RBC ghost membrane sample preparation and phosphopeptide enrichment
Ghosted RBCs were spun at 14,000 rpm for 15 min at 4^{o}C to pellet membranes. Membrane pellets were washed with 1 mL 50 mM ammonium bicarbonate (pH 8.0) with vortexing and were then spun at 14,000 rpm for 30 min at 4^{o}C. The supernatant was then removed and 500 ∝L of 50 mM ammonium bicarbonate with ∝L 0.2% acid-labile surfactant (ALS-1) in 50 mM ammonium bicarbonate (pH 8.0) was added. Samples were subjected to probe sonication three-times for 5 sec with cooling on ice between and insoluble material was cleared by centrifugation at 14,000 rpm for 30 mins at 4^{o}C. Samples were normalized to approximately 2 ∝g/∝l following a micro-Bradford assay (Pierce Bioscience), and were reduced with a final concentration of 10 mM dithiothreitol at 80^{o}C for 20 min. Samples were then alkylated with a final concentration of 20 mM iodoacetamide at room temperature for 45 min and trypsin was added to a final ratio of 1-to-50 (w/w) enzyme-to-protein and allowed to digest at 37^{o}C for 18 hr. To remove ALS-1, samples were acidified to pH 2.0 with neat TFA, incubated at 60^{o}C for 2 hrs and spun at 14,000 rpm to remove hydrolyzed ALS-1. Samples were either subjected directly to LC-MS analysis or subjected to a TiO_2 based phosphopeptide enriched protocol.

To enrich for phosphorylated peptides prior to LC-MS analysis, either 1,125 ∝g and 970 ∝g of total digested protein from RBC ghosts co-incubated with recombinant active ERK2 experiments and experiments using epinephrine-treated cells, respectively, were brought to near dryness using vacuum centrifugation and then resuspended in 200 ∝L of 80% acetonitrile, 1% TFA, 50 mg/ml MassPrep Enhancer (pH 2.5) (Waters Corp. Milford, MA). Samples were loaded onto an in-house packed TiO_2 spin column (Protea Biosciences) with a 562 ∝g or 485 ∝g binding capacity for active ERK2 treated or epinephrine treated experiments, respectively. Samples were washed twice with 200 ∝L 80% acetonitrile, 1% TFA, 50 mg/ml MassPrep Enhancer (pH 2.5) followed by two washes with 200 ∝L 80% acetonitrile, 1% TFA (pH 2.5). Retained peptides were eluted twice with 100 ∝L 20% acetonitrile, 5% aqueous ammonia (pH 10.0), acidified to pH 3 with neat formic acid and then brought to dryness using vacuum centrifugation. Prior to LC-MS analysis, each sample was resuspended in 20 ∝L 2% acetonitrile, 0.1% TFA, 25 mM citric acid (pH 2.5).

Label-free quantitative phosphoproteomic analysis of RBC ghost
Chromatographic separation of phosphopeptide enriched or non-enriched samples was performed on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C_{18} 75 µm I.D. × 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Five µL injections of each sample were trapped for 5 min on a 5 µm Symmetry C_{18} 180 µm I.D. × 20 mm column at 20 µl/min in 99.9% A. The analytical column was then switched in-line and the mobile phase was held for 5 min at 5% B before applying a linear elution gradient of 5% B to 40% B or 5% B to 30%B over 90 min at 300 nL/min for ERK2 treated experiments or epinephrine treated experiments, respectively. The analytical column was connected to fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10 µm tip orifice and coupled to the mass spectrometer through an electrospray interface.

MS data from each phosphopeptide enriched sample was acquired on a Thermo LTQ-Orbitrap XL mass spectrometer operating in positive-ion mode with an electrospray voltage of 2.0 kV with real-time lockmass correction on ambiant polycyclodimethylsiloxane (m/z 445.120025) enabled. The instrument was set to acquire a precursor MS scan from *m/z* 400–2,000 with r =

60,000 at *m/z* 400 and a target AGC setting of 1e6 ions. Each sample was analyzed four-times, one of which was used for additional qualitative identifications only and was not included in the quantitative analysis, with product ions above a threshold of 500 counts were acquired for the top 5 most intense ions in the linear ion trap. Maximum fill times were set to 1000 ms for full MS scans acquired in the OT and 250 ms for MS/MS acquired in the linear ion trap, with a CID energy setting of 35% and a dynamic exclusion of 60 s for previously fragmented precursor ions. Multistage activation (MSA) for neutral losses of 98.0, 49.0, and 32.33 Da was enabled to enhance fragmentation of phosphorylated peptides. MS data for non-phosphopeptide enriched samples was acquired on a Waters Synapt HDMS operating in positive-ion mode with an electrospray voltage of 3.0 kV. Each sample was analyzed three times in a data-independent (MS^E) mode of acquisition with 0.9 sec cycle times alternating between low collision energy (6 V) and high collision energy ramp (15 to 40 V). One additional data-dependent (DDA) analysis using a 0.9 sec MS scan followed by MS/MS acquisition on the top 3 ions with charge greater than 1 was acquired to increase the number of qualitative identifications. MS/MS scans for each ion used an isolation window of approximately 3 Da, a maximum of 4 seconds per precursor, and dynamic exclusion for 120 seconds within 1.2 Da.

Label-free quantitation and integration of qualitative peptide identifications was performed using Rosetta Elucidator (v 3.3, Rosetta Inpharmatics, Seattle, WA). All raw LC-MS/MS data within an experiment were imported and subjected to chromatographic retention time alignment using the PeakTeller® algorithm with a minimum peak time width set to 6 s, alignment search distance set to 4 min and the refine alignment option enabled. Quantitation of all measurable signals in the precursor MS spectra (excluding LC-MS analysis intended only for additional qualitative identifications), was performed by Elucidator by calculating either peak volume (area under curve) for Synapt HDMS data files or peak height for LTQ-Orbitrap data files.

Qualitative peptide identifications from all phosphopeptide enriched samples and DDA analysis of non-phosphopeptide enriched samples were made by generating DTA files for all precursor ions, which had associated MS/MS spectra. DTA files were submitted to Mascot (Matrix Science, Boston, MA) and searched against a *Homo sapien* protein database downloaded from SwissProt concatenated with the sequence-reversed version of each entry. MS^E data were independently processed within ProteinLynx Global Server 2.4 (Waters Corp) and searchable files were then submitted to the IdentityE search engine (Waters Corp). Search tolerances of 20 ppm precursor and 0.8 Da product ions were applied for LTQ-Orbitrap data and 20 ppm precursor and 0.04 Da product ions were applied for Synapt HDMS data files with lock-mass correction on m/z 785.8426 (doubly-charged Glu-1–Fibrinopeptide ion) enabled. All data were searched using trypsin specificity with up to two missed cleavages with a static modification of Carbamidomethylation (+57.0214 Da on C) and dynamic modifications of oxidation (+15.9949 Da on M). Dynamic search modifications of phosphorylation (+79.9663 Da on STY) and of deamidation (+1.008 Da on NQ) were employed for phosphopeptide enriched sample and non-phosphopeptide enriched samples, respectively. Peptides FDR were determined by adjusting the Mascot peptide ion score threshold to allow a 1% occurrence of peptides from reverse protein entries for phosphopeptide enriched experiments, or by using PeptideProphet algorithim scores which corresponded to a 2% peptide false discovery rate for non-phosphopeptide enriched experiments.

Database search results and spectra have been uploaded in the form of Scaffold 3 files (.sf3, Proteome Software, Inc) to the Tranche database (https://proteomecommons.org/tranche/)

under the group “RBC Ghost Membrane Phosphoproteome” with the following links (if a password is requested, it is *rbcphos*). The data presented are data of proteins related to the paper.

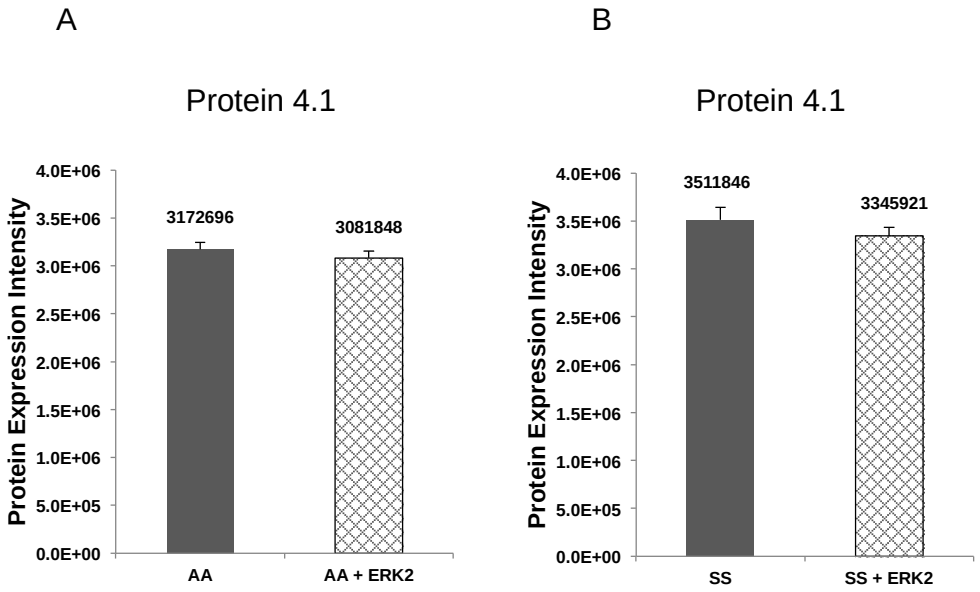

Figure S1. Protein 4.1 levels measured by unbiased label-free quantitative proteomics normalized to levels in (A) AA RBCs or (B) SS RBCs

AA and SS RBC ghosts, and AA and SS RBC ghosts co-incubated with recombinant active ERK2. Protein 4.1 levels remain constant in AA RBCs between the two conditions, and also in SS RBCs between the two conditions.