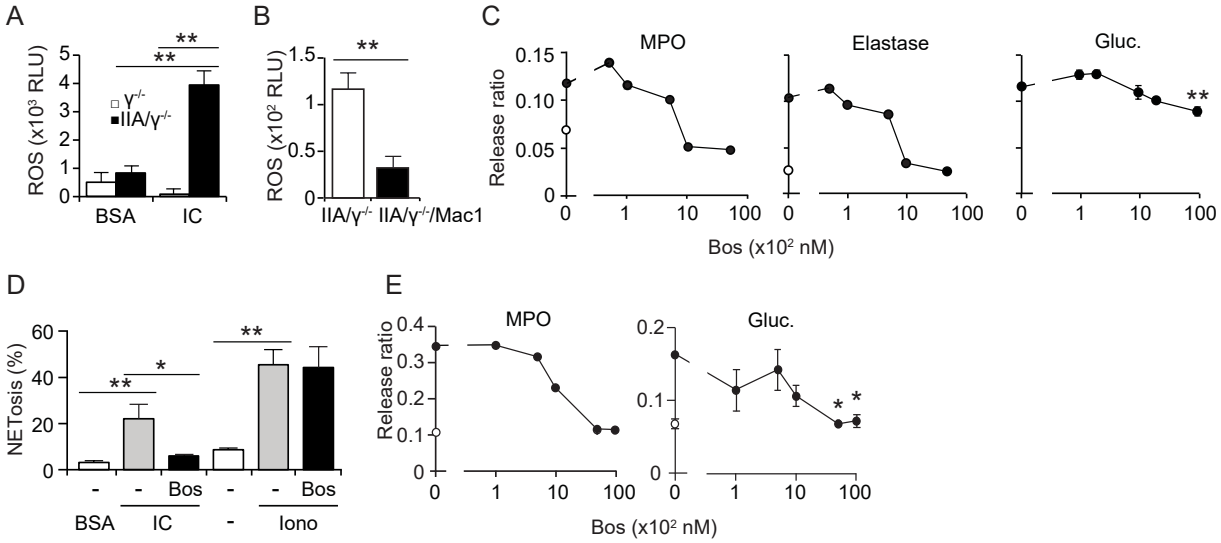
LIST OF SUPPLEMENTARY MATERIALS

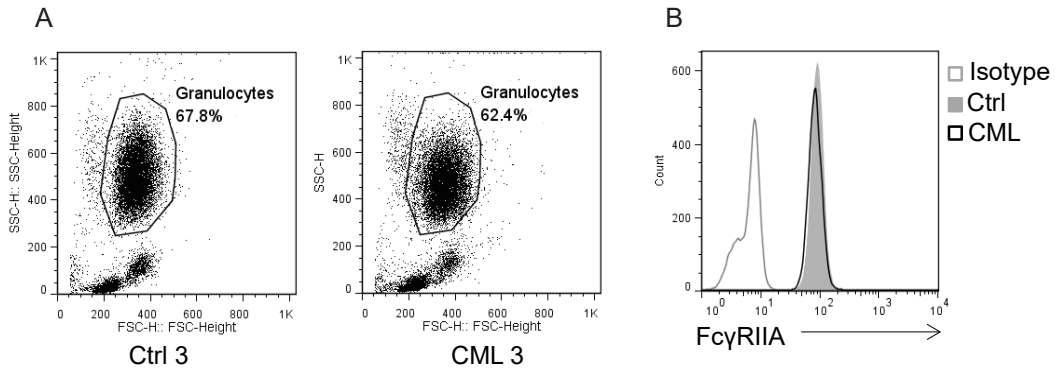
SUPPLEMENTARY FIGURES

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

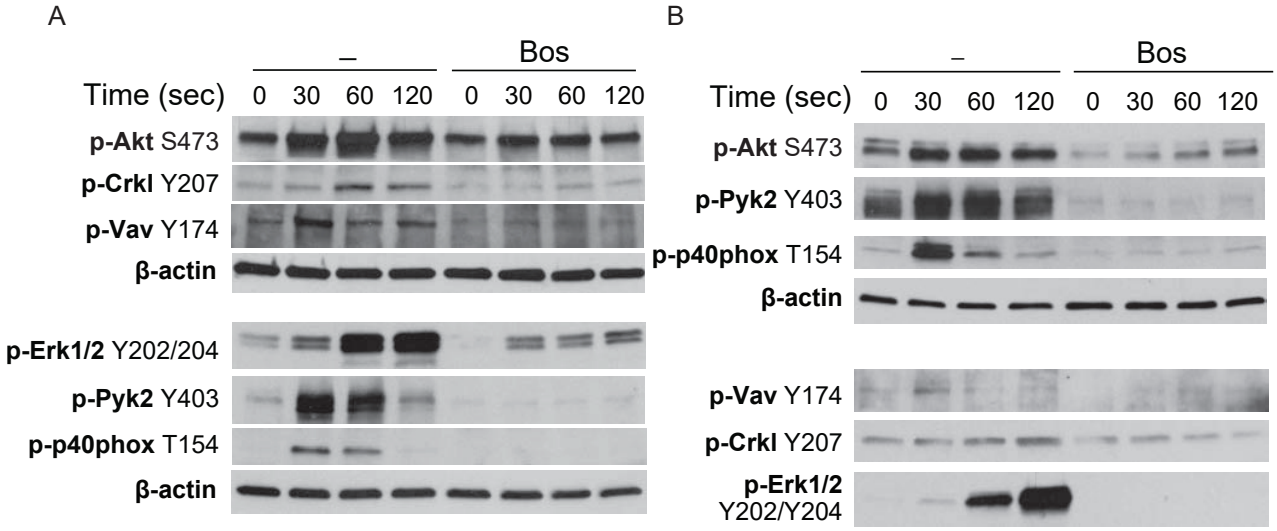
Supplementary Figure S1



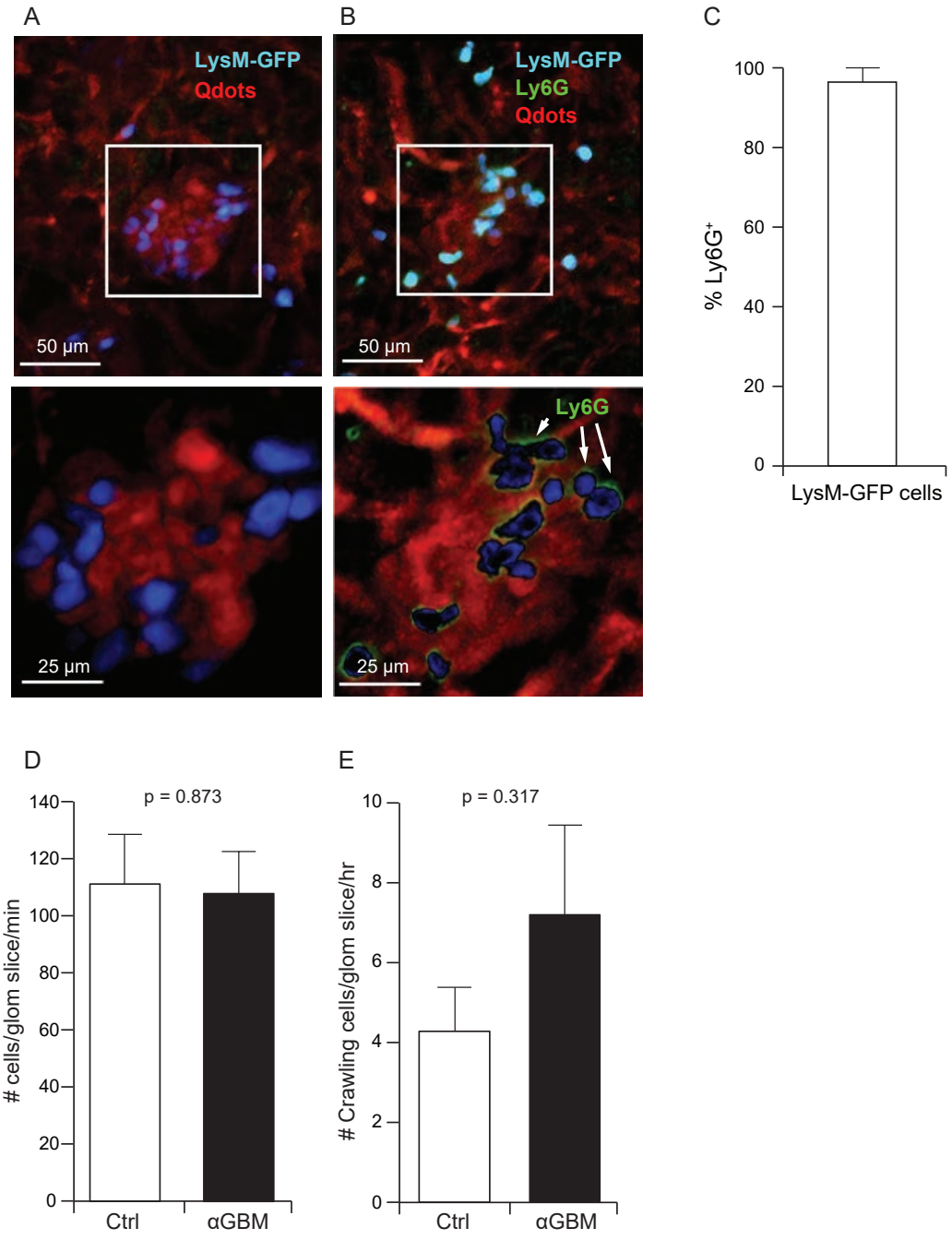
Supplementary Figure S2



Supplementary Figure S3

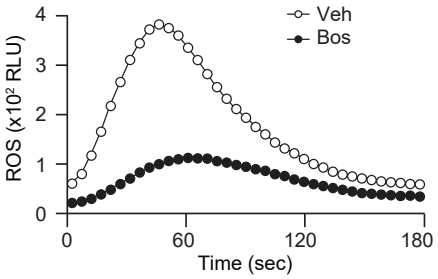


Supplementary Figure S4



Supplementary Figure S5

A



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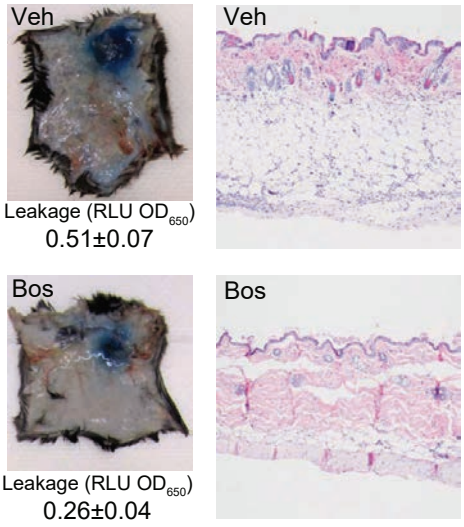

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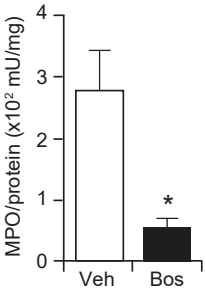

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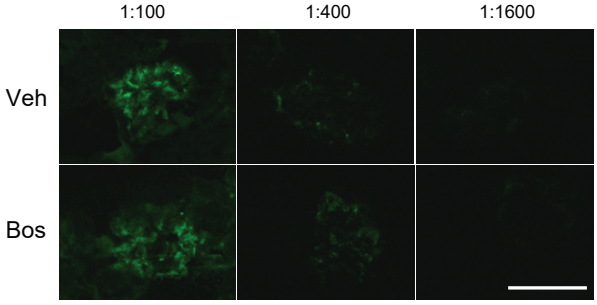

Treatment	Vehicle	Bosutinib
αGBM antibody	1:3200	1:3200

E

		WBC	Neutrophils	
		x10³/μL	x10³/μL	%
Average	Vehicle	3.0±1.0	1.2±0.5	36.8±5.0
	Bosutinib	5.6±0.7	3.2±0.7	55.8±7.7
p value		*0.08*	*0.06*	*0.10*

SUPPLEMENTARY FIGURE LEGENDS

FIGURE S1 (Related to Figure 1 and 2). **Role of FcγRIIA and Mac-1 in reactive oxygen species generation following adhesion to immobilized ICs and the effect of bosutinib on IC-induced enzyme and NETs release. A)** FcγRIIA^{+}/γ$^{-/-}$ (IIA/γ$^{-/-}$) and γ-chain-deficient (γ$^{-/-}$) mouse BMNs were dispensed on BSA- or BSA-anti-BSA immune complex (IC)-coated plates to evaluate ROS generation as in our primary compound screen with the fluorescence probe in a 384 well format plate (n=4). **B)** IIA/γ$^{-/-}$ and Mac1-deficient FcγRIIA^{+}/γ$^{-/-}$ (IIA/Mac1$^{-/-}$/γ$^{-/-}$) mice BMNs were put on BSA-anti-BSA IC-coated plates and ROS generation was measured in a 96 well format plate as in A) (n=3). **C)** IIA/γ$^{-/-}$ mice BMN MPO (left) and elastase (middle) and β-glucuronidase (right) release was assessed on BSA- or IC-coated plates following pretreatment of cells with vehicle (0) or indicated concentrations of Bos. Open circle is BMNs on BSA. (n=1 for MPO and elastase and n=4 for β-glucuronidase **D**) Vehicle (-)- or Bos-pretreated FcγRIIA^{+}/γ$^{-/-}$ peripheral blood neutrophils were incubated with BSA, ICs or ionomycin (Iono) for 4 hrs followed by DAPI staining. The percent of cells with released DNA was determined. An unpaired two-tailed t-test was performed compared to vehicle (-)-treated samples on IC or vehicle (-)-pretreated samples treated with Iono (n=4). **E)** Human peripheral blood neutrophil MPO (left) and β-glucuronidase (right) release was assessed on BSA- or IC-coated plates following pretreatment of cells with vehicle (0) or indicated doses of bosutinib (n=1 for MPO and n=4 for β-glucuronidase). Open circle is human neutrophils on BSA. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, 1-way ANOVA followed by Dunnett's multiple comparisons test for **A** and **C-E** (**E**, to assess dose responsiveness of bosutinib compared to vehicle) and 2-tailed, unpaired t test for **B**.

FIGURE S2 (Related to Figure 2). **Representative flow cytometric profile of peripheral blood neutrophils from a control and a CML patient.** Following RBC lysis, samples were incubated with mouse anti-human CD32 FITC or IgG1 isotype control, fixed and analyzed by flow cytometry. Granulocytes were gated based on FSC vs SSC. **A)** Representative dot plot shows gating scheme of granulocytes and the % granulocytes in the CML patient and matched control blood samples. **B)** Representative histogram shows similar FcγRIIA expression between the CML patient and control samples.

FIGURE S3 (Related to Figures 1 and 2). **Western blot analysis of FcγRIIA signaling following bosutinib treatment. A)** Western blot of phosphorylation (p-) of indicated proteins in FcγRIIA+/γ$^{-/-}$ mouse BMNs with vehicle (-) or bosutinib (Bos) and subjected to FcγRIIA cross-linking for the times in seconds. **B)** Western blot analysis of indicated proteins in human peripheral blood neutrophils with vehicle or Bos after FcγRIIA cross-linking. β–actin served as a loading control for A) and B).

FIGURE S4 (Related to Figure 5). ***In vivo*** **labeling of Ly6G on LysM-GFP cells during two-photon time- lapse imaging and analysis of neutrophil entry and crawling frequency in the kidney. (A)** Control LysM-GFP cells (blue) in Q-Dot 565 (red) labeled glomerular capillaries 1h after anti-GBM serum administration. **(B)** LysM-GFP (blue) cells labeled anti-Ly6G antibodies (shown as green). Double positive neutrophils are shown in glomerular capillaries 1h after anti-GBM serum administration. (A and B, bottom panels) To more clearly demonstrate Ly6G cell surface staining on LysM-GFP cells, an image subtraction calculator (Fiji, Image J) was used to eliminate GFP signal (blue channel) bleed through into the PE (green) channel. Ly6G positive (green) staining appears "membrane bound" (B, bottom panel, white arrows). **(C)** 10 glomeruli were examined and the percentage of LysM-GFP cells positive for Ly6G are shown (82/83 LysM-GFP cells were double positive). Two-Photon excitation=980nm and fluorescence emission was collected using 495nm, 560nm, 635nm emission filters. **(D-E)** The number of neutrophils entering and crawling in glomerular capillaries in the steady-state and after αGBM-induced injury was assessed by intravital microscopy. Neutrophil trafficking was analyzed on two distinct time scales by capturing 10 consecutive 10s video-rate recordings (40msec/frame) at 1 min intervals for a total of 10 min. Individual video-rate recordings were used to count the total number of neutrophils entering glomeruli (single optical

section, ~2.5μm). Then the first 30 frames of each video-rate recording were averaged and used to construct a time-lapse movie to identify crawling cells. Neutrophils were scored as crawling if they had polarized morphology and appeared to migrate for at least 3 frames at 4-8μm/min. **(D)** Neutrophil entry rates per minute in a glomerular slice in control and αGBM treated mice. **(E)** The number of crawling cells in glomerular slices per hour in control and αGBM mice. Percentage of crawling cells in both control and αGBM is <0.2% (Control = 0.06%, αGBM = 0.11%). Data is mean ± SEM. P-values were calculated from a two-tailed Mann-Whitney U test.

FIGURE S5 (Related to Figure 8). **Effect of bosutinib on FcγRIIA induced ROS *in vivo*, the Reverse Passive Arthus (RPA) reaction, antibody deposition in models of glomerulonephritis and peripheral blood leukocyte counts. A)** FcγRIIA cross-linking induced ROS generation was evaluated in mouse peripheral blood leukocytes harvested from FcγRIIA^{+}/γ$^{-/-}$ mice 4 hours after oral administration of 150 mg/kg bosutinib (Bos) or vehicle (Veh). Representative profile of 3 independent experiments is shown. **B-C)** Mice were treated subcutaneously with anti-BSA and intravenously with BSA and Evans blue dye. The skin was harvested 4hrs later. **B)** Representative pictures of skin and quantitation of Evans blue dye extracted from the excised skin are shown (left). Representative H&E stained tissue sections show skin thickness indicative of dermal edema only in vehicle treated sample (right). **C)** Neutrophil accumulation in the skin tissue was reduced in bosutinib treated mice as quantitated by MPO content (normalized to total protein). n= 4 mice per group. Data are mean ± SEM. *p < 0.05, 2-tailed unpaired t-test. **D)** Immunofluorescence was performed on 5μm frozen sections. Semiquantitative assessment of glomerular anti-GBM rabbit IgG deposition was performed by determining the end point positive titer for detection of staining using serial dilutions (1:100, 1:400, 1:1600, 1:3200, 1:6400) of a fluorophore conjugated anti-rabbit for anti-GBM IgG. 5-10 glomeruli were examined per section. All morphometric assessments were performed in a blinded protocol. The table provides the end point titer at which staining was observed for the indicated conditions. Representative images are shown. N=4 per group. The scale bar is 100μm. **E)** Peripheral leukocyte counts in blood samples taken at day 14 of the anti-GBM nephritis model. WBC, white blood cells. n=3-4 mice per group. An unpaired two-tailed t-test comparing the two groups was performed.

Movie S1. (Related to Figure 5). Two photon time lapse imaging in untreated LysM-GFP mice of neutrophil (green) behavior in glomeruli (red, Q-Dot 565). Scale bar=30μm.

Movie S2 and S3. (Related to Figure 5). Two photon time-lapse imaging in LysM-GFP mice of neutrophil (green) behavior in glomerular capillaries (red, Q-Dot 565) 1.5-2.0hr after an intravenous injection of anti-GBM antibody. Scale bar=30μm.

Movie S4. (Related to Figure 6). Video-rate imaging in untreated LysM-GFP mice of neutrophil (green) trafficking behavior in glomerular capillaries (red, Q-Dot 565). Scale bar=30μm.

Movie S5. (Related to Figure 6). Video-rate imaging in LysM-GFP mice of neutrophil (green) trafficking behavior in glomerular capillaries (red, Q-Dot 565) 1.5-2.0hr after an intravenous injection of anti-GBM antibody. Scale bar=30μm.

Movie S6. (Related to Figure 7). Two-photon time lapse imaging of adoptively transferred neutrophils (blue, vehicle or green/yellow, bosutinib treated) in glomeruli (red, 70 kD Texas-Red conjugated-dextran) 1.5-2.0hrs after intravenous injection of anti-GBM antibody. 15 minutes prior to imaging, mice were injected intravenously with FcγRIIA/γ$^{-/-}$ neutrophils treated *ex vivo* with vehicle or bosutinib and labeled with Cell Tracker Blue CMF2HC or Cell Tracker Green CMFDA respectively. Neutrophil behavior can be observed in two adjacent glomeruli. Scale bar=20μm.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Neutrophil reactive oxygen species based high throughput small molecule screen

Each compound was dissolved in DMSO at a concentration of 10μM. For a primary screen, 0.1mg/mL poly-L-lysine (Sigma-Aldrich) followed by 2.5% glutaraldehyde (Sigma-Aldrich) were dispensed onto 384-well black flat bottom plates (Corning). The plates were washed and incubated with 1mg/ml BSA (Sigma-Aldrich) followed by 40μg/mL rabbit anti-BSA antibody (B7276; Sigma-Aldrich). Mature murine neutrophils were isolated from the bone marrow of FcγRIIA^{+}/γ$^{-/-}$ mice and suspended in 100uM homovanillic acid/ 5U/ml peroxidase IV (Sigma-Aldrich)/ 1mM EDTA (Boston BioProducts)/ HBSS (Thermo Scientific) assay solution (pH 7.5). 3x10^{5} cells in 30 μL per well were loaded with a Wellmate automated dispenser (Thermo Scientific). Immediately thereafter, ICCB library compounds dissolved in 33nL DMSO were dispensed into the cell suspensions using a Compound Transfer Robot (Epson). After 60 minutes in a humidified incubator at 37°C, fluorescence levels were read at Ex/Em 321/421 nm with Envision2 (PerkinElmer). PP2 (Sigma-Aldrich), a Src inhibitor, and DMSO (vehicle) were used as positive and negative controls, respectively. The average value of the duplicate positive (PP2, Src inhibitor) and negative (DMSO, vehicle) controls at 60 min was assigned a value of 1 and 0, respectively. The average value of the library compound was normalized proportionally to these control values on the same plate to calculate an "inhibitory score". Compounds with inhibitory scores of >0.95 were regarded as hit compounds and cherry picked for the secondary screen.

FcγR crosslinking–induced generation of ROS

10^{6} BMNs suspended in PBS without Ca^{2+}/Mg^{2+}, were incubated with 10 μg/ml mouse anti-hFcγRIIa (clone IV.3; StemCell Technologies) on ice for 30 minutes and then washed and incubated with 500 ng/ml mGM-CSF (BioLegend) for 15 minutes. After pretreatment with indicated reagents or DMSO as vehicle control at 37 °C for 30 minutes, luminol (50 μM) in PBS with Ca^{2+}/Mg^{2+} was added, followed by the addition of goat anti-mouse F(ab')$_2$ (36 μg/ml; #112-006-072; Jackson ImmunoResearch). ROS generation (expressed in relative light units, RLU) was continuously monitored using a 6-channel bioluminat LB-953 luminometer (Berthold). For human PMNs, 5x10^{5} cells were analyzed without GM-CSF treatment. For PMA induced ROS generation, mouse BMNs pretreated with bosutinib for 30 minutes were stimulated with 100 ng/ml PMA (Sigma-Aldrich) and ROS generation was then monitored. The peak level of ROS for each condition was normalized to the average for the vehicle (DMSO) treated control sample. All experiments were done in duplicate.

Neutrophil Degranulation

3x10^{6} mouse BMNs or 1x10^{6} human PMNs were added to 24-well plates coated with BSA and then anti-BSA and incubated at 37 °C for 30 min. After centrifugation at 300 g at 4 °C, 100 μl supernatant was taken for analysis and cell pellets were lysed with 0.1% 500 μl Triton-X (Sigma-Aldrich). For myeloperoxidase (MPO) analysis, the substrate 3,3',5,5'-tetramethyl-benzidine (TMB, Thermo) was added to the supernatant and the cell lysate, followed by stop solution and optical absorbance at 450 nm was measured. For neutrophil elastase, 20 μM MeO-Suc-Ala-Ala-Pro-Val-AMC (Cayman) was added, and for glucuronidase, 1 mM 4-methylumbelliferyl-β-D-glucuronide hydrate (Sigma-Aldrich) in 0.05% Triton-X and 0.1 M Na acetate buffer (pH 3.5) was added to the supernatant, samples were incubated overnight at 37 °C and fluorescence intensity was read at Ex/Em 355/460 nm. The enzyme release is reported as the ratio of enzyme in the supernatant to the total enzyme in cell lysates. Treatment of cells with 5 μg/ml cytochalasin B at 37 °C for 5 min followed by 5 μM fMLP at RT for 15 minutes served as a positive control.

Western blot analysis

After incubation with 10 μg/ml mouse anti-hFcγRIIa (clone IV.3; StemCell Technologies) followed by mGM-CSF or hGM-CSF, 1X10^{5} cells were suspended in PBS-Ca^{2+}/Mg^{2+}, treated with 100 nM bosutinib

or DMSO for 30 min at 37°C, treated with 1 μM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma-Aldrich) at 4°C for 10 min, and lysed and prepared for SDS-PAGE and western blot analysis. Antibodies used were: rabbit anti-Abl1, rabbit anti-p-Crkl (Y207; #3181; Cell Signaling), rabbit anti-p-p40phox (T154; #4311; Cell Signaling), rabbit anti-p-Akt (S473; #9271; Cell Signaling), rabbit anti-p-Pyk2 (Y402; #3291; Cell Signaling), anti-p-Erk1/2 (T202/Y204; #9101; Cell Signaling), anti-p-Pak1/2 (T423/T402; #2601; Cell Signaling); rabbit anti-p-Vav (Y174; sc-16408-R; Santa Cruz); and β-actin (A5316; Sigma-Aldrich).

RBC and hIgG1/streptavidin bead preparation for Biomembrane Force Probe (BFP) experiments

Human red blood cells (RBCs) were isolated from whole blood of healthy volunteers by finger prick according to protocols approved by the Institutional Review Board of Georgia Institute of Technology. The RBCs were then biotinylated by covalently linking with biotin-PEG3500-SGA (JenKem USA) and then swollen by incubating with nystatin (Sigma-Aldrich) for 1 hour at 0°C (1). To make hIgG1/streptavidin (SA) coated beads, borosilicate glass beads (Duke Scientific) were covalently linked with mercapto-propyl-trimethoxy silane (United Chemical Technologies). Purified hIgG1 was first incubated with NHS-PEG-MAL (JenKem USA), and the complex together with streptavidin-maleimide (Sigma-Aldrich) was then incubated with the glass beads for covalent linking (1).

Two-photon intravital microscopy in the glomerulus

Briefly, mice were anesthetized, and placed on a heating pad to maintain constant body temperature. A kidney was exteriorized and immobilized in a custom-made holder. The imaging system was a multi-photon microscope (Prairie Technologies) equipped with a ×20 water immersion objective (Olympus), driven by a MaiTai Ti:sapphire laser (Spectra-Physics) tuned between 770nm and 850nm for multi-photon excitation and second-harmonic generation or a custom-built multi-photon imaging system (2) driven by a Chameleon Vision II Ti:sapphire laser (Coherent) tuned to 915nm. Vessels were visualized by intravenous injection of 70 kD Texas-Red conjugated-dextran (Invitrogen) or Qtracker® 565 (ThermoFisher scientific). Emitted light and second-harmonic signals were detected through 450/80-nm, 525/50-nm and 630/120-nm bandpass filters or alternatively using 495nm and 560nm dichroic mirrors (SEMRock) in series to separate the Qtracker, GFP and SHG signals and collect them with non-descanned detectors for the generation of three-color images. Post-acquisition image analysis, volume rendering and four-dimensional time-lapse videos were performed using Imaris software (Bitplane). Each mouse preparation was recorded for either 168 seconds (400 frames) with 0.42 second per frame, 10 min at 13 seconds per frame for a 225x250x51μm volume or, in some cases, 25 frames per second (in a single plane) for 500 frames total. For lower time resolution imaging experiments, cells captured in only a single frame were excluded from the analysis, as two frames are required to calculate the duration of an adhesion event (i.e. a cell in two consecutive frames has a duration of 0.42s). Cells that were present at the start of the experiment and remained in place for the entire 168 sec video were excluded from the analysis, since their actual dwell time could not be measured. Alternatively, Imaris was used to track individual LysM-GFP$^+$ cells in glomeruli. Cell capture was measured by counting the total number of cell tracks and dwell time by measuring cell track lengths or durations.

Cutaneous RPA reaction

Mice were given an oral administration of bosutinib or vehicle. After 30 min, anesthetized female mice were injected intradermally with rabbit IgG anti-bovine serum albumin Abs (60 μg/30 μl; B7276; Sigma-Aldrich), followed immediately thereafter by an i.v. injection of BSA (500 μg/mouse; Sigma-Aldrich) as described (3). The intradermal injection of PBS served as a negative control. In cases where edema was measured, the BSA solution contained 0.15% Evans blue dye (Sigma-Aldrich). The skin was harvested 3 hours later. Edema was evaluated by measuring the vascular leak of Evans Blue as described (3) and edema formation was measured by subtracting the absorbance in the PBS-injected site from that of the

IgG-challenged site in the same mouse. For tissue neutrophil accumulation, MPO activity in supernatants of homogenized tissue was measured with TMB substrate kit (Pierce). MPO was normalized to protein content measured with BCA Protein Assay Kit (Pierce).

Nephrotoxic (anti-GBM) nephritis model

Mice were preimmunized subcutaneously with 0.05 mg rabbit IgG (#31325; Thermo Scientific) in Freund incomplete adjuvant (Thermo) and nonviable desiccated Mycobacterium tuberculosis H37Ra (Difco, Michigan). Mice were injected intravenously with 50 μl heat-inactivated, filter-sterilized rabbit NTS (day 0). Mice were treated with oral administration (gavage) of bosutinib (150 mg/kg) or a vehicle solution daily from day 0 to 21. Spot urine and peripheral blood samples were collected at indicated time points after NTS injection. Kidneys from euthanized mice were harvested for histological analysis at day 14 (for neutrophil counts) or 21 (for histology).

Urine analysis

Using spot urine samples, urine albumin (BETHYL Laboratories) and creatinine (Cayman) were evaluated by ELISA and a biochemical method, respectively, and expressed as a ratio of urine albumin to creatinine (4).

Histological analysis and scoring

For kidneys, paraffin-embedded sections were stained with periodic acid-Schiff and H&E for histological analysis and anti-NIMP-R14 antibody (#ab2557; Abcam) to identify neutrophils. All histological analyses were done blinded to the identity of the samples. Glomerular crescent formation and tubulointerstitial injury were scored as previously described (5). Crescent formation is presented as the percentage of glomeruli with crescents in the field of view. Glomerular neutrophil accumulation was quantified in 5 high-power fields (at least 30 glomerular cross sections) and presented as neutrophils per glomerular cross-section.

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Figure 3B.

Arrows are next to the blots that were used.

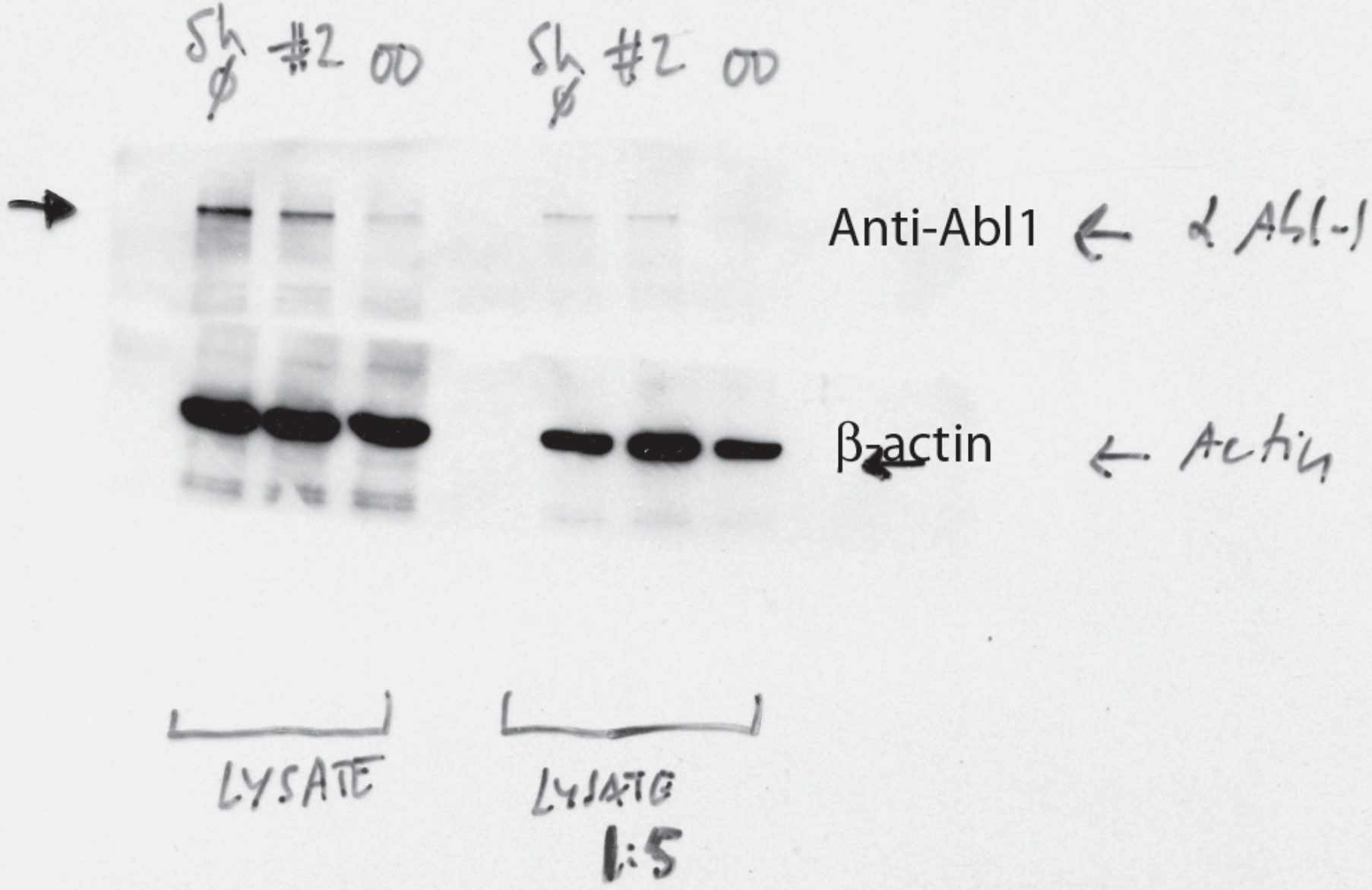

Figure 3F, left panel
(mw, molecular weight marker)

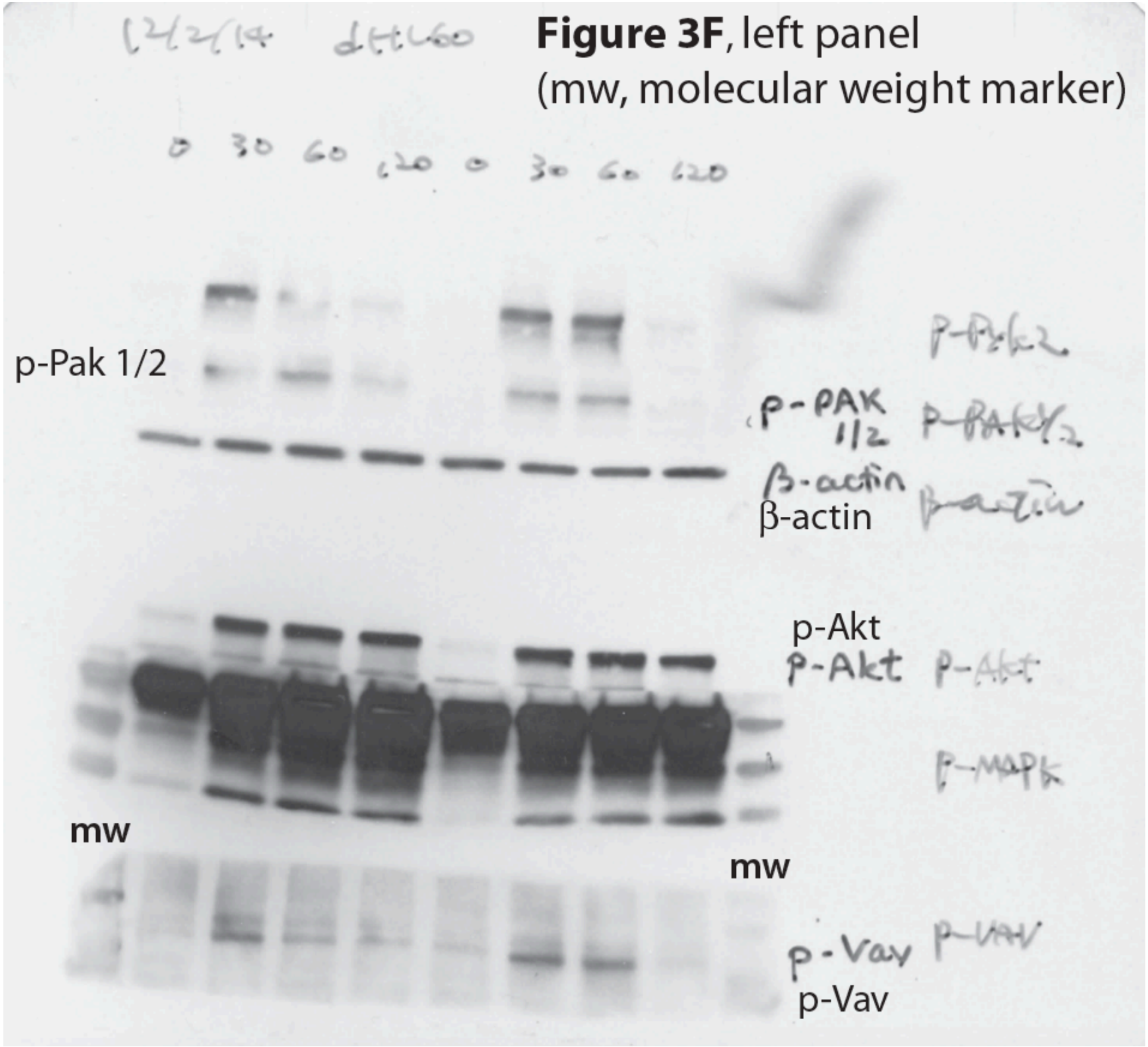

Figure 3F, right panel

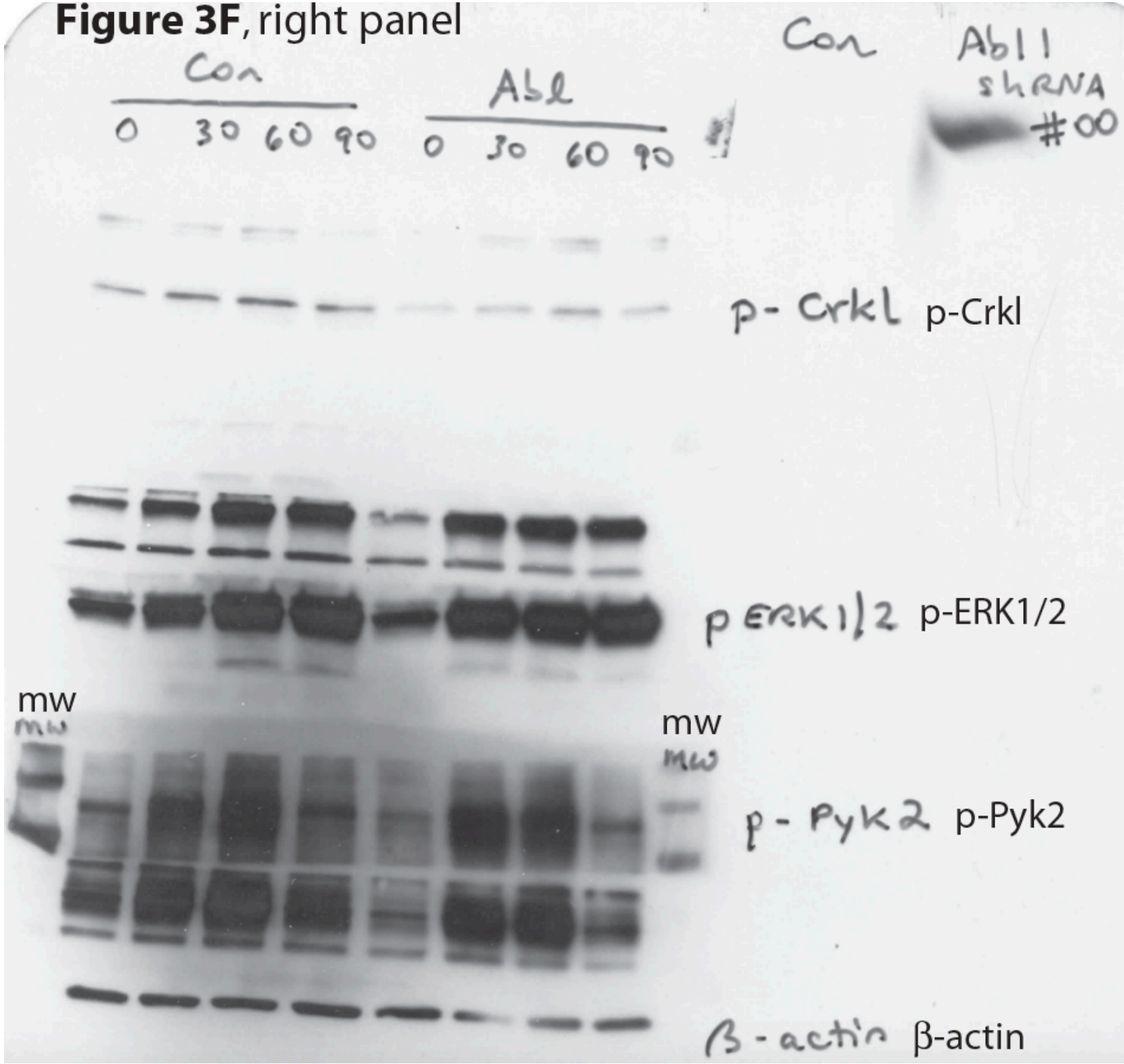

Figure S3A top panel

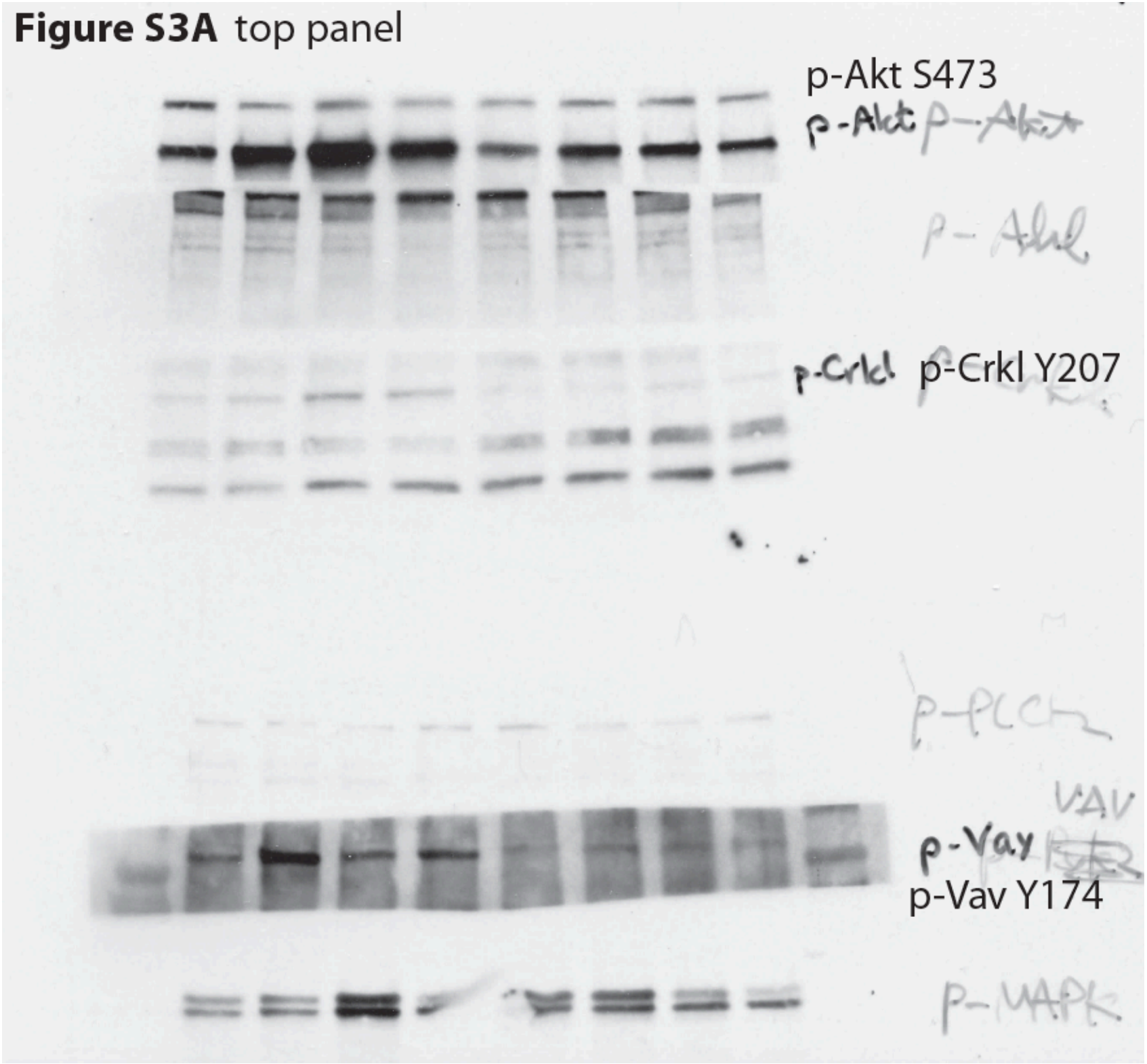

Figure S3A, bottom panel

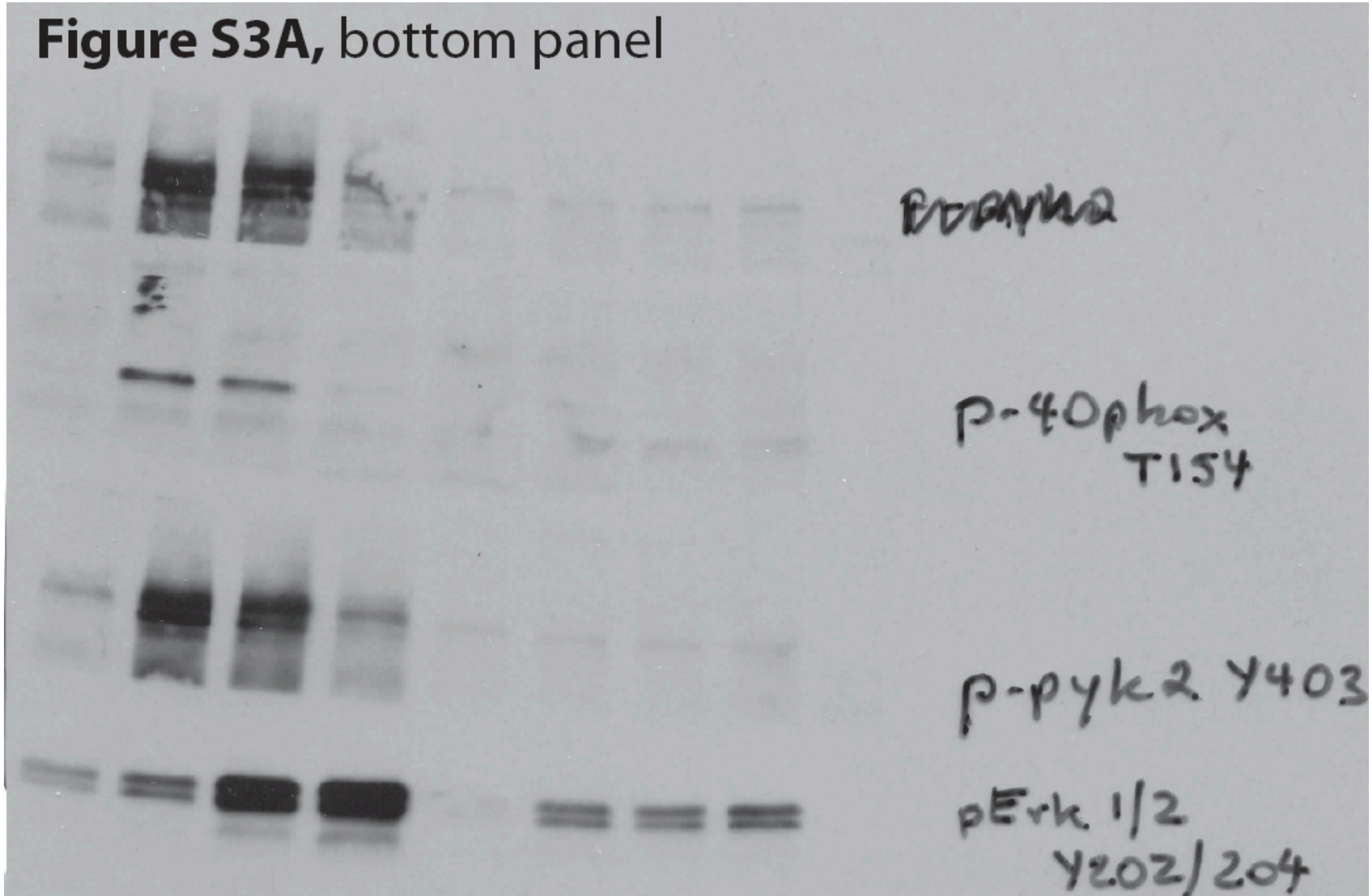

Figure S3A, actin

Figure S3B, top panel

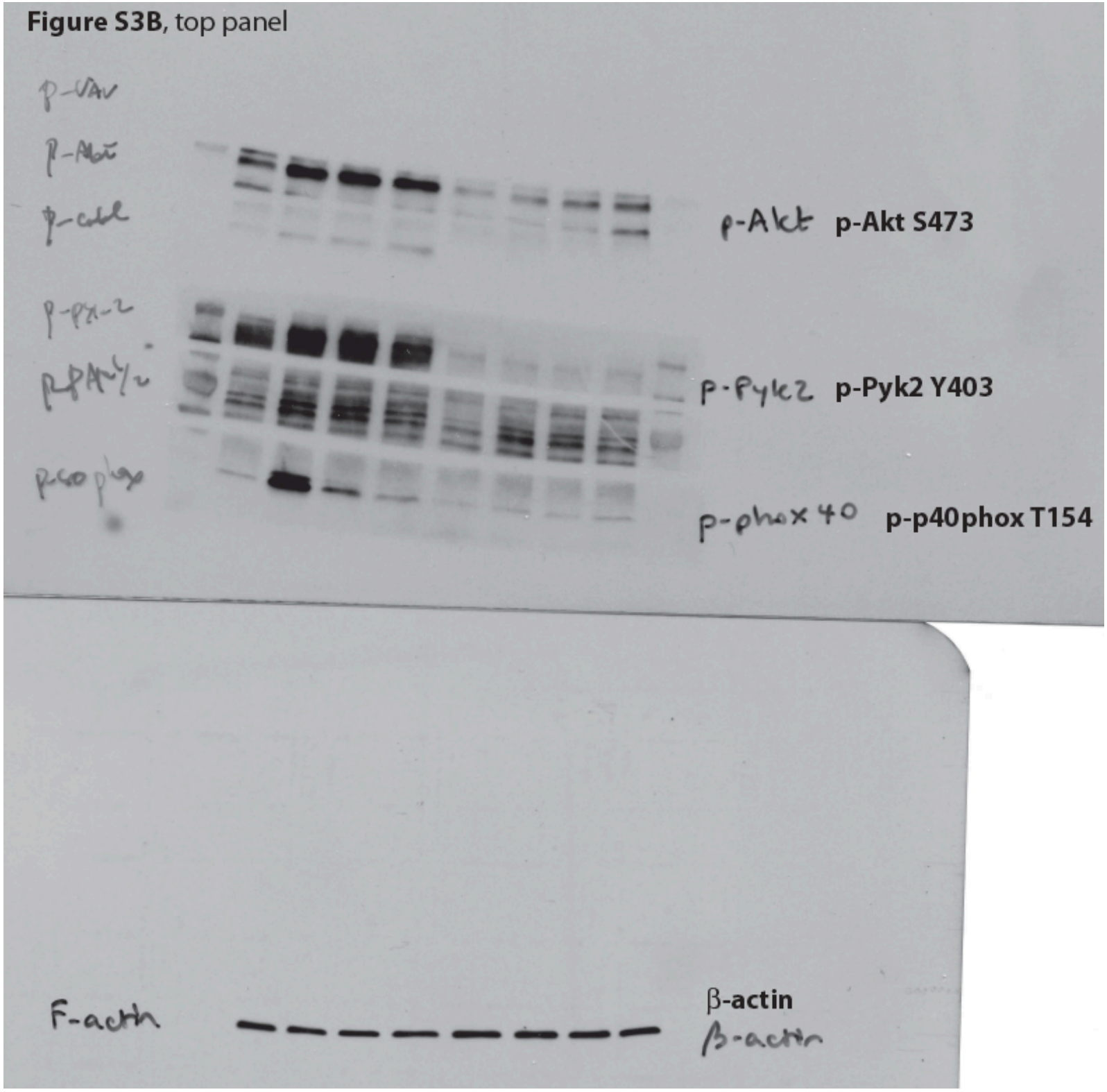

Figure 3SB, bottom panel