ONLINE METHODS

Reagents

Urea (Sigma Ultra, #U0631), iodoacetamide (IAM) (Sigma Ultra, #I1149), TRIZMA hydrochloride (T2694), DL-dithiothreitol (DTT) (Fluka, #43817), and commercial mouse plasma (P9275) were obtained from Sigma Aldrich (Saint Louis, MO). DTT (Thermo Scientific, #20291), and mass spectrometry grade acetonitrile and water (Optima LCMS, A955-4 and W6-4, respectively) were purchased from Fisher Scientific (Pittsburg, PA). Synthetic heavy and light peptides were purchased from Sigma Aldrich (St. Louis, MO), New England Peptide (Gardner, MA), and PPR Ltd (Hampshire, UK).

Mouse Models

All mouse work was performed under IACUC regulations as approved by the FHCRC animal use committee. The breast cancer model used was the doxycycline-inducible, bitransgenic MMTV-rtTA/TetO-NeuNT model, with plasma collected from one cohort (20 pairs) representing pre-clinical (not grossly visible and non-palpable tumors, <0.3 cm), and two cohorts (20 and 19 pairs respectively) representing clinically apparent (1 cm) tumors. Corresponding non-tumor bearing controls were transgenic for TetO-NeuNT only. To avoid bias, breast cancer experimental and control mice were paired at weaning and matched with respect to age, sex, litter, cage, and treatment protocols. Both groups of mice received doxycycline (2 mg/mL + 5% sucrose) in the drinking water starting at 8 weeks of age. Experimental and control mice were euthanized back-to-back on the same day. All mice were euthanized with CO₂ and blood was collected by cardiac puncture using a 1 cm³ syringe with a 23G needle. Blood samples were placed in K₃EDTA tubes and plasma was isolated by centrifugation at 2000×g for 5 minutes. Plasma was immediately transferred to cryovials and frozen at -80 °C.

Wild type mice of strain FVB were used to induce confounding conditions representing subacute inflammation and necrosis, chronic inflammation resembling arthritis, and angiogenesis. For the subacute inflammation model, surgical sponges of size 10×10 mm² were injected with 1% Carrageenan (Sigma C1867-5G) and implanted subcutaneously into the right flank. Mice were sacrificed 3 weeks after implantation. For chronic inflammation, bovine collagen type II (CII) was emulsified with complete Freund’s adjuvant at a final concentration of 4 mg/mL. A total of 0.1 mL was injected intradermally near the base of the tail. Twenty-one days later a booster of CII emulsified with incomplete Freund’s adjuvant was also injected near the base of the tail. Mice were monitored every 2-3 days for the development and progression of arthritis. For the angiogenesis model, matrigel plus FGF was injected subcutaneously into the right flank. Mice were sacrificed 3 weeks after injection. Untreated mice were used as controls. Confounding condition mice were matched with respect to age, litter, and sex. All mice within a confounding experimental condition were sacrificed on the same day, and all confounding conditions and controls were sacrificed within a three week period. Plasma was collected as described above.

Peptide Quality Control (QC)

An LCT Premier time-of-flight mass spectrometer (Waters Corporation, Milford, MA) was interfaced with a nanoLC system (Agilent 1100, Santa Clara, CA) for LC-MS analysis of peptide purity. The nanoLC system was equipped with an Eksigent Endurance autosampler. Solvents were 0.1% formic acid (mobile phase A) and acetonitrile / 0.1% formic acid (mobile phase B). The system was connected to an IntegraFrit trap column (2.3 cm x 100 μm, New Objective, Woburn, MA) and a RP-18 monolithic column (15 cm x 100 μm, Chromolith CapRod, Merck KGaA, Darmstadt, Germany) via a microcross connector (Upchurch Scientific). The trap was packed in-house with Atlantis C18 material (5 μm particle size, Waters Corporation) at a pressure of 500 psi. Samples were loaded onto the trap column at 1.2 μL/min with 2% B for 12 minutes. The LC column flow rate was decreased to 0.8 μL/min, and peptides were eluted over a 10 minute gradient that was developed from 2 to 40% B. Peptides were injected at 300 fmol on-column. Low- or non-responding peptides were re-injected at 1500 fmol on-column. The TOF-MS was operated in the positive ion mode. The capillary voltage applied was 2000 V, and the source temperature was 100 °C. The m/z range was 400-1600 amu with a 1.0 second scan time and a 0.05 second interscan delay time. Peptides were evaluated for purity by measuring a base peak at the expected m/z ratio with no additional impurity peaks present.

Depletion and Digestion of Pooled Samples for AIMS and SQ-SRM
A pool of plasma was generated by combining equal protein mass from each of 20 individual animals. For AIMS analysis, a pool of plasma from 20 tumor-bearing mice was prepared. For SQ-SRM analysis, a pool of plasma was prepared from 20 control mice in addition to a pool from 20 tumor-bearing mice. The protein concentration of individual plasma samples was determined using the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL). Abundant proteins (albumin, immunoglobulin G (IgG), α1-antitrypsin, immunoglobulin M (IgM), transferrin, haptoglobin, and fibrinogen) were depleted using the Seppro IgY-M7 LC10 column system (Sigma Aldrich, St. Louis, MO). Plasma samples were diluted 6× in IgY dilution buffer, filtered (0.22 µm), then injected onto an IgY column attached to a BioCad Vision HPLC system (ABSciex, Foster City, CA). The unretained fraction was collected for multiple injections and pooled. The depleted protein was then desalted and buffer exchanged to 10 mM Tris, pH 8 using Amicon Ultra 5 kDa molecular weight cut-off (Millipore, Billerica, MA) filters and protein concentration determined by BCA prior to trypsin digestion.

Depleted plasma was diluted 10× in digestion buffer (10 mM Tris pH 8, 6.0 M urea, and 20 mM dithiothreitol (DTT) (prepared fresh), denatured, and reduced at 37 °C for 30 minutes. Samples were then alkylated with 50 mM iodoacetamide (IAM) at room temperature in the dark for 30 minutes. After alkylation, samples were diluted 10× again in 10 mM Tris, pH 8, and digested with Trypsin Gold (Promega, Madison, WI) at a protein-to-enzyme ratio of 50:1 (w/w) overnight at 37 °C. The reaction was quenched by adding formic acid to a final concentration of 1%. The digests were desalted with Discovery SPE RP reverse phase C18 columns (Supelco). Briefly, the columns were rinsed with 3 washes of 80% acetonitrile / 0.1% formic acid (FA) and equilibrated with 4 washes of 0.1% FA. The sample was applied to the column, washed 4× with 0.1% FA, and eluted with 2 washes of 50% acetonitrile / 0.1% FA, followed by 2 washes of 80% acetonitrile / 0.1% FA. Finally, the sample was concentrated by vacuum centrifugation until the volume was approximately 20 µL.

**Strong Cation Exchange Fractionation (SCX)**

For AIMS and SQ-SRM studies, depleted and digested plasma was subjected to strong cation exchange (SCX) chromatography to generate 10 fractions. SCX fractionation was carried out on a BioBasic (250 mm x 1 mm x 5 µm) column (Thermo Scientific, Waltham, MA). Solvents were 5 mM KH₂PO₄ / 5% acetonitrile pH 3.0 (buffer A) and 5 mM KH₂PO₄ / 500 mM KCl / 5% acetonitrile pH 3.0 (buffer B). 400 µg of protein was loaded onto the column in 100% buffer A for 5 minutes at 50 µL/min, then eluted with the following gradient: 0 to 10% buffer B for 15 min, 10 to 40% buffer B for 30 min, and 40 to 70% buffer B for 35 min. Fractions were collected every minute, starting at 1 minute, and pooled to give approximately equal mass of total peptide per fraction based on a UV trace. 48 fractions were generated, and ten SCX pools were made, with SCX pools 3-9 submitted as-is for Orbitrap mass spectrometry analysis. The volumes of SCX pools 1 and 2 were dried down to approximately 500 µL using a SpeedVac due to large pool volumes. Fraction 10 was desalted using C18 spin columns (Nest Group, Southborough, MA) before analysis due to the high salt concentration at the end of the gradient. Nominal sample concentrations were calculated to ensure that fractions were in a 0.2-0.4 µg/µL range.

**Accurate Inclusion Mass Screening (AIMS)**

A complete description of the AIMS analysis including LC configuration and gradient methods is presented in Supplementary Section 2. Briefly, we targeted 16,961 proteotypic peptides from 1,144 of the original 1,908 candidate biomarkers. The +2 and +3 charge states for each of the peptides were used to generate 21 inclusion lists for targeted analysis on a hybrid LTQ-Orbitrap mass spectrometer (Thermo, Bremen, Germany) based on the number of precursors that could be monitored in one run. Each list was divided into 10 segments based on the predicted retention time of the desired peptides (see below for details) with each segment containing an inclusion list of 500 precursor ions. Each run method contained 6 scan events. The first scan event was a full MS survey scan done in the Orbitrap (automatic gain control (AGC) target value 1e6, resolution 60K, and injection time 150 ms) and scan events 2 through 6 were data-dependent MS/MS spectra acquisitions in the linear ion trap (target value of 1e4, isolation width of 2 m/z, collision energy of 35%, and injection time of 100 ms). The MS/MS scans were solely dependent on the observance of up to 500 ions present in the parent mass inclusion list. The precursor ion selection for MS/MS was set to +/- 10 ppm around the m/z values in the parent mass list. Dynamic exclusion settings included a repeat count of 1, repeat duration
of 15 sec, exclusion list size of 100, and exclusion duration of 15 sec. Charge state screening was used allowing only +2 and +3 charged peptides to be selected for MS/MS. To limit false parent mass MS/MS triggers, the monoisotopic precursor selection and non-peptide monoisotopic recognition were both enabled. Lists were segmented based on empirically observed or predicted retention times for the target peptides. Based on retention time, the peptides were divided within each run into 10 non-overlapping segments of 2000 seconds each with one inclusion list containing 500 precursor ions (two charge states per peptide) per each segment. Due to the uncertainty of RT prediction, peptides were programmed in the nearest two adjacent segments. The most hydrophobic peptides (i.e. latest eluting peptides) were confined to the last segment only. In addition to the targeted AIMS analysis, shotgun data-dependent runs were also acquired. Further details are provided in Supplementary section 2.

**Semi-quantitative Selected reaction monitoring (SQ-SRM)**

The same LC configuration and eluting gradient for the AIMS experiments was used in order to schedule the SQ-SRM targets using the empirical retention time obtained from the AIMS experiments (see Supplemental section 2 for details). A hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP, ABSciex) with a nano electrospray interface was used for the SRM analyses. The instrument was controlled by Analyst 1.5 software (ABSciex) and operated in the positive ion mode. Typical instrument settings included a spray voltage of 2.3 kV, a nebulization gas setting (GS1) of 15, an ion source temperature of 150° C, and Q1 and Q3 set to unit/unit resolution (0.7 Da). The scheduled SRM option was used for all data acquisition with about 1,100 transitions per run, a target scan time of 3 seconds, and a 15 minute SRM detection window. Transition lists included 191 transitions from the 56 housekeeping peptides in addition to the candidate peptide transitions. All SQ-SRM runs were performed in triplicate. SRM data were processed using MultiQuant 1.1 (ABSciex) with the MQ algorithm for peak integration. A 7-point smooth with a peak-splitting factor of 2 and the default MultiQuant values for noise percentage and baseline subtraction window were used. Transitions for the peptide of interest were required to have similar retention times (±7.5 min) in normal and cancer samples and multiple transitions to the same peptide were required to have the same retention time within an LC-MS run. All data were manually inspected to ensure correct peak detection and accurate integration. A signal-to-noise threshold of 8 was used to distinguish robust signals from the background. Further details are provided in Supplementary section 3.

**Response Curves**

Two types of response curves were generated by QSRM and SISCAPA-SRM (Supplementary sections 4 and 5), one for which the light peptide concentration was varied (forward curve) and one for which the heavy isotopically labeled peptide concentration was varied (reverse curve). The latter was used to assess the lower limits of detection and quantification in cases where endogenous light peptide caused the forward response curve to level off before the signal intensity had reached noise levels (asterisks in Tables 1 and 2).

The response curves for biomarker candidates in the QSRM samples consisted of 11 points, with 1:4 serial dilutions. The starting concentration varied for each analyte, based on the expected concentration of the endogenous peptide (Supplementary Excel Worksheet S4b). The internal standard concentrations also varied based on the endogenous peptide concentration. Depleted and digested mouse plasma (Sigma Aldrich, St. Louis, MO) was prepared as a diluent for each response curve point. A list of peptides and the associated transitions and instrument parameters for the biomarker candidates is presented as part of Supplementary Excel Worksheet S4a. The optimized transitions for each biomarker candidate were configured into a single multiplex assay by scheduling the transitions by retention time. Scheduled SRMs were set up using a 420 sec SRM detection window and a 1.5 sec cycle time. Three technical repeats (separate LC-SRM-MS injections) were performed at ten concentration points (and a blank sample). The best transition (determined by highest signal-to-noise and lowest background levels) for each target was selected as the basis for the quantitative assay, and the remaining transitions were used as ‘qualifiers’ to ensure a high specificity (see Supplemental Excel Worksheet S4c for a list of transitions used in quantitation).

The response curves for the SISCAPA samples consisted of 11 points (Supplementary Excel Worksheet S5b) starting with 500 fmol/µL in a capture and subsequent 1:4 serial dilutions, and the internal standard
concentration was 10 fmol/μL. Three capture replicates were performed for each concentration point, and 10 μL of digested mouse plasma (Sigma Aldrich, St. Louis, MO) was used for each capture. A list of peptides and the associated transitions and instrument parameters for each analyte in the 31-plex SISCAPA method is presented in Supplementary Excel Worksheet S5c. The captures and further sample processing steps were performed as for the pre-clinical and confounding samples below, using 1 μg of anti-peptide antibody for each analyte. The best transition (determined by highest signal-to-noise and lowest background levels) for each target was selected as the basis for the quantitative assay, remaining transitions were used as ‘qualifiers’ to ensure a high specificity (see Supplemental Excel Worksheet S5d for a list of transitions used in quantitation).

SRM data were processed using MultiQuant 1.1 (ABSciex) with the MQL algorithm for peak integration. A 3-point smooth with a peak-splitting factor of 2 and the default MultiQuant values for noise percentage and baseline subtraction window were used. Multiple transitions for the peptide of interest were required to have the same retention time as the internal standard (Supplementary Excel Worksheets S4c and S5d). All data were manually inspected to ensure correct peak detection and accurate integration. Integration results were exported to the program R for linear regression and statistical analysis. Linear regression was used to fit the ten serial dilution data points for each curve. Regression was performed using a 1/y weighting on all points having a correlation coefficient of >0.98. Precision was determined by measuring the coefficient of variation (CV, standard deviation divided by the mean) and expressed as a percent. Limits of detection (LOD) and limits of quantitation (LOQ) for each target were obtained by using the average of the blank measurements plus three times the standard deviation of the noise (for LOD) and ten times the standard deviation of the noise (for LOQ). To ensure the most reliable measurements, an additional constraint was applied to the LOQ where the average CV at the two concentration points nearest the calculated LOQ must be less than 25%.40, 41 For assays where the condition was not met, the next highest concentration point where CV < 25% was considered the LOQ for that target.

Verification Studies Using a 57-plex SRM-MS Assay

Individual plasma samples for the 57-plex Q-SRM verification studies were depleted using a Seppro IgY-M7 LC2 column system (Sigma Aldrich, St. Louis, MO) as described above, with the following additional considerations. To avoid bias in sample processing, individual samples were processed in 3 batches, based on the mouse cohorts: preclinical, clinically apparent, and confounding. Pre-clinical and clinically apparent cohort samples were processed as cage-matched case and control pairs, while confounding condition samples were randomly assigned numbers one through ten, and individual samples were assigned to groups via these numbers. Pairs or groups were depleted as described above. Blanks were run between each sample. UV traces were used to confirm uniformity of depletion.

Following depletion, individual plasma samples were digested as follows; 9 M urea, 300 mM Tris, pH 8, and 20 mM DTT were added to a final concentration of 6 M, 150 mM, and 13.3 mM, respectively. Denaturation, reduction, alkylation, and digestion were carried out as described above. After digestion, the samples were desalted with 10 mg Oasis C18 columns (Waters Corporation, Milford, MA) as described above, and bound peptides were eluted with 3 washes of 80% acetonitrile/ 0.1% formic acid. A mixture containing each of the 57 stable isotope-labeled peptides was prepared at different concentrations, based on the expected concentration of the endogenous peptide in each sample. Samples for LC-SRM-MS analysis were prepared by combining the depleted and digested sample with the mixture of 57 peptides.

Depleted and digested plasma samples were analyzed on an Eksigent nanoLC-Ultra 2Dplus (Eksigent Technologies, Dublin, CA) coupled to a 5500 QTRAP (ABSciex, Foster City, CA) mass spectrometer. Mobile phases consisted of 0.1% formic acid in water (A) and 90% acetonitrile with 0.1% formic acid (B). 15 μL of sample (corresponding to about 1.5 μL of depleted plasma) was loaded onto a 0.2 × 5 mm Chromolith CapRod RP-18e Trap column (EMD Chemicals, Gibbstown, NJ) for 3 min at 10 μL/min with 5% mobile phase B. The peptides were then separated by a 0.05 × 150 mm Chromolith CapRod RP-18e column (EMD Chemicals) by the following gradient method: hold 5% B for 6 min, gradient from 10 to 30% B for 30 min, gradient from 30 to 40% B for 5 min, gradient from 40 to 90% B for 1 min, hold 90% B for 5 min, re-equilibrate at 5% B for 14 min. The flow rate was 600 nL/min. The trap column was back-flushed during the last 5 minutes of a run using 5% B
at 10 µL/min. The LC system was coupled to the 5500 QTRAP mass spectrometer by an Advance CaptiveSpray source (Michrom Bioresources, Auburn, CA). The MS was used in positive ion mode with typical source parameters consisting of a 1200 V ion spray voltage, curtain gas setting of 10, nebulizer gas setting of 0, and an interface heater temperature of 110 °C. For each run, the acquisition of 57 peptides was scheduled using a 420 sec SRM detection window and a 1.5 sec cycle time. Six transitions were monitored for each peptide (3 light and 3 heavy transitions), see Supplementary Excel Worksheet S4a for a complete list of transitions. A Skyline document (mouseQSRM targets.sky) containing peptide sequences and SRM transitions is also available as a supplementary file. Each sample was run in triplicate. SRM data were processed using MultiQuant 1.1 (ABSciex) with the MQL algorithm for peak integration. A 3-point smooth with a peak-splitting factor of 2 and the default MultiQuant values for noise percentage and baseline subtraction window were used. Multiple transitions for the peptide of interest were required to have the same retention time as the internal standard. All data were manually inspected to ensure correct peak detection and accurate integration. Integration results were exported to the program R for linear regression and statistical analysis. More details are provided in Supplementary section 6.

Verification Studies using the 31-plex SISCAPA-SRM-MS assay
Sample digestion and SISCAPA enrichment were performed in triplicate (using 10 µL of plasma per replicate) for each individual mouse as follows. Ten microliters of plasma was diluted with 300 mM Tris, pH 8 containing 9 M urea and 30 mM dithiothreitol to yield 6 M and 20 mM final concentrations of urea and dithiothreitol, respectively. The plasma was incubated for 30 minutes at 37 °C, and 500 mM iodoacetamide was added to yield a final concentration of 40 mM. The samples were then incubated in the dark for 30 minutes at room temperature, diluted 10 times with 100 mM Tris, pH 8 and incubated with trypsin (V5280, Promega, Pittsburgh, PA) at 37 °C for 16 hours using an enzyme-to-substrate ratio of 1:50. The pH of the digests was lowered by addition of formic acid to a final 1% concentration. For the clinically apparent and confounding condition mouse plasma samples, a mixture containing 200 fmol of each of the 31 stable isotope-labeled peptides was added. Oasis C18 resins (Waters, Milford, MA) were conditioned with 3 x 0.4 mL of 80% acetonitrile in 0.1% formic acid and equilibrated with 4 x 0.4 mL of 0.1% formic acid. To lower nonspecific binding to the resins, the resins were additionally conditioned with 10 µL of a human plasma tryptic digest. Subsequently, the mouse plasma samples were loaded onto the resins and the resins were washed using 4 x 0.4 mL of 0.1% formic acid. The desalted peptides were eluted from the columns with 3 x 0.4 mL of 80% acetonitrile in 0.1% formic acid. Samples were dried by vacuum centrifugation and frozen at -80 °C until use. For the pre-clinical mice, Oasis C18 resins were conditioned with acetonitrile and formic acid as above and the mouse plasma digests were loaded onto the columns. The columns were washed with 4 x 0.4 mL of 0.1% formic acid and the peptides were eluted with 3 x 0.4 mL of 80% acetonitrile in 0.1% formic acid. The eluted peptides were dried, reconstituted with 0.1% formic acid containing 200 fmol of each of the 31 stable isotope-labeled peptides, and loaded once again on their respective C18 resins. The washing and elution steps were repeated as above and the samples were dried and frozen at -80 °C.

Immuno-enrichment of the peptides by SISCAPA was carried out in 96-well polypropylene plates (Fisher Scientific, Pittsburg, PA) as described previously, with 31 anti-peptide polyclonal antibodies multiplexed for each capture. For each individual replicate, 10 µL of digested plasma were reconstituted in 30 µL of phosphate buffered saline (PBS), and the pH of the samples was adjusted to neutral. For the pre-clinical and confounding mouse plasma samples, a mixture containing 1 µg of each of the 31 anti-peptide antibodies was added and PBS and CHAPS were added to reach a 100 µL final capture volume for each sample in each well (0.03% final CHAPS concentration). The samples were incubated overnight with gentle mixing at 4 °C. Subsequently, the plate was put into a KingFisher magnetic bead processing platform (Thermo Fisher, Waltham, MA). Forty-five microliters of protein G-immobilized magnetic beads (MyOne, Invitrogen, Carlsbad, CA) were added to the samples and mixed for 2 hours at room temperature. The beads were then washed twice with PBS containing 0.03% CHAPS and once with 1/100 PBS + 0.03% CHAPS. The peptides were eluted using 13 µL of 5% acetic acid containing 0.03% CHAPS and the eluates were frozen at -80 °C until analysis by liquid chromatography-selected reaction monitoring-mass spectrometry (LC-SRM-MS). For the peptide enrichment of the clinically apparent mouse plasma samples, 1 µg of each of the 31 anti-peptide antibodies was incubated with 10 µL of
plasma peptides, 45 μL of protein G-immobilized magnetic beads, and PBS + 0.03% CHAPS at the same time in a total capture volume of 120 μL. The plate was taped to a tumbler and incubated overnight at 4 °C while tumbling to keep the beads in suspension. The washing and elution steps were performed as above for the pre-clinical and confounding samples, with a 25 μL elution volume.

The LC system consisted of an Eksigent 2DLC system (Eksigent Technologies, Dublin, CA) configured with a nano autosampler and two external 10-port microvalves. Mobile phases consisted of 0.1% formic acid in water (A) and 90% acetonitrile with 0.1% formic acid (B). 10 μL of sample was loaded onto a 0.3 × 5 mm PepMap Acclaim C18 trap column (Dionex, Sunnyvale, CA) for 1.5 min at 10 μL/min with 3% mobile phase B. The peptides were then separated using a 0.075 x 100 mm column (PicoFrit, New Objective, Woburn, MA) packed in-house with 3 μm ReproSil-Pur C18-AQ particles (Dr. Maisch GmbH, Ammerbuch, Germany). The PicoFrit column had a 10 μm inner diameter emitter tip. A 12 minute gradient was developed from 3 to 50% B using 300 nL/min, after which solvent B was increased to 90% over 2 minutes and held at 90% for 3 minutes. The column was subsequently re-equilibrated at 3% B for 17 minutes (for 8 of the 17 minutes, the flow rate was increased to 400 nL/min to speed up the equilibration). The trap column was back-flushed during the last 2 minutes of a run using 3% B at 10 μL/min. The LC system was connected via a nanoelectrospray interface to a 4000 QTRAP (ABSciex, Foster City, CA) that was used in positive ion mode. Typical source parameters consisted of a 2300 V ion spray voltage, curtain gas and nebulizer gas settings of 15 and 12, respectively, and an interface heater temperature of 150 °C. For each run, the acquisition of 31 peptides was scheduled using a 90 sec SRM detection window and a 0.5 sec cycle time. Six transitions were monitored for each peptide (3 light and 3 heavy transitions), see Supplementary Excel Worksheet S5c for a complete list of transitions. A Skyline document (mouseImmunoSRM_targets.sky) containing peptide sequences and SRM transitions is also available as a supplementary file.

SRM data were processed using MultiQuant 1.1 (ABSciex) with the MQL algorithm for peak integration. A 3-point smooth with a peak-splitting factor of 2 and the default MultiQuant values for noise percentage and baseline subtraction window were used. Multiple transitions for the peptide of interest were required to have the same retention time as the internal standard. All data were manually inspected to ensure correct peak detection and accurate integration. Integration results were exported to R for linear regression and statistical analysis. More details are provided in Supplementary section 6.

Enzyme-Linked Immunosorbent Assays
Analyses of four mouse plasma proteins were performed using commercially available Enzyme-Linked Immunosorbent Assay kits purchased from two sources: R&D Systems, Minneapolis, MN (Milk fat globule-EGF factor 8 (Mfge8), cat #: DY2805), Lipocalin-2 (Lcn2), cat #: MLCN20), and Chitinase 3-like1 (Chi3L1, cat #: MC3L10) and RayBio, Norcross, GA (E-Cadherin (Cdh1), cat #: ELH-ECadherin-001). The samples and standards were processed according to the manufacturers’ instructions with the following plasma dilutions: 40, 400, 80, and 100 times for Mfge8, Lcn2, Chi3L1, and Cdh1, respectively.

Western blot. Pools of plasma were prepared by taking 30 μL from 5 tumor-bearing mice and their respective matched controls. The plasma pool was mixed with lithium dodecyl sulfate buffer and reducing agent (Invitrogen) and loaded at 30 μg per lane. The samples ran in a SDS-PAGE gel (4-12% Bis-Tris in MOPS Buffer or 3-8% Tris-Acetate in Tris-Acetate Buffer) and transferred to a Nitrocellulose Membrane (0.45 μm pore size). The blots were blocked with 5% non-fat dried milk, washed twice in phosphate buffered saline (PBS) (with 0.1% Tween20) then treated with the primary antibody and incubated overnight in 4 °C. The following day the blots were washed twice in PBS (with 0.1% Tween20) then treated with the secondary antibody linked with horseradish peroxidase (HRP) (IgG) for an hour at room temperature. After the secondary incubation, the blots were washed twice in PBS (with 0.1% Tween20) and treated with an enhanced chemiluminescent (ECL) substrate (LumiGlo, cat # 7003, Cell Signaling Technologies, Beverly, MA) for ten minutes in room temperature. After the ECL substrate treatment, the blots were exposed to X-ray film.

Statistical Analysis
For ROC analyses, the following steps were performed: Step 1: The transition having the highest intensity signal among all the transition ions for a given peptide was chosen as the quantifying transition, given that it also met the following two criteria: 1) the mass accuracy between the observed and theoretical mass of the transition ion was within +/- 50 ppm, and 2) the HPLC retention time difference between the heavy and light labeled peptides’ transition peaks (at the apex) did not exceed 0.1 second. In case the two criteria were not met, the second highest intensity transition was chosen as the quantifying transition (given that it met the two criteria). Step 2: Ratios of light to heavy peak areas for the quantifying transition ion of a peptide were calculated, and the median value of three replicates for each mouse sample was used in further calculations (given that the ratios were above the LOQ, which was defined as the average of three blank measurements plus ten times the standard deviation of the measurement). If data above the LOQ were available for only two of three replicates, the average of the two replicate values was used, and if data for only one of three replicates was available, then that one value was used. In case no data were available for all three replicates of a mouse sample, a value equal to the LOQ for the given transition was imputed as the median value for the three replicates. The following receiver operating characteristic (ROC) curve calculations were thus performed on a total of 20 measurements (whether actually measured or imputed), 10 from 10 individual tumor-bearing mice and 10 from 10 individual control mice. Step 3: Given a total of 20 values per protein (based on each protein’s peptide values), an ROC plot was generated for each protein by calculating the sensitivity and specificity for all possible cutoff (threshold) values. The sensitivity depended on the fraction of tumor-bearing samples that were correctly identified as tumor-bearing at a particular cutoff value, and the specificity depended on the fraction of control samples that were correctly identified as control at that particular cutoff value (Ma, S., Huang, J. Bioinformatics 2005, 21, 4356-4362). Sensitivity is indicated on the ROC curves as the true positive rate, and the false positive rate given on the graphs was calculated as 1 - specificity. We subsequently calculated the area under the curve (AUC) for each ROC curve, which is reported as a measure of the ability of the protein to discriminate between tumor-bearing and control samples (the higher the AUC, the better the ability to discriminate).

For descriptions of additional statistical analyses, the reader is referred to the Supplementary data section devoted to each stage of the pipeline for a description of the statistical analysis used at each stage.

Public Availability of Data
All data used by or generated during this study have been deposited in the public domain. TRANCHE hash codes for the plasma AIMS and shotgun data files are given in Supplementary Table S2.1, and the results are presented in Supplementary Excel Worksheet S2. All plasma verification study SRM-MS and immuno-SRM-MS results are presented in Supplementary Excel Worksheets S6a-f, and the integrated data files are also available (See Supplementary Sections 4-5 for TRANCHE hash codes). Additionally, Skyline documents for implementing the 57-plex SRM (mouseQSRM_targets.sky) and the 31-plex SISCAPA (mouselmmunoSRM_targets.sky) assays are available as Supplementary data.