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(54) Title: METHODS OF TREATING HEMOGLOBINOPATHIES

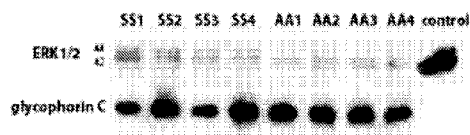


Fig. 1A

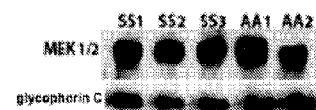
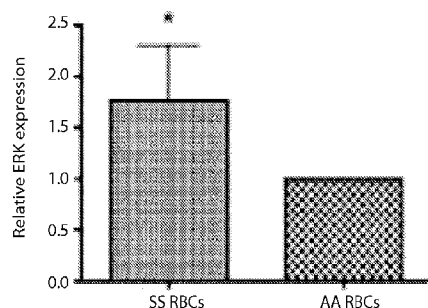


Fig. 1B



(57) Abstract: Methods of alleviating the symptoms of hemoglobinopathies, including, but not limited to, sickle cell disease, β -thalassemia, and hemoglobin H disease are provided. In some embodiments, the methods comprise administering an inhibitor selected from an ERK inhibitor, a MEK inhibitor, and a Raf inhibitor. Methods of inhibiting adhesion of sickle red blood cells to endothelial cells are also provided.

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METHODS OF TREATING HEMOGLOBINOPATHIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application claims the benefit of priority of United States Provisional Patent
5 Application No. 61/480,157, filed April 28, 2011, which is incorporated herein by reference in its
entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

(001) This invention was made with government support under K01-DK065040 awarded
by the National Institutes of Health: National Institute of Diabetes and Digestive and Kidney
10 Diseases. The government has certain rights in the invention.

BACKGROUND

(002) Vaso-occlusive phenomena and hemolytic anemia are the clinical hallmarks of
sickle cell disease (SCD). Sickle (homozygous hemoglobin S, SS) red blood cell (RBC)-based
15 adhesion and vaso-occlusive events likely initiate and/or exacerbate the profound vasculopathy
present in SCD.^{1,2} SS RBCs possess unusually active signaling pathways that contribute to a panoply
of abnormalities, including RBC adhesion to the endothelium and vaso-occlusion.²⁻⁴ Vaso-occlusion
results in recurrent painful episodes and a variety of serious organ system complications that can
lead to life-long disabilities and even death.

20 (003) Cell adhesion is a multistep cellular process that is regulated by complex
extracellular and intracellular signals, which may differ from one cell type to another. We have
previously shown that abnormal SS RBC interaction with the endothelium and with leukocytes can
be induced via stimulation of β_2 adrenergic receptors (ARs) by the stress hormone epinephrine.⁴⁻⁶
Such stimulation activates the intracellular cyclic adenosine monophosphate (cAMP)/protein kinase
25 A (PKA) pathway.⁴ β ARs are prototypic G protein-coupled receptors (GPCRs), whose signaling
properties are largely mediated by activation of stimulatory GTP-binding proteins (Gs proteins),
which in turn activate adenylate cyclase (AC), leading to generation of cAMP, and the subsequent
activation of PKA. The cAMP/PKA pathway can modulate the mitogen-activated protein kinase
(MAPK)/extracellular signal-regulated kinase (ERKs) cascade.⁷ PKA has been reported to stimulate
30 B-Raf, while inhibiting c-Raf. Therefore, the activity of downstream signaling proteins, such as
MEKs and ERKs, could be either enhanced or inhibited depending on the balance of c-Raf and B-Raf

activation.^{8,9} The cellular functions mediated by β ARs can also be independent of adenylyl cyclase activation and involve other mediators instead.^{10,11}

(004) The functions attributed to ERK1/2 at both cellular and physiological levels are diverse, including modulation of proliferation, differentiation, apoptosis, migration, and cell adhesion.¹²⁻¹⁵ Physiologically, ERK1/2 is required for immune system development, homeostasis and antigen activation, memory formation, heart development, and responses to many hormones, growth factors and insulin. Most of these previous studies have involved only nucleated cells, including erythroid cells, in which erythropoietin (EPO) is the primary regulatory cytokine of this pathway.¹⁶ However, aberrations in ERK1/2 signaling are known to occur in a wide range of pathologies, including cancer, diabetes, viral infection, and cardiovascular disease.

SUMMARY

(005) In some embodiments, methods of alleviating at least one symptom of a hemoglobinopathy in a patient is provided. In some embodiments, a hemoglobinopathy is selected from sickle cell disease, β -thalassemia, and hemoglobin H disease. In some embodiments, a hemoglobinopathy is sickle cell disease. In some embodiments, at least one symptom is selected from vaso-occlusion, acute painful episodes, chronic hemolysis (aplastic crises), avascular necrosis, infection, end-organ damage, and erythroid hyperplasia.

(006) In some embodiments, methods of inhibiting adhesion of sickle red blood cells to endothelial cells in a patient are provided. In some embodiments, methods of inhibiting adhesion of sickle red blood cells to leukocytes in a patient are provided. In some embodiments, methods of inhibiting formation of multicellular aggregates in a patient with sickle cell disease are provided. In some embodiments, methods of inhibiting adhesion of leukocytes to endothelial cells in a patient with sickle cell disease are provided.

(007) In some embodiments, a method comprises administering at least one inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.

(008) In some embodiments, the inhibitor is a MEK inhibitor. In some embodiments, the MEK inhibitor is selected from U0126, RDEA119, GSK1120212, PD98059, PD-334581, GDC-0973, CIP-137401, ARRY-162, ARRY-300, PD318088, PD0325901, CI-1040, BMS 777607, AZD8330, AZD6244, AS703026. In some embodiments, the inhibitor is an ERK inhibitor. In some embodiments, the ERK inhibitor is AEZS-131. In some embodiments, the inhibitor is a Raf inhibitor. In some embodiments, the Raf inhibitor is selected from sorafenib tosylate, GDC-0879, PLX-4720, regorafenib, PLX-4032, SB-590885-R, RAF265, GW5074, XL281, and GSK2118436.

(009) In some embodiments, a method of inhibiting adhesion of sickle red blood cells to endothelial cells is provided. In some embodiments, a method of inhibiting adhesion of sickle red blood cells to leukocytes is provided. In some embodiments, a method of inhibiting formation of multicellular aggregates in the presence of sickle red blood cells is provided. In some
 5 embodiments, a method of inhibiting adhesion of leukocytes to endothelial cells in the presence of sickle red blood cells is provided.

(0010) In some embodiments, a method comprises contacting sickle red blood cells with an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.

(0011) In some embodiments, the inhibitor is a MEK inhibitor. In some embodiments, the
 10 MEK inhibitor is selected from U0126, RDEA119, GSK1120212, PD98059, PD-334581, GDC-0973, CIP-137401, ARRY-162, ARRY-300, PD318088, PD0325901, CI-1040, BMS 777607, AZD8330, AZD6244, AS703026. In some embodiments, the inhibitor is an ERK inhibitor. In some embodiments, the ERK inhibitor is AEZS-131. In some embodiments, the inhibitor is a Raf inhibitor. In some embodiments, the Raf inhibitor is selected from sorafenib tosylate, GDC-0879, PLX-4720,
 15 regorafenib, PLX-4032, SB-590885-R, RAF265, GW5074, XL281, and GSK2118436.

DESCRIPTION OF THE FIGURES

(0012) **Figure 1.** ERK undergoes activation in sickle (SS) but not normal red blood cells (RBCs). A and B. Fifty μ g of membrane protein ghosts (SS RBC ghosts, n=4, lanes: SS1, SS2, SS3 and
 20 SS4; and normal RBC ghosts, n=4, lanes: AA1, AA2, AA3 and AA4) were used per lane. Western blots of protein ghosts were stained with antibodies against ERK1/2, glycophorin C as a loading control, and MEK1/2 (n=3 for SS RBC ghosts, lanes: SS1, SS2 and SS3; and n=2 for normal RBC ghosts, lanes: AA1 and AA2). A. ERK1/2 and MEK1/2 are highly expressed in both SS and normal RBCs and are bound to the RBC plasma membrane. B. Quantitative analysis of the data (normalized
 25 according to glycophorin C expression) presented as relative ERK1/2 expression compared to normal RBCs ($p < 0.05$ for SS vs normal RBCs, n=4 for each). C and D. Normal RBCs (n=3, lanes: 1, 2, 3 and 4) and SS RBCs (n=3, lanes: 5, 6, 7 and 8) were sham-treated (lanes 1 and 5), incubated for 1 min with 20 nM epinephrine (epi) (lanes 2 and 6), pre-treated with the MEKI, U0126, followed by epi treatment (lanes 4 and 8), or treated with U0126 alone (lanes 3 and 7). Mouse 3T3/A31
 30 fibroblast lysate was used as a positive control (lane 9). One hundred μ g of SS and normal RBC ghost proteins were used per lane. Western blots were stained with antibodies against ERK and phosphoERK. C. ERK1/2 is phosphorylated at baseline in SS RBCs, and undergoes increased phosphorylation by epi stimulation. ERK in normal RBCs is not phosphorylated and completely

failed to undergo increased phosphorylation after epi stimulation. D. Quantitative analysis of the data is presented as fold change in ERK phosphorylation. * $p < 0.01$ compared to untreated cells.

** $p < 0.001$ compared to epi-treated SS RBCs. E and F. ERK immunoprecipitated from sham-treated (lanes: 1, 2, 5, and 6) and epi-treated (lanes: 3, 4, 7, 8, 11, 12, 13 and 14) SS RBCs was incubated without MBP (lanes: 1, 3, 5, 7, 11 and 13) or with MBP (lanes: 2, 4, 6, 8, 12 and 14) as a substrate for ERK, with equal protein amounts per assay condition. Commercial active recombinant human ERK2 was incubated without MBP (lanes: 9 and 16) or with MBP (lanes: 10 and 15) as negative and positive controls, respectively. E. Immunoblots indicate that the activity of ERK is conserved and functional in SS RBCs and epi can intensify its activity. SS RBCs obtained from four different patients were tested. F. Quantitative analysis of the data is presented as fold change in ERK phosphorylation ($n=4$). $p=0.0286$ compared to non-treated cells.

(0013) **Figure 2.** ERK activation in SS RBCs involves the cAMP/PKA pathway and the tyrosine kinase $p72^{syk}$ and is sensitive to the effect of $G\alpha_i$ protein. SS RBCs (panels A, B, C and D), and reticulocyte-enriched and -depleted (mature) SS RBCs (panel E) were sham-treated, treated with forskolin (panel A), epi (panels B and C), the protein kinase A inhibitor (PKAi), 14-22 amide, (panel B), or Pertussis toxin (PTx) (panels C and D) in the presence or absence of the MEK inhibitor (MEKi) U0126 (panels A, B and D), piceatannol (panel D) or damnacanthal (panel D). RBC proteins were blotted with antibodies against ERK and phosphoERK. Quantitative analysis of the blots is presented as fold change in ERK phosphorylation. A and B. ERK undergoes phosphorylation via the cAMP/PKA pathway. A. ERK undergoes increased phosphorylation after RBC incubation with forskolin, which is inhibited by U0126 ($n=3$). * $p < 0.05$ compared to untreated cells. ** $p < 0.01$ compared to forskolin-treated SS RBCs. B. Phosphorylation of ERK is increased by epi, and this increase was abrogated by either 14-22 amide or U0126 ($n=3$). * $p < 0.01$ compared to untreated cells. ** $p < 0.01$ compared to epi-treated SS RBCs. C. ERK phosphorylation in SS RBCs is enhanced by inactivation of the $G\alpha_i$ protein. PTx at either 1 or 2 $\mu\text{g/ml}$ increased basal ERK phosphorylation ($n=9$). *: $p < 0.001$ compared to non-treated cells; †: $p < 0.05$ compared to epi-treated SS RBCs. D. The tyrosine kinase $p72^{syk}$ is implicated in ERK phosphorylation. PTx at 2 $\mu\text{g/ml}$ upregulated ERK phosphorylation, an effect that was blocked by piceatannol. Conversely, damnacanthal failed to block ERK phosphorylation induced by PTx ($n=3$). * $p < 0.01$ compared to untreated cells. $p < 0.01$ compared to PTx-treated SS RBCs. E. ERK1/2 is phosphorylated at baseline in both reticulocyte-enriched and reticulocyte-depleted (mature) SS RBCs ($n=2$).

(0014) **Figure 3.** ERK signaling modulates both SS RBC adhesion to endothelial cells and ICAM-4 phosphorylation. A and B. Activation of ERK signaling up-regulates SS RBC adhesion to the

endothelium. SS RBCs were sham-treated, stimulated with epi for 1 min or forskolin, pre-incubated with U0126 followed by epi or forskolin, or treated with U0126 alone. Adhesion of SS RBCs to HUVECs was tested in intermittent flow condition assays. Results are presented as % adherent SS RBCs at a shear stress of 2 dynes/cm². Error bars show SEM of four different experiments. A. *: $p < 0.001$ compared to sham-treated; **: $p < 0.001$ compared to epi-treated. B. *: $p < 0.001$ compared to sham-treated; **: $p < 0.001$ compared to forskolin-treated. C and D. The MEK/ERK signaling cascade is involved in ICAM-4 (LW) serine phosphorylation. Panel C. Inorganic ³²P radiolabeled intact SS RBCs were incubated in the absence (lane 1) or presence (lanes 2, 3, 4, 5 and 6) of serine/threonine protein phosphatase inhibitors (SPI), followed or not (lanes 1 and 2) by treatment with epi (lanes 3, 4, 5 and 6). In lanes 4, 5 and 6, SS RBCs were preincubated with PKAI, MEKI or PKAI+MEKI with SPI followed by epi treatment, respectively. The cpm are representative of three different experiments, calculated by subtraction of cpm present in a lane (not shown) containing immunoprecipitates using immunoglobulin P3 from cpm obtained using anti-LW (ICAM-4) mAb for immunoprecipitation under each set of conditions indicated. *: $p < 0.05$ and $p < 0.001$ for SPI-treated and SPI+epi-treated vs. sham-treated, respectively; **: $p < 0.001$ compared to SPI+epi-treated SS RBCs. Total LW loaded in each lane was detected using nitrocellulose membranes of phosphorylated LW blotted with anti-LW mAb. Panel D. SS RBCs were incubated without (lanes 1 and 3) or with epi (lanes 2 and 4). Lanes 1 and 2 were immunoprecipitated with P3. Lanes 3 and 4 were immunoprecipitated with anti-LW mAb; all lanes for panel D were immunostained with anti-LW mAb.

(0015) **Figure 4.** SS RBC adhesion is associated with the extent of ERK activation. A and B. Adhesion of SS RBCs to endothelial cells is related to the duration of cell stimulation with epinephrine. Adhesion of RBCs to HUVECs was tested in both intermittent flow and flowing condition assays, and results are presented as % adherent RBCs at a shear stress of 2 dynes/cm² and number of adherent RBCs/mm², respectively. Normal and SS RBCs were sham-treated, or stimulated with epi for 1 min or 30 min. *: $p < 0.001$ compared to sham-treated SS RBCs; **: $p < 0.001$ compared to epi-treated SS RBCs. Error bars show SEM of four different experiments. C. cAMP production in SS RBCs is associated with the time of cell stimulation with epinephrine. RBCs were treated with IBMX (for basal cAMP levels), followed or not with epi (for 1 min or 30 min) or forskolin. The specific effect of epi and forskolin on cAMP accumulation was obtained by subtracting basal cAMP levels from the total cAMP levels. The basal cAMP production and specific amounts of cAMP due to epi or forskolin stimulation were normalized as fmol cAMP/10⁸ RBCs. D and E. ERK phosphorylation is dependent on the time of SS RBC exposure to epinephrine. SS RBCs

were sham-treated or treated with epi for 1 or 30 min, U0126, or U0126 followed by epi for 1 or 30 min. Immunoblots of RBC proteins with antibodies against ERK and phosphoERK (panel D) and quantitative analysis of the data presented as fold change in ERK phosphorylation (panel E) are shown. ERK underwent increased phosphorylation after 1 min exposure to epi, while

5 phosphorylation decreased with longer (30 min) cell exposure to epi (n=4). *: $p < 0.01$ compared to non-treated cells; **: $p < 0.01$ and $p < 0.001$ for epi-treated for 30 min and U0126+epi-treated for 1 min vs cells treated with epi for 1 min, respectively (panel E). F. Inorganic ^{32}P radiolabeled intact SS RBCs were incubated in the presence (lanes 1, 2 and 3) or absence (lane 4) of SPI, followed (lanes 2 and 3) or not (lane 1) by treatment with epi for 1 min (lane 2) or 30 min (lane 3). The cpm are

10 representative of three different experiments, calculated by subtraction of cpm present in a lane (not shown) containing immunoprecipitates using immunoglobulin P3 from cpm obtained using anti-LW (ICAM-4) mAb for immunoprecipitation under each set of conditions indicated. *: $p < 0.01$ compared to sham-treated; †: $p < 0.05$ compared to SPI+epi (30 min)-treated SS RBCs. G. Prolonged cell exposure to epinephrine negatively affects phosphorylation of adenylate cyclase-associated

15 protein 1. RBC ghosts isolated from SS and normal RBCs treated with epi for 1 and 30 min were enriched in phosphopeptides, then subjected to a label-free quantitative phosphoproteomics analysis. Phosphorylation of both serine and threonine on CAP1 in SS RBCs decreased with increased time (1 min vs 30 min) of cell exposure to epi, while an increase in the abundance of these phosphopeptides was observed in normal (AA) RBCs after 30 min exposure to epi. Each data

20 point is an average of three analytical replicate measurements with error bars indicating standard deviations.

(0016) **Figure 5.** Phosphorylation of protein 4.1 is induced via the ERK signaling pathway. Sham-treated or U0126-treated SS and normal (AA) RBCs ghosts co-incubated with or without recombinant active ERK2 (ERK2) were enriched in phosphopeptides, followed by a label-free

25 quantitative phosphoproteomics analysis. Treatment of SS RBCs with U0126 caused a significant decrease in doubly phosphorylated peptide within protein 4.1. Addition of ERK2 to the U0126-treated SS RBC ghosts increased the abundance of this phosphopeptide back to levels observed in untreated SS RBCs. The complementary trend for this phosphorylated peptide was also observed upon the addition of ERK2 to AA RBCs sham-treated or U0126-treated.

30 (0017) **Figure 6.** Schematic depiction of proposed increased activation of ERK signaling pathway in SS RBCs. Epinephrine stimulates β_2 adrenergic receptors ($\beta_2\text{ARs}$) on SS RBCs. $\beta_2\text{ARs}$ are prototypic G_s -coupled receptors (GPCRs), whose signaling is largely mediated by activation of stimulatory GTP-binding proteins (G_s proteins), and inhibited by activation of G_i protein.

Activation of G_s proteins in turn activates adenylate cyclase (AC), leading to generation of cAMP, and the subsequent activation of PKA. The activity of downstream signaling proteins, such as MEKs and ERKs is enhanced by PKA activation. The tyrosine kinase p72^{Syk} acts synergistically with PKA to activate MEK/ERK cascade. Activation of ERK results in phosphorylation of the ERK consensus motif on the cytoskeletal proteins α - and β - adducins, and dematin; and protein 4.1 albeit not at the ERK consensus motif. Phosphorylation of cytoskeletal proteins may result in cytoplasmic membrane protein conformational changes, which could render ICAM-4 accessible to phosphorylation.

(0018) **Figure 7.** 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 active ERK2. Protein 4.1 levels remain constant in AA RBCs between the two conditions, and also in SS RBCs between the two conditions.

(0019) **Figure 8.** Adhesion of SS RBC to activated-endothelial cells involves ERK. Adhesion of SS RBCs to non-activated and TNF- α activated-HUVECs was tested in intermittent flow condition assays. Results are presented as % cells adherent at a shear stress of 2 dynes/cm² (n = 4). Confluent cultures of HUVECs were treated with 10 ng/ml TNF- α for 4 hours, washed, and then tested for their ability to support adhesion of sham-treated SS RBCs or SS RBCs treated with U0126 for 1 hour at 37°C. A. SS RBCs were sham-treated, or treated with 10 μ M U0126 MEK inhibitor. U0126 significantly inhibited SS RBC adherence to activated endothelial cells to levels below baseline adhesion of SS RBCs to non-activated HUVECs. *: $p < 0.001$ compared to sham-treated SS RBCs adherent to non-activated HUVECs; **: $p < 0.001$ compared to sham-treated SS RBCs adherent to TNF- α -activated HUVECs. Error bars show SEM of 4 different experiments. B. SS RBCs were sham-treated, or treated with 1 μ M RDEA119, 10 μ M RDEA119, 10 μ M AZD6244 or 10 μ M GSK1120212. All MEK inhibitors blocked SS RBC adhesion to activated HUVECs compared to sham-treated SS RBC adhesion to activated or non-activated HUVECs. *: $p < 0.0001$ compared to sham-treated SS RBC adherent to non-activated or TNF- α -activated HUVECs. Error bars show SEM of 3 different experiments.

(0020) **Figure 9.** Effect of the MEK inhibitor U0126 on sickle red cell adhesion to the vascular endothelium and vaso-occlusion in nude mice. Microscopic observations of postcapillary venules were conducted through implanted dorsal skin-fold window chambers after infusion of human sickle RBCs into the tail vein of nude mice using 20X magnification. Vessels without adherent cells appear gray, due to the fluorescence of rapidly moving RBCs. TNF- α was injected prior to infusion of the MEK inhibitor U0126 or human sickle RBCs, to induce inflammation, since sickle red cell adhesion to activated endothelium in vitro is markedly up-regulated compared to

sickle cell adhesion to non-activated endothelium. A. Mice (n=5) were injected with 0.02 mg/kg TNF- α intraperitoneally 4 hours before infusion into the tail vein of fluorescently-labeled human sickle RBCs. Human sickle RBCs showed striking adhesion to postcapillary venules, with permanent vaso-occlusion at junctions, although it was also observed in straight non-junctional venular segments as indicated by arrows. B. Mice (n=5) were injected with 0.02 mg/kg TNF- α intraperitoneally 3 hours and 30 min before infusion into the tail vein of placebo (vehicle, 0.4% dimethyl sulfoxide (DMSO) in normal saline). Thirty minutes later, placebo-treated mice were infused into the tail vein with fluorescently-labeled human sickle RBCs. Infusion of human sickle RBCs resulted in marked RBC adhesion to postcapillary endothelium, with intermittent occlusion of vessels and permanent blockage of some vessel segments, especially at junctions. These data indicate that intravenous administration of placebo did not prevent human sickle red blood cell adhesion and vasoocclusion. C and D. Mice were injected with 0.02 mg/kg TNF- α intraperitoneally 3 hours and 30 min before infusion into the tail vein of U0126 (2 mg/kg in 0.4% DMSO in normal saline, n=5) (C), or U0126 (0.2 mg/kg in 0.04% DMSO in normal saline, n=1) (D). Thirty minutes later, U0126-treated mice were infused with fluorescently-labeled human sickle RBCs. Human sickle RBCs showed little adhesion, which was observed only in few venular segments without promotion of frank vaso-occlusion as indicated by arrows. Scale bar = 150 μ m. E. Effect of U0126 on % venular length occupied by SS RBCs. Video frames showing > 30 vessel segments were used to quantify the length of venules occupied by SS RBCs in animals treated as described in Panels B, C and D. The values were averaged among groups of animals to represent the mean % venular length occupied by SS RBCs. Error bars show SEM. *: $p < 0.001$ vs placebo for vessel diameter $\leq 25 \mu$ m; **: $p < 0.05$ vs placebo for vessel diameter $> 25 \mu$ m.

(0021) Figure 10. MEK inhibitors prevent SSRBC adhesion in inflamed vessels and vaso-occlusion *in vivo*. Human sickle RBCs were sham-treated or treated with the MEK inhibitor RDEA119 or U0126 *ex vivo*, washed extensively, and then infused into the tail vein of nude mice pretreated with TNF- α . Microscopic observations of venules were conducted through a dorsal skin-fold window chamber using 10X and 20X magnifications, after infusion of fluorescently labeled human SS RBCs. Vessels without adherent cells appear gray, due to rapidly moving fluorescent RBCs. Figure 10A. Infusion of sham-treated SS RBCs (n=5) resulted in marked RBC adherence in inflamed venules and vaso-occlusion as shown by arrows. Figure 10B. U0126-treated SS RBCs (n=5) show rare adhesion in inflamed vessels as indicated by arrows, but no apparent vaso-occlusion. Figure 10C. Treatment of human SSRBCs with RDEA119 (n=5) dramatically decreased human SS RBC adhesion as indicated by arrows and prevented vessel obstruction. Scale bar = 150

μm. Figure 10D. Effect of RDEA119 treatment of SS RBCs on % venular length occupied by treated SS RBCs. Video frames showing > 30 vessel segments were used to quantify the length of venules occupied by SS RBCs in animals treated as described above. The values were averaged among groups of animals to represent the mean % venular length occupied by SS RBCs. Error bars show SEM of 5 different experiments for each treatment. *: $p < 0.001$ vs sham for vessel diameter ≤ 25 μm; **: $p < 0.05$ vs sham for vessel diameter > 25 μm.

(0022) Figure 11. MEK inhibitors inhibit epinephrine-stimulated SS RBC-mediated neutrophil adhesion to endothelial cells. SS RBCs were treated with epinephrine (Epi), or pre-incubated with U0126, RDEA119, AZD6244 or GSK1120212 followed by treatment with Epi, and then washed. Native neutrophils (PMNs) from healthy donors were then co-incubated with treated-SS RBCs, and assayed for their ability to adhere to HUVECs. Adhesion of PMNs was much higher when cells were co-incubated with Epi-treated SS RBCs compared to adhesion of naïve PMNs not co-incubated with SS RBCs. However, all MEK inhibitors tested were equally able to markedly reduce the effect of epinephrine-stimulated SS RBCs on PMN adhesion to HUVECs when compared to adhesion of epinephrine-activated SS RBC-mediated PMN adhesion. *: $p < 0.0001$ compared to native PMNs; and **: $p < 0.0001$ compared to PMN adhesion mediated by epinephrine-treated SS RBCs. Error bars show SEM of 4 different experiments.

(0023) Figure 12. (A) AScore site localization scoring distributions across all 375 unique phosphorylated peptides from RBC ghost preparations. (B) Coefficient of variation (%CV) distribution of measured phosphopeptide peak heights from triplicate LC-MS analysis of a treatment group following accurate-mass and retention time alignment. Error bars indicate standard deviation within each %CV bin across all eight treatment groups.

(0024) Figure 13. Selected ion chromatogram (A) and AUC quantitation (B) of 173-VADPDHDHTGFLTE[pY]VATR-191 ($[M+3H]^3+$ 741.999 m/z) (SEQ ID NO:1) the active form of Mitogen-Activated Protein Kinase-1 (GN= MAPK1, ERK2), across 24 LC-MS injections. This peptide was qualitatively identified with a maximum mascot ion score of 63.3 and a site localization Ascore of 41 +/- 12.

(0025) Figure 14. A set of graphs is showing the distribution of types of phosphorylated residues and phosphorylated residues per peptide (A) and the biological functions of the phosphorylated proteins (B).

(0026) Figure 15. Two-dimensional (2D) agglomerative cluster analysis of Z-score transformed (ie magnitude of significance of change) phosphopeptide intensities across eight unique RBC treatment groups. Person correlations were used as the measure of similarity (-1

dissimilar, +1 identical). (A) RBC membrane fractions from healthy (AA) and sickle-cell (SS) patients pre-treated with or without the MEK inhibitor, U0126, and subsequently with or without activated ERK2. (B) Analysis performed only on SS or AA RBC treatment groups.

(0027) Figure 16. ERK1/2 signaling modulates glycophorin A serine phosphorylation.

5 Inorganic ^{32}P radiolabeled intact SS RBCs were incubated in the absence (lane 1) or presence (lanes 2, 3 and 4) of serine/threonine protein phosphatase inhibitors (SPI), not followed (lanes 1 and 2) or followed by treatment with epinephrine (epi) (lanes 3 and 4). In lane 4, SS RBCs were preincubated with MEK1/2 inhibitor U0126 prior to treatment with SPI followed by epi treatment. The cpm are representative of three different experiments, calculated by subtraction of cpm present in a lane
10 (not shown) containing immunoprecipitates using immunoglobulin P3 from cpm obtained using anti-glycophorin A mAb for immunoprecipitation under each set of conditions indicated. *: $p < 0.05$ and $p < 0.001$ for SPI-treated and SPI+epi-treated vs. sham-treated, respectively; **: $p < 0.001$ compared to SPI+epi-treated SS RBCs. Total glycophorin A loaded in each lane was detected using nitrocellulose membranes of phosphorylated glycophorin A blotted with anti-glycophorin A mAb.
15 PhosphorImager analysis of immunoprecipitated ^{32}P -radiolabeled glycophorin A and negative control immune complexes showed that glycophorin A of non-stimulated SS RBCs (Figure 2, lane 1) is modestly phosphorylated at baseline. Treatment of SS RBCs with serine phosphatase inhibitors (SPI) (lane 2) slightly increased glycophorin A phosphorylation by 1.9 ± 0.1 -fold ($p < 0.05$, $n=3$), suggesting that increased glycophorin A phosphorylation is a result of serine phosphorylation, as
20 tyrosine phosphatase inhibitors were not present. Epinephrine in the presence of SPI had a stronger effect on glycophorin A phosphorylation (2.93 ± 0.35 -fold increase over sham-treated SS RBCs; $p < 0.001$) (lane 3). However, treatment of SS RBCs with the MEK/12 inhibitor U0126 significantly decreased the combined effect of epinephrine and SPI on glycophorin A phosphorylation (lane 4) compared to cells treated with epinephrine and SPI ($p < 0.001$) (lane 3).

25

DETAILED DESCRIPTION

(0028) Preliminary studies have suggested that the mitogen-activated protein kinase (MAPK)/the extracellular signal-regulated kinase (ERK1/2) is present at higher abundance in sickle red blood cells (SS RBCs) than in normal RBCs and is bound to the cytoplasmic membrane. The
30 present inventors have shown that RK1/2 is active in enucleated SS RBCs, and that triggering this kinase promotes activation of signaling pathways and consequent RBC adhesion to the endothelium. Stimulation of β_2 adrenergic receptors ($\beta_2\text{ARs}$) on SS RBCs by epinephrine for a brief period of time increases activation of the ERK1/2 signaling cascade, which is involved in

phosphorylation of the RBC adhesion receptor ICAM-4 and protein 4.1. The present inventors also found that the ERK consensus motifs on dematin and α - and β -adducins undergo increased serine phosphorylation, indicating that these cytoskeletal proteins are substrates for ERK.

(0029) ERK has been implicated in erythropoietin-induced erythroid cell proliferation and survival,²⁹ and the present inventors have now demonstrated that the activity of this kinase and its upstream signal are conserved in mature SS RBCs. In some instances, ERK1/2 is hyperactive without stimulation of SS RBCs, and increased activation of this kinase can increase within 1 minute of SS RBC exposure to epinephrine. In contrast, in normal RBCs, despite the abundance of ERK1/2, ERK is not active at baseline and fails to become phosphorylated/activated with epinephrine or forskolin stimulation. The inability of ERK1/2 to undergo activation in normal RBCs suggests that the activity of ERK itself and/or at least one of the upstream effectors required for ERK activation is lost. Indeed, investigators have previously described that RBCs undergo maturation-related loss of multiple protein kinase activities, including PKA, PKC, and casein kinases.³⁰ In contrast, although SS RBCs are also fully differentiated, the present inventors have found that preservation of ERK activity and its downstream signaling molecules appears to be involved at least in the abnormal activation of RBC adhesive function.

(0030) Our data further implicate involvement of the protein G_s and cAMP/PKA as upstream mediators in activation of ERK and its downstream signal transduction pathway. Our findings are consistent with studies by Schmitt and Stork⁷ demonstrating that isoproterenol stimulation of endogenous β_2 ARs activated ERK in HEK293 cells via a cAMP-dependent PKA pathway, and this ERK pathway was insensitive to the effect of PTx, which inactivates the protein G α_i . In addition to PKA, we have also identified a role for the tyrosine kinase p72^{Syk} in activation of ERK in SS RBCs, while excluding involvement of p56^{lck}-related Src family tyrosine kinases. Thus, in SS RBCs, PKA and the tyrosine kinase p72^{Syk} are implicated in ERK activation, acting most likely in concert to regulate the MEK/ERK signaling pathway.

(0031) The engagement of epinephrine-stimulated ERK in regulation of SS RBC adhesion to the endothelium suggests that the MEK/ERK signal can promote an adhesive, vaso-occlusive pathology. It is also apparent from the data herein that epinephrine-induced adhesion of SS RBCs to non-activated endothelial cells requires ICAM-4 phosphorylation, which occurs via the cAMP/PKA/ERK signaling pathway. Furthermore, the adhesive function of SS RBCs appeared to be related to the extent of ERK and ICAM-4 phosphorylation/activation, since all three similarly increased or decreased depending on the time of cell exposure to epinephrine. Additionally, basal cAMP levels, the upstream effector of MEK/ERK, were much higher in SS RBCs than in normal cells,

suggesting that the increased level of cAMP in SS RBCs reflects at least in part the persistence of the abnormal ERK activation and RBC adhesive phenotype. However, although epinephrine increased cAMP levels in only 50% of the SCD patient samples tested, cAMP production, which seems to be needed to activate ERK signaling in these sickle cells, was also influenced by the duration of cell exposure to epinephrine. This may be explained at least in part by the dramatic decrease in the abundance of phosphopeptides within CAP1 in SS RBCs due to continued cell exposure to epinephrine stimulation. PKA might also exert a negative feedback loop through activation of phosphodiesterases, resulting in cAMP hydrolysis switching off downstream signaling because of the extended cell exposure to epinephrine (Rochais F, *J Biol Chem.* 2004). CAPs are not only involved in adenylate cyclase (AC) association, but in actin binding, SH3 binding, and cell morphology maintenance as well (Hubberstey AV, FASEB, 2002; and Bertling E, *Mol. Biol. Cell*, 2004). Previous observations of increased normal RBC membrane filterability after epinephrine treatment for 20 min (Tuvia S, *J. Physiol.*, 1999), explain the enhanced phosphorylated CAP1 in normal RBCs after 30 min epinephrine exposure. Furthermore, Shain et al.³¹ suggested that maintenance of altered cell morphology required persistent increased cAMP levels due to continuous β AR stimulation. In contrast, our data suggest that when an increase in ERK activation occurs within 1 min of cell exposure to epinephrine, persistent β_2 AR stimulation has a negative effect on ERK activation and consequently the RBC adhesive function. Based on this analysis, it is expected that inhibition of b-Raf or c-Raf will result in similar effects in SS RBCs as these are additional upstream activators in this pathway.

(0032) The data herein also define the putative downstream targets of ERK in RBCs. Label-free quantitative phosphoproteomics analysis implicates ERK2 in phosphorylation of protein 4.1 and shows that the ERK consensus motifs on dematin and adducins α and β undergo increased phosphorylation in the presence of this kinase. Dematin is also a substrate for PKC and PKA, and PKA-induced dematin phosphorylation completely abolishes its actin bundling capability.^{32, 33} Alternatively, rapid phosphorylation of α - and β -adducins by PKC at Ser-726 and Ser-713, respectively (Manno S, *J Biol Chem*, 2005) leads to decreased F-actin capping and dissociation of spectrin from actin, implicating adducin phosphorylation in cytoskeletal remodeling.³⁴ Furthermore, studies have previously shown that protein 4.1 phosphorylation, induced by cAMP-dependent kinase at Ser-331 and protein kinase C at Ser-312 documented after 20 min of cell stimulation (Manno S, *J Biol Chem*, 2005), results in a significant reduction in both the ability of protein 4.1 to promote spectrin binding to F-actin and in spectrin-protein 4.1 binding.³⁵ Thus, phosphorylation of cytoskeletal proteins and proteins of the junctional complexes by ERK in SS RBCs

may also lead to cytoskeletal deorganization, which in turn, could potentially render ICAM-4 accessible to undergo phosphorylation, and to then mediate adhesion to the endothelium, or to affect its adhesivity with an as yet undetermined kinase. In fact, other investigators have shown that cell adhesion can be regulated by an intricate network of signaling molecules, which are responsible for guiding their interaction with substrate mainly via cytoskeleton rearrangement.³⁶ A schematic overview of the proposed β_2 -AR signaling pathway in SS RBCs is shown in Figure 6.

(0033) Finally, while aberrant ERK activation may arise in other pathologies, the present inventors are the first to describe atypical ERK activation in SS RBCs and its involvement in the abnormal RBC adhesion to the endothelium. Abnormal activation of ERK in SS RBCs may therefore be associated with the pathophysiology of sickle cell disease, making the MEK/ERK pathway a therapeutic target for preventing and treating vaso-occlusion. Various MEK and ERK inhibitors are currently being investigated in phase II clinical trials as therapeutic agents in cancer. The present invention provides methods of alleviating the symptoms of hemoglobinopathies, such as sickle cell disease and β -thalassemia, comprising administering MEK and/or ERK inhibitors.

(0034) The phospho-proteomic analysis presented in the Examples suggests that aberrant ERK activation may also be involved in additional symptoms and RBC defects associated with sickle cell disease. SS RBCs are characterized by a panoply of abnormalities, including polymerization of deoxygenated HbS, persistent oxidative membrane damage associated with HbS cyclic polymerization, abnormal activation of membrane cation transports, cell dehydration, and cytoskeletal dysfunction. In particular, the Examples demonstrate that ERK alters the phosphorylation state of proteins that may be involved in maintaining mechanical stability of RBC and may lead to a reduction in shear resistance as well as effect RBC shape, flexibility, anion transport and protein trafficking. Thus, MEK/ERK inhibition may result not only in amelioration of vaso-occlusion, but also other symptoms of sickle cell disease.

Definitions

(0035) The subject matter disclosed herein is described using several definitions, as set forth below and throughout the application.

(0036) Unless otherwise noted, the terms used herein are to be understood according to conventional usage by those of ordinary skill in the relevant art. In addition to the definitions of terms provided below, it is to be understood that as used in the specification, embodiments, and in the claims, "a", "an", and "the" can mean one or more, depending upon the context in which it is used.

(0037) As used herein, "about," "approximately," "substantially," and "significantly" will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is used, "about" or "approximately" will mean up to plus or minus 10% of the particular term and "substantially" and "significantly" will mean more than plus or minus 10% of the particular term.

(0038) As used herein, the terms "include" and "including" have the same meaning as the terms "comprise" and "comprising."

(0039) As used herein, the terms "patient" and "subject" may be used interchangeably and refer to one who receives medical care, attention or treatment. As used herein, the term is meant to encompass a person diagnosed with a disease such as a hemoglobinopathy or at risk for developing a hemoglobinopathy (e.g., a person who may be genetically homozygous or heterozygous for a sickle cell-causing mutation, but is not symptomatic). A "patient in need thereof" may include a patient having, suspected of having, or at risk for developing a hemoglobinopathy or symptoms thereof.

(0040) As used herein, the term "treatment," "treating," or "treat" refers to care by procedures or application that are intended to alleviate symptoms of a disease (including reducing the occurrence of symptoms of the disease). Although it is preferred that treating a condition or disease such as a hemoglobinopathy will result in an improvement of the condition, the term treating as used herein does not indicate, imply, or require that the procedures or applications are at all successful in alleviating symptoms associated with any particular condition. Treating a patient may result in adverse side effects or even a worsening of the condition which the treatment was intended to improve. Treating may include treating a patient having, suspected of having, or at risk for developing a hemoglobinopathy or symptoms thereof.

(0041) As used herein the term "effective amount" refers to the amount or dose of the agent, upon single or multiple dose administration to the subject, which provides the desired effect in the subject under diagnosis or treatment. The disclosed methods may include administering an effective amount of the disclosed agents (e.g., as present in a pharmaceutical composition) for treating a hemoglobinopathy in the patient, whereby the effective amount alleviates symptoms of the hemoglobinopathy (including reducing the occurrence of symptoms of the hemoglobinopathy).

(0042) An effective amount can be readily determined by the attending diagnostician, as one skilled in the art, by the use of known techniques and by observing results obtained under analogous circumstances. In determining the effective amount or dose of agent administered, a

number of factors can be considered by the attending diagnostician, such as: the species of the patient; its size, age, and general health; the particular symptoms or the severity of the hemoglobinopathy; the response of the individual patient; the particular agent administered; the mode of administration; the bioavailability characteristics of the preparation administered; the
 5 dose regimen selected; the use of concomitant medication; and other relevant circumstances.

(0043) The phrase "alleviates at least one symptom," as used herein, means that a particular treatment results in a lessening of at least one symptom of a disease. Such lessening of a symptom may be a qualitative or quantitative reduction in the severity of the symptom, or may be a reduction in the number of occurrences of the symptom; even though each occurrence may be as
 10 severe as it was before the treatment (one or more occurrences may also be less severe).

Nonlimiting exemplary symptoms of sickle cells disease include vaso-occlusion, acute painful episodes, chronic hemolysis (aplastic crises), avascular necrosis, infection, end-organ damage, acute chest syndrome, leg ulceration, priapism, and decreased life expectancy. Nonlimiting exemplary symptoms of thalassemia include hemolysis, erythroid hyperplasia, biliary tract disease, infection,
 15 leg ulcers, extramedullary hematopoiesis, increased risk for developing thromboembolic phenomena, liver and heart damage, and decreased life expectancy.

(0044) The term "hemoglobinopathy," as used herein, refers to a condition that is caused by a genetic mutation in a globin gene that results in a mutated hemoglobin α chain or β chain protein, or a condition that is caused by a genetic mutation that results in an abnormal ratio of
 20 hemoglobin α chain to β chain or crossover fusion products of 2 globin genes. Nonlimiting exemplary hemoglobinopathies include sickle cell disease (including, but not limited to, homozygous for hemoglobin S and a variety of sickle cell syndromes that result from inheritance of the sickle cell gene in compound heterozygosity with other mutant beta globin genes, including, but not limited to, hemoglobin SC disease (HbSC), sickle beta(+) thalassemia, sickle beta(0) thalassemia,
 25 sickle alpha thalassemia, sickle delta beta(0) thalassemia, sickle Hb Lepore, sickle HbD, sickle HbO Arab, and sickle HbE), β -thalassemia (including, but not limited to, β -thalassemia major (also known as Cooley's anemia) and β -thalassemia intermedia, and hemoglobin H disease (α -thalassemia with $\alpha^+ - \alpha^0$ phenotype)). Nonlimiting exemplary genetic mutations that cause sickle cell disease include Hb SS, which is hemoglobin with an E6V mutation in each of the two hemoglobin β chains; Hb SC, which is hemoglobin with one β chain with an E6V mutation and one β chain with an E6K mutation;
 30 Hb SD, which is hemoglobin with one β chain with an E6V mutation and one β chain with a β 121 Glu-> Gln mutation; sickle-HbO Arab, which is hemoglobin with one β chain with an E6V mutation and one β chain with a β 121(GH4)gGlu->Lys mutation; and Hb SE, which is hemoglobin with one β

chain with an E6V mutation and one β chain with an E26K mutation. Nonlimiting exemplary genetic mutations that cause β -thalassemia include various β -mutations, such as IVS II-I, CD 36/37, CD41/42, CD 39; IVS1-6; IVS1-110, CD71/72, IVS1-5, IVS1-1, CD26, IVS2-654, CAP+1, CD19, -28, -29, IVS1-2, InCD (T-G) and CD17; and rare β -mutations, i.e. InCD (A-C), CD8/9, CD43, -86, CD15, Poly A, Poly T/C, IVS2-1, CD1, CD35/36, CD27/28, CD16, CD37, and 619bpDEL. Nonlimiting exemplary genetic mutations that cause Hb H disease include $\alpha^+ - \alpha^0$ phenotypes such as $\alpha 2$ Poly A (AATAAA $\rightarrow$ AATA--), $\alpha 2$ Poly A (AATAAA $\rightarrow$ AATGAA), and $\alpha 2$ Poly A (AATAAA $\rightarrow$ AATAAG); α^+ phenotypes such as $\alpha 2$ CD 142 (TAA $\rightarrow$ CAA), $\alpha 2$ CD 142 (TAA $\rightarrow$ AAA), and $\alpha 2$ CD 142 (TAA $\rightarrow$ TAT); and α^0 phenotypes such as $-\alpha^{3.7}$ Init CD (ATG $\rightarrow$ GTG), --^{SEA}, --^{THAI}, --^{MED II}, --^{BRIT}, --^{MED I}, --^{SA}, -- $(\alpha)^{20.5}$, and --^{FIL}.

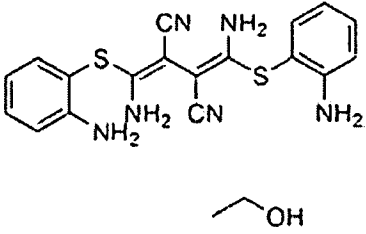
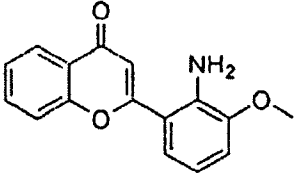
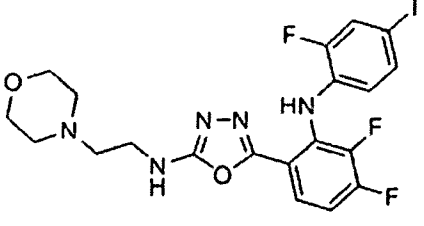
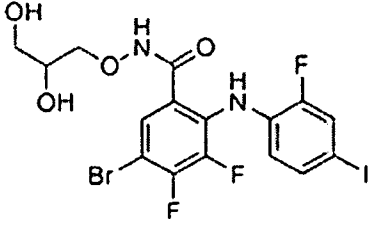
(0045) The term "MEK inhibitor," as used herein, refers to an inhibitor of MEK kinase activity. A MEK inhibitor may be any type of molecule, including, but not limited to, small molecules and expression modulators (such as antisense molecules, microRNAs, siRNAs, etc.), and may act directly on the MEK protein, may interfere with expression of the MEK protein (e.g., transcription, splicing, translation, and/or post-translational processing), and/or may prevent proper intracellular localization of the MEK protein. Exemplary MEK inhibitors include, but are not limited to, U0126, PD98059, PD-334581, GDC-0973, CIP-137401, ARRY-162, ARRY-300, PD318088, PD0325901, CI-1040, BMS 777607, AZD8330, AZD6244, RDEA119, GSK1120212 and AS703026.

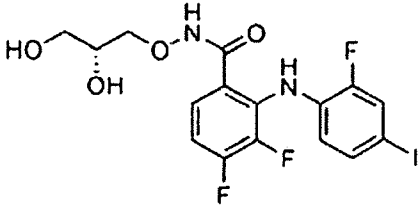
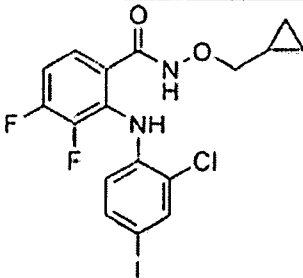
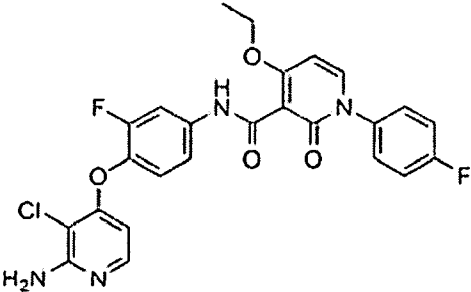
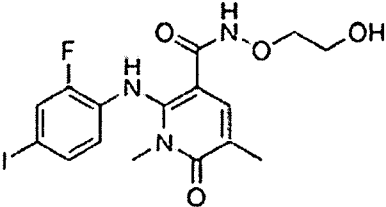
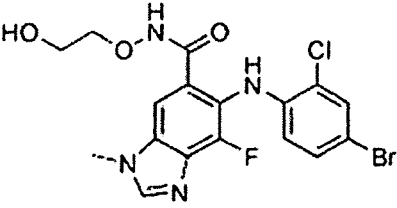
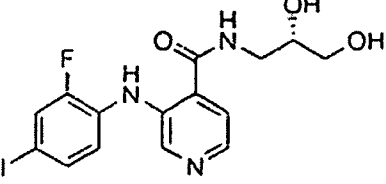
(0046) The term "ERK inhibitor," as used herein, refers to an inhibitor of ERK kinase activity. An ERK inhibitor may be any type of molecule, including, but not limited to, small molecules and expression modulators (such as antisense molecules, microRNAs, siRNAs, etc.), and may act directly on the ERK protein, may interfere with expression of the ERK protein (e.g., transcription, splicing, translation, and/or post-translational processing), and/or may prevent proper intracellular localization of the ERK protein. A nonlimiting exemplary ERK inhibitor is AEZS-131.

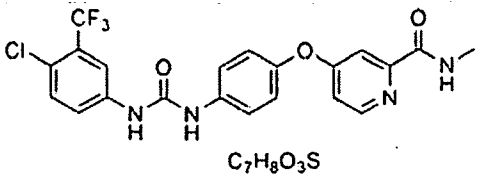
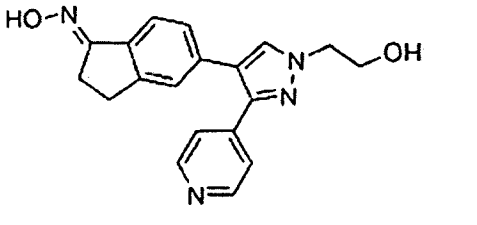
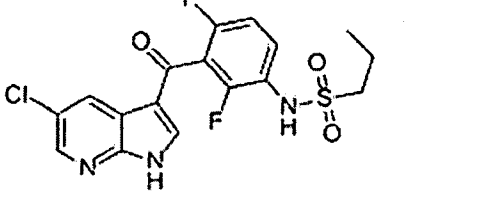
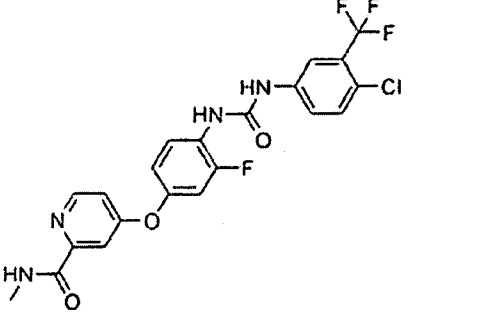
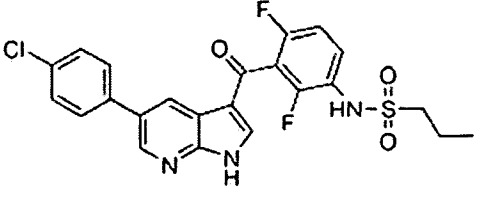
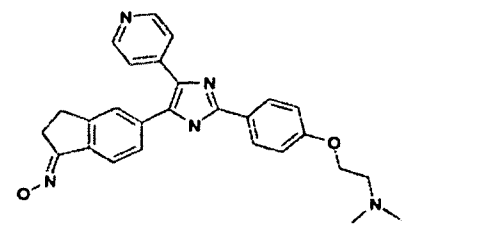
(0047) The term "Raf inhibitor," as used herein, refers to an inhibitor of b-Raf kinase activity and/or c-Raf kinase activity. A Raf inhibitor may be any type of molecule, including, but not limited to, small molecules and expression modulators (such as antisense molecules, microRNAs, siRNAs, etc.), and may act directly on the Raf protein, may interfere with expression of the Raf protein (e.g., transcription, splicing, translation, and/or post-translational processing), and/or may prevent proper intracellular localization of the Raf protein. Nonlimiting exemplary Raf inhibitors include sorafenib tosylate, GDC-0879, PLX-4720, regorafenib, PLX-4032, SB-590885-R, RAF265, GW5074, XL281, and GSK2118436.

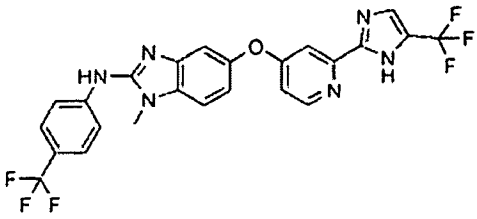
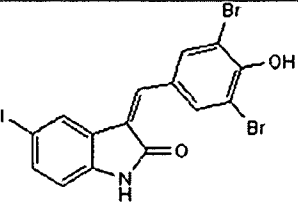
(0048) A table providing additional information on some of the exemplified MEK, ERK, and B-Raf inhibitors is provided below as Table 4.

Table 4: Non-limiting exemplary inhibitors of MEK, ERK, and/or B-Raf

Inhibitor	Alternate name(s)	Structure or source
U0126	U0126-EtOH	
PD98059		
PD-334581		 Chemical Formula: $C_{20}H_{19}F_3IN_5O_2$ Molecular Weight: 545.30
GDC-0973	XL518	Genentech
CIP-137401	CIP-1374	Allostem Therapeutics
ARRY-162		Array BioPharma / Novartis
ARRY-300		Array BioPharma / Novartis
PD318088		

PD0325901		
CI-1040	PD184352	
BMS 777607		
AZD8330	ARRY-424704 ARRY-704	
AZD6244	Selumetinib	
AS703026	MSC1936369B	
AEZS-131		Aeterna Zentaris Inc.

sorafenib tosylate	BAY 43-9006 AZ 628	 <chem>CN(C)C(=O)c1ccnc1Oc2ccc(cc2)NC(=O)Nc3ccc(cc3)C(F)(F)FCl</chem> $C_7H_8O_3S$
GDC-0879		 <chem>OCCN=NC(c1ccc2c(c1)C=CC2)c3ccc(cc3)N=O</chem>
PLX-4720		 <chem>CCS(=O)(=O)Nc1cc(F)c(cc1F)C(=O)c2c3c(c[nH]2)c4ccc(Cl)cc4</chem>
regorafenib	BAY 73-4506	 <chem>CCN(C)C(=O)c1ccnc1Oc2ccc(cc2)NC(=O)Nc3ccc(cc3)C(F)(F)FCl</chem>
PLX-4032	RG7204	 <chem>CCS(=O)(=O)Nc1cc(F)c(cc1F)C(=O)c2c3c(c[nH]2)c4ccc(Cl)cc4</chem>
SB-590885		 <chem>CN(C)COc1ccc(cc1)N2C(=N3C(=N2)C(=C4C(=CC(=C3)C(=O)N4)C5=CC=CC=C5)C6=CC=CC=C6</chem>

RAF265	CHIR-265	
GW5074		
XL281	BMS-908662	Exelixis
GSK2118436		GlaxoSmithKline

(0049) In some embodiments, methods of alleviating at least one symptom of a hemoglobinopathy in a patient are provided. Such methods comprise, in some embodiments, administering to the patient an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor. Nonlimiting exemplary hemoglobinopathies include β -thalassemia, sickle cell disease and Hemoglobin H.

(0050) For the treatment of sickle cell disease or other hemoglobinopathies, in some embodiments, at least one symptom that may be alleviated by administering the inhibitors described herein is selected from vaso-occlusion, acute painful episodes, chronic hemolysis (aplastic crises), avascular necrosis, infection, end-organ damage, and erythroid hyperplasia. In some embodiments, alleviating a symptom of sickle cell disease means reducing the amount, frequency, duration or severity of the symptom. For example, for vaso-occlusion, in some embodiments, alleviating the symptom includes reducing the average size of the vaso-occlusions and/or reducing the number of vaso-occlusions. Further, alleviating a symptom may or may not result in a reduction in the discomfort experienced by the patient as a result of the symptom. That is, in some embodiments, while the number and/or average size of vaso-occlusions may be reduced following a treatment described herein, the patient may or may not experience a similar reduction in acute pain caused by vaso-occlusion.

(0051) In some embodiments, when vaso-occlusion is alleviated by administration of an inhibitor described herein, acute painful episodes are also alleviated (i.e., the number and/or severity is reduced). In some embodiments, when vaso-occlusion is alleviated by administration of an inhibitor described herein, hemolysis is also alleviated. In some embodiments, vascular

endothelial injury is alleviated by administration of an inhibitor described herein. In some embodiments, when hemolysis is alleviated by administration of an inhibitor described herein, the incidence of infections is reduced. In some embodiments, when hemolysis is alleviated by administration of an inhibitor described herein, erythroid hyperplasia is also alleviated. In some
5 embodiments, when vaso-occlusion and/or hemolysis are alleviated by administration of an inhibitor described herein, end-organ damage is also alleviated.

(0052) In some embodiments, methods of inhibiting adhesion of sickle red blood cells to endothelial cells are provided. In some embodiments, methods of inhibiting adhesion of sickle red blood cells to leukocytes are provided. Such methods comprise, in some embodiments, contacting
10 the sickle red blood cells with an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.

(0053) In some embodiments, methods of inhibiting adhesion of sickle red blood cells to endothelial cells in a patient are provided. In some embodiments, methods of inhibiting adhesion of sickle red blood cells to leukocytes in a patient are provided. Such methods comprise, in some
15 embodiments, administering to the patient an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.

(0054) In some embodiments, a method comprises administering to the patient, or contacting a sickle red blood cell with, a MEK inhibitor. Nonlimiting exemplary MEK inhibitors include U0126, PD98059, PD-334581, GDC-0973, CIP-137401, ARRY-162, ARRY-300, PD318088,
20 PD0325901, CI-1040, BMS 777607, AZD8330, AZD6244, RDEA119, GSK1120212 and AS703026. In some embodiments, a method comprises administering to the patient, or contacting a sickle red blood cell with, an ERK inhibitor. A nonlimiting exemplary ERK inhibitor is AEZS-131. In some embodiments, a method comprises administering to the patient, or contacting a sickle red blood cell with, a Raf inhibitor. In some embodiments, the Raf inhibitor inhibits b-RAF. In some
25 embodiments, the Raf inhibitor inhibits c-Raf. In some embodiments, the Raf inhibitor inhibits both b-Raf and c-Raf. Nonlimiting exemplary Raf inhibitors include sorafenib tosylate, GDC-0879, PLX-4720, regorafenib, PLX-4032, SB-590885-R, RAF265, GW5074, XL281, and GSK2118436.

In some embodiments, a method comprises administering to the patient, or contacting a sickle red blood cell with a combination of two or more inhibitors selected from a MEK inhibitor, an
30 ERK inhibitor, and a Raf inhibitor. The two or more inhibitors may be co-administered. Co-administration indicates the inhibitors may be administered in any order, at the same time or as part of a unitary composition. The two inhibitors may be administered such that one inhibitor is

administered before the other with a difference in administration time of 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, 1 day, 2 days, 4 days, 7 days, 2 weeks, 4 weeks or more.

(0055) Administration to a subject may include formulating the therapeutic agents, such as a MEK inhibitor, an ERK inhibitor, and/or a B-Raf inhibitor, with pharmaceutically acceptable carriers and/or excipients, etc., to form pharmaceutical compositions. Suitable formulations for therapeutic compounds are available to those skilled in the art. Administration may be carried out by any suitable method, including intraperitoneal, intravenous, intramuscular, intrathecal, subcutaneous, transcutaneous, oral, nasopharyngeal, or transmucosal absorption among others. The dosage for a particular subject may be determined based on, for example, the subject's weight, height, and/or age; the severity of the subject's disease or symptoms; the length of treatment and/or number of doses anticipated in a particular regimen; the route of administration; etc.

(0056) The following examples are illustrative and are not intended to limit the disclosed subject matter. All references cited herein are incorporated herein by reference in their entireties.

EXAMPLES

Example 1: Materials and Methods

(0057) *Endothelial cells.* Primary human umbilical vein endothelial cells (HUVECs) were grown as monolayers in EBM2 medium (Lonza Walkersville, Inc., Walkersville, MD) supplemented with EGM2 (Lonza Walkersville) as described previously.⁴ EC passage was accomplished with trypsinization, as required. Cells were used until they reached the 5th passage. For flow chamber experiments, HUVECs were cultured until they reached confluence on clear glass slides precoated with 2% gelatin.

(0058) *Antibodies.* Antibodies used included the following monoclonal and polyclonal antibodies (Abs, as purified immunoglobulin [Ig] unless otherwise noted): BS46 (mouse anti-ICAM-4, generously provided by Dr. Jean-Pierre Cartron, INSERM Unité 665, Paris, France);¹⁷ and mouse anti-phospho-myelin basic protein (Millipore, Temecula, CA); mouse anti-human transferrin receptor (BD Biosciences, San Jose, CA); and mouse anti-human glycophorin C produced in our laboratory. Rabbit anti-human ERK1/2 was from Upstate, Charlottesville, VA; rabbit anti-human phospho-ERK1/2 was from Cell Signaling Technology, Danvers, MA; and rabbit anti-human MAPK kinase (MEK1/2) was from Sigma-Aldrich, St. Louis, MO. The murine myeloma protein P3x63/Ag8 (P3 ascitic fluid, diluted 1:500) was used as a non-reactive control murine Ig for mAbs.¹⁸ In all studies, Abs were used at saturating dilutions unless otherwise indicated.

(0059) **Collection, preparation and treatment of RBCs.** Sick cell patient donors had not been transfused for at least three months, had not experienced vaso-occlusion for three weeks, and were not on hydroxyurea. Fresh blood samples from patients homozygous for hemoglobin S and from healthy donors were collected into citrate tubes. Blood was used within less than 24 h of collection. Packed RBCs were separated as previously described in detail.⁵ RBCs were separated from the buffy coat containing leukocytes and platelet-rich plasma by gravity at 4° C for at least 2 h. Plasma and buffy coat were removed by aspiration, and RBCs were washed four or five times in sterile PBS with 1.26 mM Ca²⁺, 0.9 mM Mg²⁺ (pH 7.4). Packed RBCs were analyzed for leukocyte and platelet contamination using an Automated Hematology Analyzer Sysmex K-1000 (Sysmex, Co.,
10 Cobe, Japan).

(0060) Aliquots of packed RBCs were treated with various reagents to affect cAMP signaling or protein phosphorylation. Sham-treated RBCs were incubated with the same buffer and vehicle, but without the active agent. Unless otherwise indicated, RBCs were treated at 37°C with one or more of the following reagents: 20 nM epinephrine (Sigma-Aldrich, St. Louis, MO) for 1 or 30
15 min; 2 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, Sigma) for 2 h; 80 μM forskolin (Sigma) for 30 min; 1 or 2 μg/ml Pertussis toxin (PTx, Calbiochem, La Jolla, CA); 5 μM MEK1/2 inhibitor (MEKI, U0126, Calbiochem); 30 nM protein kinase A inhibitor (PKAI) 14-22 amide (Calbiochem); 10 μM damnacanthal (Enzo Life Sciences International, Inc., Plymouth Meeting, PA); or 10 μM piceatannol (Enzo Life Sciences International, Inc.) for 1 h. Treated RBCs were then
20 washed 5 times with 4 ml PBS with Ca²⁺ and Mg²⁺. Normal RBCs were used as controls. Prior to adhesion assays, treated RBCs were labeled with PKH 26 red fluorescent cell linker kit (Sigma), following the manufacturer's instructions.

(0061) For *in vitro* adhesion assays, human SS RBCs were sham-treated with buffer and vehicle alone or treated at 37° C with the MEK inhibitor, U0126 (Calbiochem, La Jolla, CA) at 10 μM
25 for 1 h, followed or not by treatment with 20 nM epinephrine for 1 min or 80 μM forskolin for 30 min. Cells were then washed three times with 5 ml PBS with Ca²⁺ and Mg²⁺. Prior to adhesion assays, washed treated SS RBCs were labeled with PKH 26 red fluorescent cell linker kit (Sigma-Aldrich, St. Louis, MO), following the manufacturer's instructions.

(0062) For some *in vivo* adhesion studies, packed SS RBCs were fluorescently labeled with
30 the dye Dil (Molecular Probes Inc., Eugene, OR), following the manufacturer's instructions. Dil was used in our previous *in vivo* studies and by other investigators, and this dye have no effect on RBC suspension viscosity and RBC survival in circulation (Unthank JL et al. Microvasc. Res. 1993; 45:193-210; Zennadi et al., Blood 2007). Cell morphology was checked by microscopy.

(0063) **Western blot.** Treated packed RBCs were lysed with hypotonic buffer (5 mM Na_2HPO_4 + 1 mM EDTA + 0.1% NaN_3 , pH 8) containing 2 mM phenylmethylsulfonylfluoride (PMSF, Sigma), phosphatase inhibitor cocktail (Sigma) and protease inhibitor cocktail (Sigma). Protein separation by polyacrylamide gel electrophoresis using equal amounts of total RBC membrane ghost proteins per lane, after correcting total protein measurements for residual hemoglobin content, and Western blot¹⁹ using the appropriate Ab were then performed. Mouse 3T3/A31 fibroblast lysate was used as a ERK1/2 positive control for immunoblots. For total ERK1/2, membranes blotted with anti-phosphoERK Ab were stripped and reexposed to Western blotting using anti-ERK1/2 Ab. Bands were analyzed densitometrically using ImageJ software downloaded from the NIH website. PhosphoERK1/2 data were normalized according to total ERK1/2 and are presented as fold change in ERK phosphorylation.

(0064) **MAP kinase activity assay.** Treated packed SS RBCs were lysed for 20 min at 4°C with lysis buffer (10 mM EDTA, 20 mM Tris, 110 mM NaCl, pH 7.5) containing 2 mM PMSF, 1% Triton X-100, phosphatase inhibitor cocktail (Sigma) and protease inhibitor cocktail (Sigma). ERK1/2 was immunoprecipitated with anti-ERK1/2 antibody at 4°C, and immune complexes were obtained using protein A-agarose (Amersham Biosciences Corp., Piscataway, NJ). ERK1/2 immunocomplex was examined for ERK1/2 activity using myelin basic protein (MBP) at 2 mg/ml (Millipore) as a substrate and ATP as a phosphate donor with equal protein amounts per assay condition. For the negative control, an equal volume of water was substituted for ERK1/2 substrate. Commercial active recombinant human ERK2 was used (Sigma) as a positive control. The reaction mixture was incubated for 20 min at 30°C, followed by protein separation and immunoblotting using anti-phosphoMBP mAb (Millipore).

(0065) Non-radiolabeled RBC ghosts isolated from packed RBCs sham-treated, or treated with U0126 or epinephrine for 1 or 30 min were separated by mass spectrometry, and then subjected to Label-Free quantitative phosphoproteomic analysis after phosphopeptide enrichment (see below).

(0066) **Reticulocyte enrichment.** Reticulocytes were separated from mature SS RBCs using anti-transferrin receptor mAb and goat anti-mouse IgG-coated micro-bead affinity columns (MACS, Miltenyi Biotec, Inc, Auburn, CA), following the manufacturer's instructions.

(0067) **Flow chamber assays.** Graduated height flow chambers were used to quantify adhesion of RBCs to HUVECs substantially as previously described in detail.^{4, 20} In some adhesion studies, slides coated with HUVECs were treated with human recombinant TNF- α at 10 ng/ml for 4 hours. Slides coated with HUVECs treated or not with TNF- α were then washed three times with 20

ml HBSS with 1.26 mM Ca²⁺, 0.9 mM Mg²⁺ (Gibco, Grand Island, NY) warmed previously to 37°C and then fit into a variable height flow chamber. The flow chamber was mounted on the stage of an inverted phase contrast microscope (Diaphot, Nikon Inc., Melville, NY) connected to a thermoplate (Tokai Hit Co., Ltd., Japan) set at 37°C. Cells were observed using a video camera (RS photometrics,) attached to the microscope and connected to a Macintosh G4 computer. RBC (3 ml) suspended at 0.2% (vol/vol) in HBSS with Ca²⁺, Mg²⁺ were infused into the flow chamber and allowed to adhere to the slide for 10 min without flow. Before exposure to flow, a minimum of three fields at each of seven different locations along a line oriented normal to future flow were examined for the total number of fluorescent cells. Fluid flow (HBSS with Ca²⁺, Mg²⁺) was then started using a calibrated syringe pump. After exposure to flow, the fields were again examined and the number of adherent cells counted. The fraction of adherent cells was presented as (number of cells attached after exposure to flow)/(cells present per field before flow). The wall shear stress was calculated as:

$$\tau_w = \frac{6\mu Q}{wH(x)^2}$$

τ_w = wall shear stress (dyne/cm²); Q = volumetric flow rate (cm³/s); μ is media viscosity, w is the width of the flow channel, and H(x) is the height of the flow chamber as a function of position along the microscope slide. Several investigators have shown that blood flow in small vessels may be continuous, with shear stresses of 1-2 dynes/cm², or flow may be intermittent. Our data were obtained using both intermittent and continuous flow conditions.

(0068) ***³²P erythrocyte labeling, anti-ICAM-4 immunoprecipitation and detection of phosphorylation.*** Packed RBCs depleted of endogenous ATP stores and ³²P-labeled as previously described,²¹ were incubated with phosphatase inhibitor cocktail (Sigma) in the presence or absence of MEKI U0126, PKAI, or a combination of both U0126 and PKAI, prior to 1 or 30 min treatment with epinephrine. Cells were then washed 4 times. ICAM-4 protein immunoprecipitation, and total and phospho-ICAM-4 detection were performed as previously described in detail.⁴ To further confirm that the immunoprecipitates were specific for ICAM-4, anti-ICAM-4 mAb and the negative control immunoglobulin P3 were used to immunoprecipitate ICAM-4 from non-radiolabeled SS RBCs incubated in the presence or absence of epinephrine. Blots were then immunostained with anti-ICAM-4 mAb.

(0069) ***Whole Cell cAMP Accumulation:*** Whole cell cAMP accumulation was assayed to assess the functional capacity of the RBC β_2 -ARs to stimulate the production of cAMP. Washed packed RBCs were pre-treated with IBMX to define basal cAMP accumulation, followed by treatment with epinephrine for 1 min or 30 min, or forskolin. Samples were placed on ice,

stimulation was halted, and cells fixed by the addition of 12.5 mM EDTA. Cell samples were boiled, clarified by centrifugation and assayed for cAMP content by radioimmunoassay as described previously.²² Basal cAMP production was subtracted from the total cAMP produced by the cells. The amounts of cAMP were then normalized as fmol cAMP/10⁸ RBCs.

5 (0070) **Statistical analysis.** Data were compared using parametric analyses (GraphPad Prism 4 Software, San Diego, CA), including repeated and non-repeated measures of analysis of variance (ANOVA). One-way ANOVA analyses were followed by Bonferroni corrections for multiple comparisons (multiplying the *p* value by the number of comparisons). A *p* value < 0.05 was considered significant.

10 (0071) **RBC ghost membrane sample preparation and phosphopeptide enrichment.**
Ghosted RBCs were spun at 14,000 rpm for 15 min at 4°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°C. The supernatant was then removed and 500 µL of 50 mM ammonium bicarbonate with µL 0.2% acid-labile surfactant (ALS-1) in 50 mM ammonium
15 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°C. Samples were normalized to approximately 2 µg/µl following a micro-Bradford assay (Pierce Bioscience), and were reduced with a final concentration of 10 mM dithiothreitol at 80°C for 20 min. Samples were then alkylated with a final concentration of 20 mM iodoacetamide
20 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°C for 18 hr. To remove ALS-1, samples were acidified to pH 2.0 with neat TFA, incubated at 60°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 TiO₂ based phosphopeptide enriched protocol.

25 (0072) To enrich for phosphorylated peptides prior to LC-MS analysis, either 1,125 µg and 970 µg 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 µL of 80% acetonitrile, 1% TFA, 50 mg/ml MassPrep Enhancer (pH 2.5) (Waters Corp. Milford, MA). Samples were loaded onto an
30 in-house packed TiO₂ spin column (Protea Biosciences) with a 562 µg or 485 µg binding capacity for active ERK2 treated or epinephrine treated experiments, respectively. Samples were washed twice with 200 µL 80% acetonitrile, 1% TFA, 50 mg/ml MassPrep Enhancer (pH 2.5) followed by two washes with 200 µL 80% acetonitrile, 1% TFA (pH 2.5). Retained peptides were eluted twice with

100 μ 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 μ L 2% acetonitrile, 0.1% TFA, 25 mM citric acid (pH 2.5).

(0073) *Label-Free quantitative phosphoproteomic analysis of RBC Ghost.*

5 Chromatographic separation of phosphopeptide enriched or non-enriched samples was performed on a Waters NanoAquity UPLC equipped with a 1.7 μ m BEH130 C₁₈ 75 μ m I.D. X 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 μ L injections of each sample were trapped for 5 min on a 5 μ m Symmetry C₁₈ 180 μ m I.D. X 20 mm column at 20 μ L/min in 99.9% A. The analytical column was then switched
10 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 μ m tip orifice and coupled to the mass spectrometer through an electrospray interface.

15 (0074) 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 ambient polycyclodimethylsiloxane (m/z 445.120025) enabled. The instrument was set to acquire a precursor MS scan from m/z 400-2000 with $r =$ 60,000 at m/z 400 and a target AGC setting of 1e6 ions. Each sample was analyzed four-times, one
20 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
25 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
30 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.

(0075) 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.

(0076) 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 algorithm scores which corresponded to a 2% peptide false discovery rate for non-phosphopeptide enriched experiments.

(0077) 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*).

(0078) **Mice:** All animal experiments were carried out in accordance with protocols approved by the Duke University Animal Care and Use Committee. Female athymic homozygous nude mice (nu-/nu-) were between 8-12 weeks of age (Charles River Laboratories, Wilmington, MA).

5 (0079) **Window chamber surgery:** General anesthesia was achieved by intra-peritoneal injection of 100 mg/kg of ketamine (Abbott Laboratory, Chicago, IL) and 10 mg/kg of xylazine (Bayer, Shawnee Mission, KS). A double-sided titanium frame window chamber was surgically implanted into the dorsal skin fold under sterile conditions using a laminar flow hood. Surgery involved carefully removing the epidermal and dermal layers of one side of a dorsal skin fold,
10 exposing the blood vessels of the subcutaneous tissue adjacent to the striated muscles of the opposing skin fold, and then securing the two sides of the chamber to the skin using stainless steel screws and sutures. A glass window was placed in the chamber to cover the exposed tissue and secured with a snap ring. Subsequently, animals were kept at 32-34°C until *in vivo* studies were performed 3 days post-surgery.

15 (0080) **RBC infusions and intravital microscopy:** Murine recombinant TNF- α was dissolved in normal saline at a concentration of 0.1 mg/mL and mice bearing dorsal-skin window chamber implants were given a single intraperitoneal (IP) injection of 20 μ g/kg TNF- α and control animals received same volume of normal saline. Three hours and 30 min following TNF- α administration, either placebo [0.4% dimethyl sulfoxide (DMSO) in normal saline] or U0126 (Cell
20 Signaling Technology) (2 and 0.2 mg/kg, in 0.4% and 0.04% DMSO, respectively) was injected intravenously via tail vein of anesthetized animals. Thirty minutes later, labeled human SS RBCs (300 μ l hematocrit (Hct) 50% in PBS with Ca²⁺ and Mg²⁺) were then infused. In some experiments, animals administered with TNF- α were infused 4 hours later with washed SS RBCs sham-treated or treated with 10 μ M U0126 or 10 μ M RDEA119 for 1 hour. Animals were placed on the stage of an
25 Axoplan microscope (Carl Zeiss, Thornwood, NY); temperature was maintained at 37°C using a thermostatically controlled heating pad. RBC adhesion and blood flow dynamics were observed in subdermal vessels for at least 30 minutes using 20X and 10X magnifications. Microcirculatory events and cell adhesion were simultaneously recorded using a Trinitron Color video monitor (PVM-1353 MD, Sony) and JVC videocassette recorder (BR-S3784, VCR King, Durham, NC) connected to a
30 digital video camera C2400 (Hamamatsu Photonics K.K., Japan). Arterioles were distinguished from venules based on: 1) observation of divergent flow as opposed to convergent flow; 2) birefringent appearance of vessel walls using transillumination, which is characteristic of arteriolar vascular

smooth muscle; and 3) relatively straight vessel trajectory without evidence of tortuosity. Cell adherence was quantitated by considering cells attached to the vessel walls and immobile for 1 minute. The percentage of the length of vessels with diameters $\leq 25 \mu\text{m}$ or $> 25 \mu\text{m}$, occupied by SS RBCs was quantified as: % venular length occupied by SS RBCs = length of vessel wall with adherent cells/total length of the vessel segments analyzed X 100.

Example 2: ERK1/2 is present in mature RBCs and undergoes activation by epinephrine in SS but not normal RBCs

(0081) Recently, our preliminary data showed that ERK1/2 can be found bound to the RBC plasma membrane. The cAMP/PKA pathway is known to both activate SS RBCs to adhere abnormally to endothelial cells (ECs)⁴ and modulate the MAPK/ERK cascade. Given the importance of abnormal SS RBC adherence in SCD pathophysiology, we investigated the possibility that ERK activity is conserved in SS RBCs and inducible by epinephrine. RBC ghosts consisting of membrane fragments prepared from SS and normal (AA) RBCs were first analyzed to confirm the presence of ERK1/2 and MEK1/2, the upstream kinase of ERK1/2 activation. MEK1/2 was abundant in both SS and AA RBCs, while ERK1/2 was expressed at higher levels in SS vs AA RBCs ($p < 0.05$, Figures 1A and B). Since ERK1/2 is also well expressed by platelets and leukocytes, we examined our RBC suspensions for contamination by other blood cells. Our SS RBC preparations ($0.13 \pm 0.01 \times 10^6/\text{ml}$ RBCs) showed no contamination by platelets, but a very low level of contamination by leukocytes ($0.2 \pm 0.06 \times 10^3/\text{ml}$) was sometimes detected. However, when similar numbers of isolated sickle cell patient leukocytes were examined for the presence of ERK1/2, no detectable signal was observed (data not shown), making it apparent that the observed ERK signal was in fact derived from SS RBCs.

(0082) Our data also indicated that ERK1/2 is phosphorylated at baseline in SS RBCs, and epinephrine at a physiologic "stress" dose (20 nM)²⁹ promoted a 2.1 ± 0.1 -fold increase in ERK phosphorylation within 1 minute ($n=3$; $p < 0.001$) (Figures 1C and D). Incubation of SS RBCs with the MEKI U0126, which specifically inhibits MEK1/2, prior to epinephrine treatment, significantly inhibited the effect of epinephrine on ERK1/2 phosphorylation ($p < 0.001$) (Figures 1C and D). Because our previous data indicated that the degree of adhesive response to epinephrine stimulation varied from patient to patient,⁴ the effect of epinephrine on ERK phosphorylation was measured in samples obtained from a larger group of patients ($n=19$). Although a statistically significant increase (2 ± 0.17 -fold) in ERK phosphorylation above basal levels was observed ($P < 0.05$), SS RBCs from only 40% of patients exhibited more than 1.5-fold elevation in ERK phosphorylation by epinephrine. These patients were classified as responders. This data suggests that not all SCD

patients are susceptible to epinephrine-stimulated increased ERK phosphorylation. In contrast, ERK1/2 was never found phosphorylated in AA RBCs and failed to undergo phosphorylation by epinephrine (Figure 1C). In contrast, ERK1/2 was never found phosphorylated in normal (AA) RBCs at baseline and also failed to undergo phosphorylation after epinephrine stimulation (Figure 1C).

5 (0083) To further confirm that ERK1/2 preserved its activity in SS RBCs and that phosphorylation was indeed an indicator of ERK activation, we used the ERK specific substrate, myelin basic protein (MBP), to test the activity of ERK1/2 isolated from both sham-treated and epinephrine-treated SS RBCs, in the presence of inhibitors of PKA, PKC, Ca^{2+} /calmodulin-dependent kinase and p34^{cdc2} kinase to prevent nonspecific phosphorylation of MBP by these enzymes.²⁴

10 ERK1/2 immunoprecipitated from sham-treated SS RBCs was capable of phosphorylating MBP to some extent, while MBP phosphorylation by ERK1/2 immunoprecipitated from epinephrine-treated SS RBCs increased 2.1 ± 0.3 -fold compared to MPB phosphorylation induced by ERK1/2 isolated from sham-treated cells ($n=4$; $p=0.0286$) (Figures 1E and F). These data indicate that ERK1/2 can be already somewhat activated in SS RBCs and that epinephrine can augment its activity.

15 **Example 3: ERK1/2 in SS RBCs acts downstream of the cAMP/PKA signaling pathway**

(0084) We found that treatment of SS RBCs with forskolin, which directly activates AC to produce cAMP, promoted increased ERK1/2 phosphorylation, which was in turn prevented by MEK1 U0126, suggesting that cAMP is needed for ERK activation in SS RBCs (Figure 2A).

(0085) To determine the role of PKA in ERK phosphorylation, we used the PKA-specific inhibitor (PKAI), 14-22 amide. Treatment of SS RBCs with the PKAI, 30 nM 14-22 amide, at a concentration known to promote optimal inhibition of PKA in SS RBCs, did not significantly decrease basal ERK phosphorylation in these sickle cells (Figure 2B). However, PKAI completely blocked the effect of epinephrine on ERK phosphorylation ($p<0.01$, $n=3$). Pre-treatment of SS RBCs with a combination of PKAI and MEK1 U0126 also completely blocked ERK phosphorylation in response to

20 epinephrine stimulation ($p<0.01$) (Figure 2B). Together these data suggest that ERK1/2 activation in SS RBCs is dependent on the cAMP/PKA pathway.

(0086) In some instances, $\beta_2\text{AR}$ activation employs a $\text{G}\alpha_i$ (or $\text{G}\alpha_o$) pathway to stimulate ERK activity.⁷ We investigated whether epinephrine stimulated SS RBC $\beta_2\text{AR}$ s mediated ERK activation also involved the $\text{G}\alpha_i$, using Pertussis toxin (PTx), which inhibits $\text{G}\alpha_i$ -signaling. Inhibition of $\text{G}\alpha_i$ with

30 1 or 2 $\mu\text{g/ml}$ PTx alone significantly increased basal phosphorylation of ERK1/2 by 2.04 ± 0.1 - and 2.53 ± 0.11 -fold, respectively and combining PTx with epinephrine had no additional effect (Figure 2C, $p<0.001$). These results suggest that increased ERK1/2 phosphorylation in SCD patient samples tested is negatively affected by $\text{G}\alpha_i$ activation, or due to the direct actions of PTx.

(0087) Because direct or indirect involvement of cytoplasmic tyrosine kinases in activation of MAP kinase cascades has also been demonstrated,^{25, 26} we evaluated the contribution of tyrosine kinase-induced signaling to RBC ERK1/2 phosphorylation. ERK1/2 was phosphorylated at baseline in sham-treated SS RBCs (Figure 2D). Treatment with 2 µg/ml PTx markedly increased ERK1/2 phosphorylation. Damnacanthal, a highly potent and selective inhibitor of the tyrosine kinase p56^{lck},²⁷ did not abrogate ERK phosphorylation in response to PTx (Figure 2D). However, piceatannol, which preferentially inhibits the tyrosine kinase p72^{syk} vs p56^{lyn}, completely blocked the effect of PTx on ERK phosphorylation. Once more, U0126 blocked the effect of PTx on ERK1/2 phosphorylation. These data suggest that the piceatannol-sensitive tyrosine kinase p72^{syk} also plays a role in SS RBC ERK1/2 activation.

(0088) To determine if ERK1/2 is active only in the youngest cell population (reticulocytes), reticulocyte-enriched and -depleted (mature) SS RBCs were analyzed for kinase phosphorylation. Flow cytometric analysis showed that up to 15% of unseparated SS RBCs expressed the transferrin receptor, a reticulocyte marker. After separation, more than 95% of the reticulocyte-enriched cells expressed the transferrin receptor, while the reticulocyte-depleted population reacted with the anti-transferrin receptor antibody no more strongly than with the negative control immunoglobulin (data not shown). ERK1/2 was strongly phosphorylated in both reticulocyte-enriched and reticulocyte-depleted cells (n=2) (Figure 2E), suggesting that ERK activity is preserved in both reticulocytes and mature SS RBCs.

20 **Example 4: ERK1/2 is involved in SS RBC adhesion to endothelial cells**

(0089) Since the pharmacological agents epinephrine and forskolin modulate both SS RBC adhesion to ECs⁴ and ERK activation, we determined the contribution of MEK/ERK signaling to RBC adhesion. Epinephrine significantly up-regulated SS RBC adhesion to HUVECs at a shear stress of 2 dynes/cm² in intermittent flow condition assays ($p < 0.001$) (Figure 3A). However, U0126 completely inhibited the effect of epinephrine on SS RBC adhesion ($p < 0.001$). Treatment of SS RBCs with U0126 alone also blocked SS RBC adhesion to HUVECs (91±4.6% inhibition) when compared to adhesion of sham-treated SS RBCs ($p < 0.01$).

(0090) Forskolin also enhanced SS RBC adhesion to HUVECs at a shear stress of 2 dynes/cm² ($p < 0.001$, n=3) (Figure 3B), and this effect was blocked by U0126 (83±4% inhibition, compared to increased adhesion by forskolin alone; $p < 0.01$). This suggests that the MEK/ERK pathway contributes to up-regulation of SS RBC adhesive function to ECs.

Example 5: ERK signaling is implicated in phosphorylation of the RBC adhesion receptor ICAM-4 (Landsteiner-Wiener blood group antigen, LW)

(0091) We further explored the possibility that the ERK signaling pathway is involved in ICAM-4 (LW) phosphorylation, which mediates adhesion via binding to endothelial $\alpha\text{v}\beta 3$ integrin.⁴

5 The ICAM-4 protein possesses only one serine, one tyrosine and no threonine within the 12 amino acids of its cytoplasmic tail, and it does not contain a typical PKA target consensus motif. Nevertheless, up-regulation of SS RBC adhesion to non-activated ECs requires serine phosphorylation of the ICAM-4 receptor.⁴ PhosphorImager analysis of immunoprecipitated ³²P-radiolabeled ICAM-4 and negative control immune complexes showed that ICAM-4 of non-

10 stimulated SS RBCs (Figure 3C, lane 1) is modestly phosphorylated as previously shown. Treatment of SS RBCs with serine phosphatase inhibitors (SPI) (lane 2) increased ICAM-4 phosphorylation by 3.7 ± 0.46 -fold ($p < 0.05$, $n = 3$), suggesting that increased ICAM-4 phosphorylation is a result of serine phosphorylation, as tyrosine phosphatase inhibitors were not present. These were similar to the effects of epinephrine, although SPI-stimulation induced a significant increase (2.62 ± 0.6 -fold) in

15 ICAM-4 phosphorylation above baseline in a larger group of patients ($n = 8$) ($P < 0.05$), only half of all SS RBC samples exhibited ≥ 2 -fold elevation in ICAM-4 phosphorylation in response to SPI. Epinephrine in the presence of SPI had a stronger effect on ICAM-4 phosphorylation (7.4 ± 1.07 -fold increase over sham-treated SS RBCs; $p < 0.001$) (lane 3). Treatment of SS RBCs with either the PKAI or U0126 (lanes 4 and 5, respectively) significantly decreased the combined effect of epinephrine

20 and SPI on ICAM-4 phosphorylation compared to cells treated with epinephrine alone ($p < 0.001$) (lane 3). Treatment of SS RBCs with both PKAI and MEKI completely blocked epinephrine and SPI from up-regulating phosphorylation of ICAM-4 ($p < 0.001$) (Figure 3C, lane 6).

(0092) Immunoblots of ³²P-radiolabeled ICAM-4 immunoprecipitates from stimulated and non-stimulated SS RBCs (Figure 3C) indicated that a similar amount of ICAM-4 was

25 immunoprecipitated from these cells. Control immunoblots of immunoprecipitated ICAM-4 and the negative control complexes immunoprecipitated with P3 from stimulated and non-stimulated SS RBCs are shown in Figure 3D.

(0093) To define whether ICAM-4 is a substrate for ERK, we used non-treated packed normal RBCs as a source of ICAM-4, since ERK is inactive in these cells (Figure 1) and ICAM-4 is not

30 phosphorylated at baseline.⁴ Exposure of immunoprecipitated ICAM-4 to active recombinant ERK2 did not cause ICAM-4 phosphorylation, indicating that ICAM-4 is not a substrate for ERK (data not shown). Together, our data demonstrate that ICAM-4 in SS RBCs undergoes serine phosphorylation by a yet unknown kinase, and this process is PKA and MEK/ERK1/2 dependent.

Example 6: SS RBC adhesion is strictly related to the inception of ERK activation

(0094) Epinephrine significantly increased SS RBC adhesion to HUVECs under both intermittent and constant flow conditions after 1 min exposure ($p < 0.001$ for each), while adhesion decreased after 30 min cell exposure to epinephrine (mean decrease of all samples = $56 \pm 1.5\%$ and $73 \pm 4.7\%$ for intermittent and constant flow conditions, respectively; $p < 0.001$ for each) (Figures 4A and B). In contrast, epinephrine treatment for either 1 or 30 min had minimal effect on normal RBC adhesion to HUVECs under either intermittent or constant flow conditions (Figures 4A and B).

(0095) We also examined the effect of exposure time of SS RBCs to epinephrine on cAMP production, which appears to act upstream of ERK1/2. Basal cAMP in normal RBCs from healthy donors was significantly lower than basal cAMP in SS RBCs ($p = 0.0187$) (Figure 4C). In about 50% of the samples examined ($n = 19$), incubation of SS RBCs with epinephrine for 1 min resulted in accumulation of high levels of intracellular cAMP comparable to the levels of cAMP induced by forskolin treatment for 30 min. Although the cAMP response to epinephrine (1 min exposure time) varied among patients, as previously described,²⁸ cAMP levels uniformly declined with 30 min exposure time of SS RBCs to epinephrine ($p < 0.05$) (Figure 4C). Epinephrine exposure for 1 min had lower effect on cAMP accumulation in normal RBCs ($n = 12$) than in SS RBCs (Figure 4C) as previously shown.

(0096) Additionally, while a 1 min exposure of SS RBCs to epinephrine markedly increased ERK phosphorylation ($p < 0.01$ for epinephrine-treated for 1 min vs sham-treated), ERK phosphorylation decreased after a 30 min exposure ($p < 0.01$ for epinephrine-treated for 1 min vs 30 min) to levels observed in sham-treated cells ($p > 0.05$ for epinephrine-treated for 30 min vs sham-treated) (Figures 4D and E).

(0097) ICAM-4 phosphorylation also decreased with longer exposure time (30 min vs 1 min) of SS RBCs to epinephrine (Figure 4F). PhosphorImager analysis of immunoprecipitated ³²P-radiolabeled ICAM-4 and negative control immune complexes showed that treatment of SS RBCs with epinephrine for 1 min in the presence of SPI (lane 2) enhanced ICAM-4 phosphorylation by 1.84 ± 0.15 -fold over sham-treated cells (lane 4, $p < 0.01$). Thirty minutes exposure of SS RBCs to epinephrine in the presence of SPI (lane 3) significantly diminished the effect of epinephrine and SPI on ICAM-4 phosphorylation compared to cells treated with epinephrine for 1 min (lane 2, $p < 0.05$). Altogether, these data indicate that exposure time to epinephrine influences all these downstream effects—SS RBC adhesion, cAMP levels and phosphorylation of both ERK1/2 and ICAM-4—in a parallel fashion, suggesting that the time course of up-regulation of ICAM-4-mediated SS RBC adhesion is closely associated to the extent of ERK1/2 activation.

(0098) To identify potential proteins involved in regulation of the ERK pathway, a label-free quantitative phosphoproteomics analysis of RBC ghosts isolated from SS and normal RBCs treated with epinephrine for 1 and 30 min was undertaken. SS RBCs treated with epinephrine for 30 min showed a dramatic decrease in phosphorylation of serine 310 within adenylate cyclase-associated protein 1 (CAP1) compared to cells stimulated with epinephrine for 1 min (-4.3-fold, $p=1.54 \times 10^{-5}$) (Figure 4G). Conversely, AA RBCs exposed to epinephrine for 30 min showed an enhancement in phosphorylation of the CAP1serine vs 1 min epinephrine exposure (+1.4-fold, $p=0.008$). Threonine 307 within CAP1 also underwent a smaller yet statistically significant decrease in phosphorylation in SS RBCs exposed to epinephrine for 30 min vs 1 min epinephrine exposure (-1.4-fold, $p=4.92 \times 10^{-7}$). Our data indicate that exposure to epinephrine for a prolonged period of time negatively affects phosphorylation of CAP1 in SS but not in AA RBCs. Adenylate cyclase-associated proteins (CAPs) are known to regulate AC activation to increase cAMP levels under specific environmental conditions. We therefore suggest that a decrease in CAP1 phosphorylation in SS RBCs might down-regulate AC activity in these cells, negatively affecting signaling downstream of ERK.

Example 7: ERK signaling pathway is implicated in phosphorylation of protein 4.1

(0099) A label-free quantitative phosphoproteomics analysis was also performed to identify additional putative downstream targets of ERK by adding recombinant active ERK2 to RBC ghosts isolated from SS and normal RBCs. Because endogenous ERK is active at baseline in SS but not normal RBCs (Figure 1), SS RBCs were treated with U0126 prior to incubation of the ghosts with recombinant ERK2. We found that phosphorylation of protein 4.1 was induced in the presence of recombinant ERK2. Treatment of SS RBCs with U0126 resulted in a significant decrease (-1.7-fold, $p=1.01 \times 10^{-6}$) of a Ser540/Ser542 doubly phosphorylated peptide within protein 4.1 (Figure 5). Addition of recombinant ERK2 to the U0126-treated SS RBC ghosts increased the abundance of this phosphopeptide (+1.7-fold, $p=8.06 \times 10^{-14}$) back to levels observed in untreated SS RBCs, indicating the specificity of ERK2 as the upstream kinase. As expected, treatment of AA RBCs with U0126 did not induce a decrease of this doubly phosphorylated peptide within protein 4.1, since endogenous ERK is inactive in these cells. However, the complementary trend for this phosphorylated peptide was observed upon the addition of recombinant ERK2 to untreated and U0126-treated AA RBC ghosts, for which an increase of 2.1-fold ($p=1.5 \times 10^{-12}$ for untreated AA RBCs vs untreated AA RBCs+ERK2) and 1.7-fold ($p=4.6 \times 10^{-6}$ for U0126-treated AA RBCs vs U0126-treated AA RBCs+ERK2) were measured, respectively.

(00100) To confirm that the measured changes in phosphorylated peptide levels were not due to a difference in protein level between these treatment conditions, a non-phosphopeptide enriched proteomic analysis of AA RBC ghosts and AA RBC ghosts co-incubated with recombinant ERK2 was performed, and confirmed that protein 4.1 levels were similar between the two conditions (Figure 7A). This indicates that the observed changes in phosphopeptide abundance results from upstream kinase activity. Similar results were obtained when SS RBC ghosts and SS RBC ghosts co-incubated with recombinant ERK2 were analyzed (Figure 7B).

(00101) Collectively, these data further strengthen our findings that ERK is active in SS RBCs, and suggest that activation of the ERK cascade induces phosphorylation of the cytoskeletal protein 4.1.

Example 8: Recombinant active ERK2 phosphorylates the ERK consensus motif on dematin and adducins α and β .

(00102) To identify ERK substrates in RBCs, all phosphopeptide sequences within the dataset identified when active recombinant ERK2 was added to RBC ghosts were searched for the known ERK consensus motif, [PV]x[pST]P. Adducin- α and - β , and dematin, contained nine, seven and one unique phosphorylated peptides, respectively, with phosphorylation of residues within the ERK consensus motif. Only the statistically significant phosphopeptides with fold-changes of >1.5 are listed in Table 1. These peptides underwent a significant increase in phosphorylation in AA RBCs when recombinant ERK2 was added to the ghosts, while a decrease in phosphorylation of these peptides was observed in U0126-pretreated SS RBCs (Table 1). This suggests that the cytoskeletal proteins adducins α and β and dematin are substrates for ERK in RBCs.

(00103) **Table 1.** Motif Specific Phosphorylation by active recombinant ERK2. Fold changes in phosphorylation for peptides containing the ERK consensus motif [PV]x[pST]P were presented. Phosphorylation is up-regulated in normal RBCs (AA) with addition of active ERK2 and down-regulated in SS RBCs (SS) with addition of the MEK inhibitor U0126.

Table 1. Motif Specific Phosphorylation by Active Recombinant ERK2

Protein Description	Modified Peptide Sequence (SEQ ID NO:)	AA+ERK2 vs. AA	P value	SS+U0126 vs. SS	P value
α -adducin	[pS]PG[pS]PVGEGTGSPPK (SEQ ID NO:2)	-1.71	0.035	1.47	0.122
α -adducin	EEEAHRPP[pS]PTEAPTEASPEPAPDPAPVAEE AAPSAVEEGAAADPG[pS]DGSPGK (SEQ ID NO:3)	-1.69	0.025	1.60	0.074
β -adducin	ETAPEEPG[pS]PAK[pS]APA[pS]PVQSPAK (SEQ ID NO: 4)	-2.36	4.21E-06	1.91	3.87E-09
β -adducin	ETAPEEPGSPAK[pS]APA[pS]PVQSPAK (SEQ	-1.75	0.020	1.82	0.018

	ID NO: 5)				
β -adducin	ETAPEEPG[pS]PAK[pS]APASPVQSPAK (SEQ ID NO: 6)	-1.68	0.037	1.68	0.010
Dematin	[pS]TSPPP[pS]PEVWADSR (SEQ ID NO: 7)	-1.76	9.46E-09	1.48	4.98E-04

(00104) Fold changes in phosphorylation for peptides containing the ERK consensus motif [PV]x[pST]P were presented. Phosphorylation is up-regulated in normal RBCs (AA) with addition of active ERK2 and down-regulated in SS RBCs (SS) with addition of the MEK inhibitor U0126.

5

Example 9: ERK1/2 is involved in SS RBC adhesion to activated-endothelial cells

(00105) We have previously shown that both pharmacological agents epinephrine and forskolin upregulate SS RBC adhesion to non-activated endothelial cells via the MEK/ERK signaling pathway (Zennadi et al., Blood 2012). Treatment of SS RBCs with the MEK inhibitor U0126 alone
 10 also significantly blocked SS RBC adhesion to non-activated HUVECs ($91 \pm 4.6\%$ inhibition) when compared to adhesion of sham-treated SS RBCs ($p < 0.01$). These data suggest that increased SS RBC adhesion to non-activated endothelial cells requires activation of RBC adhesion molecules via stimulation of the MEK/ERK pathway.

(00106) Since inflammatory molecules are commonly augmented in sickle cell disease
 15 patients, and because Kaul et al. (Blood 2000;95:368-374) has reported that human SS RBCs adhered to cytokine-stimulated postcapillary endothelium in the absence of plasma, we asked whether ERK in non-stimulated SS RBCs also contributes to SS RBC adhesion to TNF- α -activated HUVECs. Treatment of HUVECs with TNF- α resulted in a significant increase in SS RBC adhesion by 2.6-fold at a shear stress of 2 dynes/cm² in intermittent flow conditions when compared to
 20 adhesion of SS RBCs to non-activated HUVECs, where less than 30% of the cells were able to adhere ($p < 0.001$) (Figure 8A). However, pre-treatment of SS RBCs with 10 μ M U0126 significantly reduced adhesion of SS RBCs to TNF- α -activated HUVECs and only $18 \pm 5\%$ of the cells adhered compared to adhesion of SS RBCs to non-activated HUVECs ($p < 0.001$). These results suggest that ERK is active in SS RBCs without prior cell-stimulation and is also involved in adhesion to activated-endothelial cells.

25 (00107) To confirm these initial data, we performed a similar experiment using three other inhibitors of MEK, RDEA119, AZD6244 and GSK1120212. These inhibitors were selected based on their good tolerability in long-term human therapeutic studies. Treatment of SS RBCs with RDEA119, AZD6244 and GSK1120212 (Figure 8B) significantly abolished SS RBC adhesion to TNF- α -activated endothelial cells to levels below baseline adhesion of SS RBCs to non-activated HUVECs

similar to the results shown for U0126 above. These data suggest that ERK signaling is involved in abnormal RBC adhesive interactions with activated-endothelium, and does NOT require activation of the RBCs to mediate RBC adhesion.

5 **Example 10: ERK contributes to SS RBC adhesion to vascular endothelium and vaso-occlusion in vivo**

(00108) The following experiment was designed to determine whether the MEK inhibitor U0126 can be used as a preventive agent of SS RBC adhesion to activated endothelium and vaso-occlusion. Human SS RBC preparations showed unmeasurable (0 cells/ μ l) leukocytes or platelets, making it unlikely that human leukocytes and platelets could participate in SS RBC adhesion and

10 vaso-occlusion in our model.

(00109) All human SS RBCs were fluorescently labeled, then washed prior to infusion into animals for observation within native intact vessels. Infusion of SS RBCs to mice injected with TNF- α resulted in marked SS RBC adhesion to vessels, predominantly in postcapillary venules, with intermittent occlusion of vessels and permanent blockage of some vessel segments (Figure 9A).

15 Vaso-occlusion occurred most frequently where vessels curved and at junctions, although it was also observed in straight non-junctional venular segments. SS RBC adhesion was even greater when mice were infused with vehicle (placebo, 0.4% DMSO in normal saline) 30 min prior to RBC infusion (Figure 9B) than that observed without vehicle infusion (Figure 9A). SS RBC adhesion to venular endothelium occurred in less than 5 min after RBC infusion, and led to the permanent

20 obstruction of small diameter vessels (Figure 9B). SS RBC adhesion was also observed in larger vessels. Vaso-occlusion also occurred most frequently where vessels curved and at junctions, although it was also observed in straight non-junctional venular segments. In contrast, SS RBCs showed very little adhesion to vascular endothelium and adhered only occasionally to small postcapillary venules when animals were infused with 2 mg/kg U0126 in 0.4% DMSO 30 min prior

25 to RBC infusion with no apparent vaso-occlusion (Figure 9C). Similarly, when animals were infused with 0.2mg/kg U0126 in 0.04% DMSO in saline 30 minutes prior to RBC infusion no apparent vaso-occlusion was observed (Figure 9D). The effect of U0126 on % venular length occupied by SS RBCs was quantified. The MEK inhibitor U0126 at 0.2 and 2 mg/kg induced a significant decrease in percentage venular length occupied by SS RBCs for vessels $\leq 25 \mu$ m in diameter ($p < 0.001$) (Figure

30 9E). However, when vessel diameter was $> 25 \mu$ m, U0126 at 0.2mg/kg did not significantly reduce the percentage venular length occupied by SSRBCs. These data strongly suggest that ERK is implicated in SS RBC adhesion to poscapillary venules promoting vaso-occlusion, and that the MEK inhibitor U0126 is able to down-regulate adhesion of subsequently infused SS RBCs and prevent

precipitation of occlusion of enflamed vessels when provided in the circulation. These data also suggest that MEK inhibitors may be able to reduce inflammation.

(00110) To further these studies, we tested whether the MEK inhibitor prevents SS RBC-induced vasoocclusion via at least its effect on SS RBCs. SS RBCs were treated with the MEK inhibitors U0126 and RDEA119 *ex vivo* prior to administration to the mice. Intravital microscopy studies showed that infusion of sham-treated human SS RBCs to nude mice treated with TNF- α (n=5), showed marked adhesion in inflamed venules and induced occlusion of small diameter (9-25 μ m) vessels (n=5). SSRBC adhesion was also observed in much larger vessels (up to 100 μ m in diameter), indicating that human SSRBC-induced vasoocclusion was not a result of trapping of human SSRBCs in vessels with diameters $\leq$ 8 μ m, since the size of human RBC is 8 μ m in diameter (Figures 10A). However, inhibition of MEK/ERK in human SSRBCs with U0126 *ex-vivo* prior to RBC infusion to animals treated with TNF- α (n=5), dramatically decreased human SSRBC adhesion and prevented vessel obstruction (Figures 10B). Similarly, treatment of human SS RBCs with RDEA119 (n=5) also dramatically decreased human SS RBC adhesion and prevented vessel obstruction (Figure 10C). The Effect of U0126 and RDEA119 on % venular length occupied by SS RBCs, was quantified. The percentage of venular length occupied by both U0126-treated SS RBCs and RDEA119-treated SS RBCs significantly decreased for vessels $\leq$ 25 μ m in diameter ($p < 0.001$ compared to sham) and vessels with a diameter > 25 μ m ($p < 0.05$ compared to sham) (Figure 10D). These data suggest that MEK inhibition with U0126 and RDEA119 improved SS RBC circulatory behavior due to amelioration of SS RBC adhesive function. Thus, our data suggest that ERK and its mechanism of action could represent a novel target for the treatment of SCD pathophysiology.

Example 11: Incubation of polymorphonuclear cells with SSRBCs results in activation of the polymorphonuclear cells

(00111) To further analyze the effect of ERK activation on SSRBCs, we treated SSRBCs with 20 nM epinephrine alone for 1 min or after pre-incubation with 10 mM U0126, RDEA119, AZD6244 or GSK1120212 for 1 hour, followed by treatment with 20 nM epinephrine for 1 min. The cells were then washed prior to admixture with fluorescently-labeled native polymorphonuclear cells (PMNs) obtained from healthy donors at a RBC:PMN ratio of 10:1. After co-incubation for 30 minutes at 37°C, cells mixtures were assayed for their ability to adhere to non-activated HUVECs. Since the only cell population visualized was red-fluorescence labeled normal PMNs, the quantitation of adherent PMNs did not include non-labeled SS RBCs or any remaining non-labeled leukocytes from SCD patients. Our data show that co-incubation of epinephrine-activated SS RBCs with naive PMNs, resulted in significant activation of PMN adhesion to non-activated endothelial

cells compared to adhesion of native normal PMNs not co-incubated with SS RBCs (Figure 11). However, blocking ERK activity in activated-SSRBCs with MEK inhibitors U0126, RDEA119, AZD6244 and GSK1120212 significantly decreased the ability of activated-SS RBCs to promote neutrophil adhesion (Figure 11). These data suggest that ERK signaling is involved in activation of SS RBC adhesion receptors involved in interactions with the endothelium and leukocytes. Downregulation of RBC adhesion receptor activity by targeting ERK may not only decrease SS RBC adhesion to the endothelium but also reduce RBC-stimulated leukocyte activation.

Example 12: Phosphoproteomic profiling of RBC membranes

(00112) However, sickle cell adhesion and vaso-occlusion alone do not account for the pathophysiology of SCD. Subsequent changes in red cell membrane structure and function and disordered cell volume control may also play an important role. Therefore, we investigated the ERK1/2 mediated RBC protein phosphorylation in SS RBC plasma membrane as compared to AA RBC plasma membrane.

(00113) *Collection, preparation and treatment of RBCs.* Human SCD patients homozygous for hemoglobin S were not transfused for at least three months, had not experienced vaso-occlusion for three weeks, and were not on hydroxyurea. Blood samples from SCD patients and healthy donors collected into citrate tubes, were used within less than 24 h of collection. Packed RBCs were separated as previously described in detail.⁶⁶ Packed RBCs were analyzed for leukocyte and platelet contamination using an Automated Hematology Analyzer K-1000 (Sysmex, Japan). For proteomics studies, aliquots of packed RBCs were treated at 37°C with 10 µM MEK1/2 inhibitor U0126 (Calbiochem, La Jolla, CA) for 1 hour. Sham-treated RBCs were incubated with the same buffer and vehicle, but without the active agent. Normal RBCs were used as controls.

(00114) *MAP kinase activity assay.* Treated packed normal and SS RBCs were lysed at 4°C with lysis buffer (10 mM EDTA, 20 mM Tris, 110 mM NaCl, pH 7.5) containing 2 mM PMSF, 1% Triton X-100, phosphatase inhibitor cocktail (Sigma) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). RBC membrane ghosts were then incubated with or without recombinant active human ERK2 (sigma) at 8.2 µg/ml with a specific activity of 700 nmole/min/mg, in the presence of inhibitors of PKA, PKC, Ca²⁺/calmodulin-dependent kinase and p34^{cdc2} kinase to prevent nonspecific protein phosphorylation by these enzymes,⁵⁷ and with ATP as a phosphate donor with equal membrane ghost protein amounts per assay condition. For the negative control, an equal volume of water was substituted for ATP. The reaction mixture was incubated for 20 min at 30°C. To stop the enzymatic reaction samples were placed on ice.

(00115) *RBC membrane ghost preparation and phosphopeptide enrichment.* Non-radiolabeled RBC membrane ghosts isolated from packed RBCs sham-treated or treated with U0126 and incubated with or without recombinant ERK2, were spun at 14,000 rpm for 15 min at 4°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°C. The supernatant was then removed and 500 µL of 50 mM ammonium bicarbonate with µL 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 min at 4°C. Samples were normalized to approximately 2 µg/µL following a micro-Bradford assay (Pierce Biotechnology, Inc), and were reduced with a final concentration of 10 mM dithiothreitol at 80°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°C for 18 hr. To remove ALS-1, samples were acidified to pH 2.0 with neat TFA, incubated at 60°C for 2 hrs and spun at 14,000 rpm to remove hydrolyzed ALS-1. Samples were either subjected to LC-MS analysis following a 10X dilution into mobile phase A or subjected to a TiO₂ based phosphopeptide enriched protocol.

(00116) To enrich for phosphorylated peptides prior to LC-MS analysis, 1,125 µg of total digested protein from RBC ghosts were brought to near dryness using vacuum centrifugation and then resuspended in 200 µL 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 TiO₂ spin column (Protea Biosciences) with a 562 µg binding capacity pre-equilibrated with 80% acetonitrile, 1% TFA (pH 2.5). For all loading, washing, and elution steps, the centrifuge was set to achieve a flow rate of no faster than 100 µL/min. Samples were washed twice with 200 µL 80% acetonitrile, 1% TFA, 50 mg/ml MassPrep Enhancer (pH 2.5) followed by two washes with 200 µL 80% acetonitrile, 1% TFA (pH 2.5). Retained peptides were eluted twice with 100 µL 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 µL 2% acetonitrile, 0.1% TFA, 25 mM citric acid (pH 2.5).

(00117) *Label-Free quantitative proteomic analysis of RBC membrane Ghosts.* Chromatographic separation of phosphopeptide enriched or non-enriched samples was performed on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C₁₈ 75 µm I.D. X 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 C₁₈ 180 μm I.D. X 20 mm column at 20 $\mu\text{L}/\text{min}$ 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 over 90 min at 300 nL/min. The analytical column was connected to fused silica
5 PicoTip emitter (New Objective, Cambridge, MA) with a 10 μm tip orifice and coupled to the mass spectrometer through an electrospray interface.

(00118) 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 ambient polycyclodimethylsiloxane (m/z 445.120025)
10 enabled. The instrument was set to acquire a precursor MS scan from m/z 400-2000 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 acquired MS/MS spectra in the ion-trap for the top 10 most abundant precursor ions and was used for additional qualitative identifications only. All other quantitative analysis acquired MS/MS spectra in the ion-trap for the top 5 most abundant precursor ions above a threshold of 500
15 counts. 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.

(00119) Non-phosphopeptide enriched data were acquired on a Waters Synapt HDMS
20 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.
25 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.

(00120) *Database searching and Label-free quantitation.* 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 either the phosphopeptide
30 enriched or non-enriched experiments 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.

(00121) 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 10 ppm precursor and 0.8 Da product ions were initially applied to LTQ-Orbitrap data and then manually refined to 4 ppm around the apex of the ppm mass error distribution from the most confident forward entries. Tolerances of 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. False-discovery rate were determined by adjusting the Mascot peptide ion score threshold to allow a 1% occurrence of peptide spectral matches from reverse protein entries for phosphopeptide enriched experiments, or by using PeptideProphet algorithm thresholds corresponded to a 2% peptide false discovery rate for non-phosphopeptide enriched experiments.

(00122) 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*).

(00123) *Glycophorin A phosphorylation and immunoprecipitation*. Packed RBCs ³²P-labeled as previously described,⁶⁸ were sham-treated, or incubated with serine/threonine phosphatase inhibitor (SPI) cocktail (Sigma) for 30 min, SPI cocktail followed by 1 min treatment with 20 nM epinephrine, or pre-incubated with 10 μM U0126 for 1 h followed by SPI cocktail, then treated with 20 nM epinephrine for 1 min. Cells were then washed 4 times. Glycophorin A immunoprecipitation using anti-glycophorin A monoclonal antibody (mAb) (Abcam, Cambridge, MA) and the negative control immunoglobulin P3, and total and phospho-glycophorin A detection were performed as

previously described in detail.⁶⁶ To confirm that the immunoprecipitates were specific for glycophorin A, anti-glycophorin A mAb and the negative control P3 were used to immunoprecipitate glycophorin A from non-radiolabeled treated SS RBCs. Blots were immunostained with anti-glycophorin A mAb.

5 (00124) *Statistical analysis.* Data were compared using parametric analyses (GraphPad Prism 5 Software, San Diego, CA), including repeated and non-repeated measures of analysis of variance (ANOVA). One-way and two-way ANOVA analyses were followed by Bonferroni corrections for multiple comparisons (multiplying the p value by the number of comparisons). A p value < 0.05 was considered significant.

10 (00125) *Label-Free Quantitative Phosphoproteomic Profiling of RBC Membranes.* Quantitation of global (non-targeted) phosphorylation events directly from human RBCs in disease-affected patients has been very limited in the literature. The most common analytical strategies have employed coupling two-dimensional gel electrophoresis of solubilized RBC proteins with either global ³²P labeling or anti-phosphotyrosine detection antibodies, followed by LC-MS/MS
15 identification of phosphoproteins from differentially expressed protein spots. In addition to the limited number of unique treatment groups, which could be directly compared within a single study, these previous approaches do not afford residue-specific quantitation of phosphorylation events as initial detection in changes in phosphorylation status measured at the protein level. This is particularly problematic for proteins containing multiple sites of phosphorylation, as each could
20 be independently modulated by different kinases or phosphatases as a function of various stimuli. In addition, different phosphorylation sites could have different effect on protein function. Although strategies such as iTRAQ, which are commonly used for phosphoproteomic quantitation from non-cell culture based systems, address some of these limitations, the reagents are still limited to a maximum of eight unique treatment groups (or unique samples), and add significant
25 cost when performing the labeling at the quantities of protein required for phosphoproteomic analysis.

(00126) Across all our samples tested, 375 unique phosphopeptides (527 total phosphorylated residues) corresponding to 155 phosphoproteins were identified at a peptide spectral match false discovery rate of 1.0%. As localization of specific phosphorylated residues is
30 critical for defining kinase specific events, all phosphopeptides were subjected to ModLoc, a probability-based localization tool implemented within Rosetta Elucidator based on the AScore algorithm (Figure 12A). (Beausoleil et al., A probability-based approach for high-throughput protein

phosphorylation analysis and site localization. *Nature biotechnology*. Oct 2006;24(10):1285-1292). Approximately 74% (348) of phosphorylated residues had ModLoc scores above 15 (>90% probability of correct localization) and 66% (310) had ModLoc scores above 20 (>99% probability of correct localization). To assess the quantitative robustness of the label-free approach, the average technical coefficient of variation (%CV) of retention-time aligned phosphorylated peptide intensities within a treatment group were calculated. The average %CV across all 375 phosphopeptides was 19.8%, with 80% of the signals having a %CVs less than 27.1% (Figure 12B). The intensity of the phosphorylated peptide V173-[pY187]-R191 within the activated site of ERK1/2 was used to assess inter-treatment group variation, including variation from TiO₂ phosphopeptide enrichment, as activated ERK2 was spiked in equal amounts to four of the eight samples. The average %CV of this phosphopeptide within any treatment group was 7.0% and across all ERK2 spiked samples was 18.1% (Figures 13A&B).

(00127) Consistent with a majority of TiO₂-enrichment based global mammalian phosphoproteomic studies, 78% (415) of the identified phosphorylated residues were localized to serines, 16% (85) to threonines, and 5% (27) to tyrosines, with an average of 1.4 phosphorylated residues per peptide (Figure 14A). Gene ontology classification of the biological function of the 155 identified phosphoproteins indicated nearly a third of the phosphoproteins were involved in binding as their primary biological function. Sub-classification of the binding category revealed over 80% of those phosphoproteins were involved in either protein binding (51%) or nucleotide binding (30%) (Figure 14B). Phosphoproteins involved in ion binding consisted 12% of the total phosphoproteins. These data were expected since our RBC samples were membrane fractions. Consistent with other RBC membrane phosphorylation studies, the phosphoproteins of SS RBC membrane ghosts with the highest number of uniquely phosphorylated peptides (>10), were ankyrin-1 of the ankyrin complex (n=33), spectrin β chain of the cytoskeleton network (n=15), and proteins of the junctional complex involved in binding integral membrane proteins to cytoskeletal proteins, including α - and β -adducins (n=22 and n=18, respectively), dematin (n=18) and protein 4.1 (n=17) (Table 2). However, with the exception of spectrin β chain, adducins α and β , and dematin, inhibition of MEK1/2 and its downstream signaling in SS RBCs with the MEK inhibitor U0126, and co-incubation of U0126-treated SS RBC membrane ghosts with active recombinant ERK2, failed to induce significant changes (decrease and increase, respectively) in the abundance of ankyrin-1 and protein 4.1 phosphopeptides (Table 2).

Table 2. Unique phosphorylated SS RBC membrane proteomes.

<u>Protein Description</u>	<u>Gene Name</u>	<u>Unique Phosphorylated Peptides</u>	<u>Unique Phosphorylated Residues</u>
Ankyrin-1	ANK1	33	26
Glyophorin A	GYPA	23	8
Alpha-adducin	ADD1	22	22
Beta-adducin	ADD2	18	
Protein 4.1	EPB41	17	10
Dematin	EPB49	16	
Spectrin beta chain	SPTB1	15	15
Band 3 anion transport protein	SLC4A1	14	10
Uncharacterized protein LOC388588	YA047	7	
GTPase-activating protein and VPS9 domain-containing protein 1	GAPVD1	5	
Lipin-2	LPIN2	5	
Serine/threonine-protein kinase WNK1	WNK1	5	
Spectrin alpha chain	SPTA1	4	
Erythroid membrane-associated protein	ERMAP	4	
Heat shock protein HSP90-alpha	HSP90A	4	
Phosphatidylinositol 4-kinase	PI4K2A	4	
TSC22 domain family protein 4	TSC22D4	4	

Unique phosphopeptides and their unique phosphorylated residues in SS RBC membrane fractions are presented.

- (00128) Previous studies have shown that protein 4.1 is extensively phosphorylated in sickle red cells. George, et al. Altered phosphorylation of cytoskeleton proteins in sickle red blood cells: the role of protein kinase C, Rac GTPases, and reactive oxygen species. *Blood Cells Mol Dis.* Jun 15

2010;45(1):41-45. Protein 4.1 phosphorylation, induced by cAMP-dependent kinase at Ser-331, results in multiple changes in RBC membrane, including significant reduction both in the ability of protein 4.1 to promote spectrin binding to F-actin and in spectrin-protein 4.1 binding. Ling et al., Modulation of red cell band 4.1 function by cAMP-dependent kinase and protein kinase C phosphorylation. *J Biol Chem.* Feb 15 1988;263(5):2209-2216. These changes weaken the binding sites for glycophorin C, XK and Duffy of the 30 kDa domain and the stability of the ternary junction complex, with possible effects on membrane mechanical stability and reduction in shear resistance to the membrane. However, while ERK1/2 signaling in SS RBCs is cAMP- and PKA-dependent, increased phosphorylation of protein 4.1 and ankyrin-1 in SS RBCs seems to not involve ERK1/2 signaling. Our data also indicate that in addition to these commonly reported phosphoproteins, several other phosphoproteins with >5 unique phosphorylated peptides were also observed (Table 2). These phosphoproteins also affect RBC shape, flexibility, anion transport and protein trafficking, and adhesion, all of which contribute to the pathophysiology of SCD.

(00129) *ERK1/2 Induces Atypical Phosphorylation of SS RBC membrane proteins.* To assess global quantitative differences between all treatment groups, data were subjected to two-dimensional agglomerative clustering of Z-score transformed (i.e. magnitude of significance of change) individual phosphopeptide intensities. This analysis revealed the most significant differentiation (most negative Pearson correlation) across all treatment groups, was the sickle versus healthy red cell phenotype, with 201 phosphopeptides being significantly up-regulated in SS vs AA RBCs at a *p*-value < 0.05 and fold-increase of >1.75 (chosen based on an alpha value corresponding to a 95% confidence interval in a statistical powering calculation). The weight of variation from the sickle state of the RBC (-0.664) was more significant than the addition of exogenous active ERK2 or the inhibition of MEK1/2 activity with the MEK1/2 inhibitor U0126, suggesting that in addition to MEK1/2/ERK1/2 phosphorylation cascades in the SS RBC, other cellular signaling pathway activities are also involved (Figure 15A). Interestingly, clustering of all phosphopeptides within only the SS RBC samples revealed the strongest differentiating factor was in the presence or absence of U0126 (Pearson correlation, -0.422), which supports the previous observation that ERK1/2 is constitutively hyperactive in these sickle RBCs and inhibiting ERK1/2's upstream activator, MEK1/2, alters a number of signaling events (Figures 15A&B). Recovery of the U0126 treatment by addition of exogenous active ERK2 resulted in the phosphorylation profile becoming more similar to the sham-treated SS RBCs. In comparison, clustering of all phosphopeptides within only the AA RBC samples revealed the strongest differentiating factor was the addition of exogenous active ERK2 (-0.489), which is consistent with the normal inactivity of

ERK1/2 in AA RBCs and suggests that ERK1/2 signaling is indeed mediating downstream phosphorylation of a number of targets (Figures 15A&B).

(00130) Putative downstream targets specific to MEK1/2-dependent activation of ERK1/2 were initially identified comparing individual phosphopeptide intensities between SS RBCs and SS RBCs treated with U0126. The MEK1/2 inhibitor U0126 was able to significantly down-regulate 36 unique RBC membrane phosphopeptides (from 22 unique phosphoproteins) in SS RBCs (Table 3). We analyzed a number of these phosphoproteins referring first to the model of red blood cell membrane functional organization proposed by Anong WA et al. who identified two major protein complexes bridging the RBC membrane to cytoskeleton network: the junctional complex formed by band 3, glycophorin C, Rh group, glucose transporter, dematin, p55, adducin, band 4.1 and 4.2 with associated glycolytic enzymes, and the ankyrin complex formed by band 3, glycophorin A, Rh group, ankyrin, and protein 4.2. Both complexes participate in anchoring the membrane to the actins, and α - and β -spectrins network, involving also other peripheral proteins as tropomyosin and tropomodulin. Here, we found that MEK1/2-dependent ERK1/2 activation in SS RBCs affected membrane-bound proteomes of both the junctional and ankyrin complexes, including dematin, α - and β -adducins, and glycophorin A. Glycophorin A was the most affected protein in SS RBCs by this pathway, which contained 11 unique phosphorylated peptides with 8 unique phosphorylated residues (6 phospho-serines and 2 phospho-threonines). The abundance of 6 of the phosphorylated residues, which was significantly downregulated with U0126 treatment of SS RBCs, was up-regulated in AA RBCs in the presence of exogenous active ERK2, suggesting that increased phosphorylation of glycophorin A by MEK1/2/ERK1/2 signaling could potentially affect SS RBC membrane properties. Glycophorin A, is the major sialoglycoprotein, and increased SS RBC adhesion to vascular endothelial cells has been postulated to result from clustering of negatively charged glycophorin-linked sialic acid moieties at the RBC surface. Enhanced SS RBC adhesion may also result from increased phosphorylation of glycophorin A by MEK1/2/ERK1/2 signaling. In addition, modulation in glycophorin A phosphorylation may also affect glycophorin A interactions with band 3, which could result in decreased in both anion transport by band 3 and band 3 trafficking.

(00131) Our data also indicated that adducin- β contained three unique phosphorylated peptides, with phosphorylation of residues within the ERK1/2 consensus motif, suggesting that the cytoskeletal protein adducin- β is a substrate for ERK1/2 in RBCs (Table 3). A significant decrease in phosphorylation of these peptides was observed in U0126-treated SS RBCs, while a significant

increase in phosphorylation was observed in both U0126-treated SS RBCs and in AA RBCs when recombinant active ERK2 was added to the membrane ghosts. However, the phosphorylated serine on either adducin- α or dematin, was not within the ERK1/2 consensus motif. Previous studies have shown that rapid phosphorylation of α - and β -adducins by PKC at Ser-726 and Ser-713, respectively, leads to decreased F-actin capping and dissociation of spectrin from actin, implicating adducin phosphorylation in cytoskeletal remodeling. Alternatively, dematin is a substrate for PKC and PKA, and PKA-induced dematin phosphorylation completely abolishes its actin bundling capability. Studies *in vitro* and *in vivo* in mice genetically lacking dematin have also shown its important role in maintaining red cell homeostasis and membrane mechanical properties.

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Table 3: Changes in Phosphorylation of Peptides in SS and AA RBCs

Protein Description	Modified Peptide	Fold Change: SS vs SS+ U0126 (Relative to SS)	p-value	Fold Change: SS+ U0126 vs SS+U0126 +ERK (Relative to SS+ U0126)	p-value	Fold Change: AA vs AA+ERK (Relative to AA)	p-value
Leucine-zipper-like transcriptional regulator 1	MAGPGST*GGQIGAAAL AGGAR (SEQ ID NO: 8)	-6.48	4.50E-02	6.70	4.54E-06		
Adenylyl cyclase-associated protein 1	SGPKPFSAPKPQTS*PSP K (SEQ ID NO: 9)	-4.79	2.00E-03	5.33	2.00E-04		
Dematin	QPLTSPGSVS*PSR (SEQ ID NO: 10)	-4.40	1.00E-03				
Alpha-adducin	QKGS*EENLDEAR (SEQ ID NO: 11)	-4.12	2.80E-45	2.42	1.25E-11		
Protein MICAL-2	VS*S*GIGAAAEVLNLY *MNDHRPKAQAT*SPDL ESMRK (SEQ ID NO: 12)	-4.06	8.44E-05				
facilitated glucose transporter member 1	QGGAS*QSDKTPEELFHP LGADSQV (SEQ ID NO: 13)	-3.37	7.99E-08				
U3 small nucleolar RNA-associated protein 15	VVHS*FDYAAS*ILSLALA HEDETIVVGMTNGILS*V KHR (SEQ ID NO: 14)	-2.93	7.39E-11	2.19	6.08E-05	2.06	9.39E-14
Leucine-rich repeats and immunoglobulin	T*HPETIIALRGMNVTLC TAVSSSDSPMST*VWR (SEQ ID NO: 15)	-2.92	1.27E-23	1.84	2.65E-05		

lin-like domains protein 2							
Transmembrane protein 151B	SPPGS*AAGES*AAGGGGGGGPGVSEELTAAAAAAADEGPAR (SEQ ID NO: 16)	-2.85	3.91E-05				
Eukaryotic translation initiation factor 4B	SQS*SDTEQQSPTSGGGK (SEQ ID NO: 17)	-2.48	4.00E-03	1.95	2.00E-02		
Spectrin beta chain, erythrocyte	QIAERPAETGPQEEEGETAGEAPVS*HHAATER (SEQ ID NO: 18)	-2.42	5.77E-04				
Glycophorin-A	KSPSDVKPLPS*PDT*DVPLS*SVEIENPETSQ (SEQ ID NO: 19)	-2.26	5.00E-03			2.12	8.04E-04
Glycophorin-A	SPSDVKPLPSPTDVPLS*SVEIENPETSQ V (SEQ ID NO: 20)	-2.18	3.00E-03			2.74	7.97E-05
60S acidic ribosomal protein P2	KEES*EES*DDDMGFGLFD (SEQ ID NO: 21)	-2.11	3.61E-08				
Glycophorin-A	SPSDVKPLPSPT*DVPLSVEIENPETSQ (SEQ ID NO: 22)	-2.00	2.59E-21				
Glycophorin-A	S*PS*DVKPLPSPTDVPLSSVEIENPETS*DQ (SEQ ID NO: 23)	-1.99	1.60E-02			1.87	3.10E-02
Glycophorin-A	S*PS*DVKPLPSPTDVPLSSVEIENPETSQ (SEQ ID NO: 24)	-1.98	8.00E-03				
Glycophorin-A	SPSDVKPLPS*PDT*DVPLSSVEIENPETSQ (SEQ ID NO: 25)	-1.96	2.13E-07				
Protein Wnt-16	HERWNCMITAAATTAPMGASPLFGYELS*SGTK (SEQ ID NO: 26)	-1.95	1.90E-02				
UV excision repair protein RAD23 homolog A	EDKS*PSEESAPTTSPESVSGSVPSSGSSGR (SEQ ID NO: 27)	-1.95	1.96E-14			2.19	4.13E-32
Glycophorin-A	SPSDVKPLPSPTDVPLSSVEIENPET*SDQ (SEQ ID NO: 28)	-1.93	1.82E-13				
Metabotropic glutamate receptor 7	LSHKPSDRPNGEAKT*ELCENVDPNS*PAAK (SEQ ID NO: 29)	-1.91	2.95E-09			2.29	7.98E-15
Beta-adducin	ETAPEEPGS*PAKS*APAS*PVQSPAK (SEQ ID NO: 30)	-1.91	3.87E-09			2.36	4.21E-06
Glycophorin-A	KS*PSDVKPLPSPTDVPLSSVEIENPETS*DQ (SEQ ID NO: 31)	-1.88	2.10E-02			1.97	5.00E-03
Beta-adducin	TESVTSGPMSPEGSPSKS*PSK (SEQ ID NO: 32)	-1.88	3.00E-02	1.85	3.00E-03		
Glycophorin-A	SPSDVKPLPSPTDVPLSS*VEIENPETSQ (SEQ ID NO: 33)	-1.87	3.30E-07				

Lipin-2	S*GGDETPSQSSDISHVL ETETIFTPSSVK (SEQ ID NO: 34)	-1.86	3.37E- 04				
Proteasome subunit alpha type-3	ESLKEEDS*DDDNM (SEQ ID NO: 35)	-1.83	5.42E- 06				
Beta-adducin	ETAPEEPGSPAKS*APAS *PVQSPAK (SEQ ID NO: 36)	-1.82	1.80E- 02	1.80	3.00E- 02	1.75	2.00E- 02
E3 ubiquitin- protein ligase UBR4	T*SPADHGGSVGSESGG SAVDSVAGEHSVSGR (SEQ ID NO: 37)	-1.80	1.02E- 07				
Spectrin beta chain, erythrocyte	LS*S*SWES*LQPEPSHP Y (SEQ ID NO: 38)	-1.80	6.00E- 03			4.41	4.02E- 21
Uncharacteriz ed protein LOC388588	DGVS*LGAVSST*EEASR (SEQ ID NO: 39)	-1.80	4.90E- 02				
Glycophorin-A	SPSDVKPLPSPDTDVPLSS VEIENPETS*DQ (SEQ ID NO: 40)	-1.77	4.00E- 03				
Spectrin beta chain, erythrocyte	LS*SS*WESLQPEPSHPY (SEQ ID NO: 41)	-1.76	5.56E- 05			2.02	9.30E- 11
Uncharacteriz ed protein LOC388588	DGVS*LGAVS*STEEASR (SEQ ID NO: 42)	-1.76	9.99E- 05			2.19	2.40E- 09
Glycophorin-A	SPSDVKPLPSPDT*DVPLS *SVEIENPETSDDQ (SEQ ID NO: 43)	-1.75	4.80E- 02				

*Indicates site of phosphorylation

(00132) MEK1/2/ERK1/2 signaling in SS RBCs induced changes within the actins/spectrins network as well, by affecting phosphorylation of β -spectrins (Table 3). Erythrocyte spectrin, the major component of the membrane skeleton, undergoes a number of naturally occurring or pathologically induced posttranslational phosphorylation via a cAMP-dependent protein kinase. ³²P-labeling studies indicate that only the β -subunit of spectrin is phosphorylated in intact erythrocyte, and phosphorylation occurs in a sequential manner where each specific site is completely phosphorylated before the next site is modified with the first phosphorylation event occurring on Ser-2114, followed by Ser-2125, Ser-2123, Ser-2128, Ser-2117, and Ser-2110. However, in situ studies by Manno et al. using intact erythrocyte membranes demonstrated that an increase in β -spectrin phosphorylation by casein kinase I causes a decrease in erythrocyte membrane mechanical stability. In addition, certain leukemia patients with elliptocytosis and poikilocytosis displayed an elevated amount of spectrin dimers coinciding with increased β -spectrin phosphorylation. Our findings are in accordance with these previous studies, and all together strongly suggest that increased phosphorylation of β -spectrin destabilizes tetramer formation and

has important *in vivo* physiological functions. Membrane skeleton also appears to regulate lateral and rotational mobility of band 3 and glycophorin A in the plane of the membrane.

(00133) Furthermore and interestingly, label-free proteomic analysis revealed that the peptide metabotropic glutamate receptor 7 (mGlu7) underwent serine phosphorylation at the ERK consensus motif (Table 3). Indeed, studies have also demonstrated that mGluR7 activation occurs via an ERK-dependent mechanism, which increased cofilin activity and F-actin depolymerization. mGlu7 acts as an autoreceptor mediating the feedback inhibition of glutamate release, and prolonged activation of this receptor potentiates glutamate release. Increased phosphorylation of mGlu7 in SS RBCs, could explain the rate of active glutamate transport in these cells, which increases 15-fold over that in normal RBCs. Significant Changes were also observed in the status of leucine-rich repeats and immunoglobulin-like domains protein 2, leucine-zipper-like transcriptional regulator 1, and glucose transporter 1, but only in membrane ghosts prepared from SS RBCs treated with U0126 or after addition of exogenous active ERK2 to these membrane ghosts (Table 3). Changes in the status of these proteins by MEK1/2/ERK1/2 signaling may potentially disturb degradation of misfolded glycoproteins and receptor ubiquitination, and affect protein transcription. Similarly and not surprisingly, ERK1/2 signaling was also found to increase phosphorylated adenylyl cyclase-associated protein 1 (CAP1) only in SS RBCs. CAP1 is known to regulate adenylyl cyclase activation to increase cAMP levels under specific environmental conditions. Indeed, basal cAMP levels are much higher in sickle than in healthy RBCs, and cAMP and PKA can act as upstream effectors of MEK1/2/ERK1/2 in SS RBCs. CAPs are also involved in actin binding, SH3 binding, and cell morphology maintenance as well. The failure of recombinant active ERK2 to significantly upregulate the abundance of the phosphorylated peptides, leucine-rich repeats and immunoglobulin-like domains protein 2, leucine-zipper-like transcriptional regulator 1 and CAP1, in healthy RBCs suggests a negative regulatory mechanism might exist in these cells to prevent activation of ERK1/2-dependent phosphorylation of these membrane proteins. PKA for instance, has been shown to exert a negative feedback loop through activation of phosphodiesterases, resulting in cAMP hydrolysis switching off downstream signaling.

(00134) *ERK1/2 is involved in phosphorylation of glycophorin A.* The pharmacological stress hormone epinephrine can modulate ERK1/2 activation in SS RBCs. Because our proteomics data showed that ERK1/2-induced changes in the phosphorylation state of glycophorin A affected numerous peptides, we determined the contribution of epinephrine-induced increased activation of ERK1/2 signaling in glycophorin A phosphorylation. PhosphorImager analysis of

immunoprecipitated ^{32}P -radiolabeled glycophorin A and negative control immune complexes showed that glycophorin A of non-stimulated SS RBCs (Figure 16, lane 1) is modestly phosphorylated at baseline. Treatment of SS RBCs with serine phosphatase inhibitors (SPI) (Figure 16, lane 2) slightly increased glycophorin A phosphorylation by 1.9 ± 0.1 -fold ($p < 0.05$, $n=3$), suggesting that increased glycophorin A phosphorylation is a result of serine phosphorylation, as tyrosine phosphatase inhibitors were not present. Epinephrine in the presence of SPI had a stronger effect on glycophorin A phosphorylation (2.93 ± 0.35 -fold increase over sham-treated SS RBCs; $p < 0.001$) (Figure 16; lane 3). However, treatment of SS RBCs with the MEK/12 inhibitor U0126 significantly decreased the combined effect of epinephrine and SPI on glycophorin A phosphorylation (Figure 16; lane 4) compared to cells treated with epinephrine and SPI ($p < 0.001$) (Figure 16; lane 3). Immunoblots of ^{32}P -radiolabeled glycophorin A immunoprecipitates from stimulated and non-stimulated SS RBCs indicated that a similar amount of glycophorin A was immunoprecipitated from these cells. Our data strongly confirm that increased glycophorin A phosphorylation is dependent on MEK1/2-dependent ERK1/2 signaling pathway in SS RBCs. It has been suggested that glycophorin contains receptors or other surface recognition sites of the erythrocyte. Although the conformation of glycophorin in the lipid bilayer is not known, it has been suggested that the glycoproteins exist as aggregates in the membrane in order to facilitate receptor function. However, we do not know whether increased phosphorylation of glycophorin A affects the state of aggregation of this glycoprotein. Recently, Shapiro and Marchesi have demonstrated that the site of phosphorylation of glycophorin is located on the cytoplasmic COOH terminal end.⁶⁵ It remains to be determined if phosphorylation plays a role in the formation of aggregates of the protein.

(00135) Indeed, ERK activation in sickle RBCs not only up-regulated sickle red cell adhesion to TNF- α activated endothelial cells in vitro, but affected proteins involved in nitric oxide transport, oxidative stress, proteins of the water channel, maintenance of the integrity of the plasma membrane and to anchor specific ion channels, ion exchangers and ion transporters in the plasma membrane, membrane morphogenesis and cytoskeletal organization, regulation of integrin-mediated signaling, and membrane integrity, permeability and polarity as well (Table 3). The MEK inhibitor U0126 down-regulates phosphorylation of ERK targets. These data suggest that ERK is involved not only in abnormal SS RBC adhesion, but affects multiple other red cell functions related but not limited to oxidative stress, hemolysis and ion transport.

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CLAIMS

1. A method of alleviating at least one symptom of a hemoglobinopathy in a patient comprising administering an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
- 5 2. The method of claim 1, wherein the hemoglobinopathy is selected from sickle cell disease, β -thalassemia, and hemoglobin H disease.
3. The method of claim 2, wherein the hemoglobinopathy is sickle cell disease.
4. The method of any one of claims 1-3, wherein the at least one symptom is selected from vaso-occlusion, acute painful episodes, chronic hemolysis (aplastic crises), avascular necrosis,
10 infection, end-organ damage, and erythroid hyperplasia.
5. A method of inhibiting adhesion of sickle red blood cells to endothelial cells comprising contacting the sickle red blood cells with an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
6. A method of inhibiting adhesion of sickle red blood cells to leukocytes comprising
15 contacting the sickle red blood cells with an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
7. A method of inhibiting formation of multicellular aggregates in the presence of sickle red blood cells comprising contacting the sickle red blood cells with an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
- 20 8. A method of inhibiting adhesion of leukocytes to endothelial cells in the presence of sickle red blood cells, comprising contacting the sickle red blood cells with an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
9. A method of alleviating at least one of acute pain, chronic hemolysis (aplastic crises), avascular necrosis, organ damage, and erythroid hyperplasia comprising contacting the
25 sickle red blood cells with an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
10. A method of inhibiting adhesion of sickle red blood cells to endothelial cells in a subject, comprising administering an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
- 30 11. A method of inhibiting adhesion of sickle red blood cells to leukocytes in a subject, comprising administering an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.

12. A method of inhibiting formation of multicellular aggregates in a subject with sickle cell disease, comprising administering an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
13. A method of inhibiting adhesion of leukocytes to endothelial cells in a subject with sickle cell disease, comprising administering an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor.
14. A method of alleviating at least one of acute pain, chronic hemolysis (aplastic crises), avascular necrosis, organ damage, and erythroid hyperplasia in a subject comprising administering an inhibitor selected from a MEK inhibitor, an ERK inhibitor, and a Raf inhibitor to the subject.
15. The method of any one of claims 10-14, wherein the subject is human.
16. The method of any one of the preceding claims, wherein the inhibitor is a MEK inhibitor.
17. The method of claim 16, wherein the MEK inhibitor is selected from U0126, PD98059, PD-334581, GDC-0973, CIP-137401, ARRY-162, ARRY-300, PD318088, PD0325901, CI-1040, BMS 777607, AZD8330, AZD6244, AS703026, RDEA119, and GSK1120212.
18. The method of any one of claims 1 to 15, wherein the inhibitor is an ERK inhibitor.
19. The method of claim 18, wherein the ERK inhibitor is AEZS-131.
20. The method of any one of claims 1 to 15, wherein the inhibitor is a Raf inhibitor.
21. The method of claim 20, wherein the Raf inhibitor is selected from sorafenib tosylate, GDC-0879, PLX-4720, regorafenib, PLX-4032, SB-590885-R, RAF265, GW5074, XL281, and GSK2118436.

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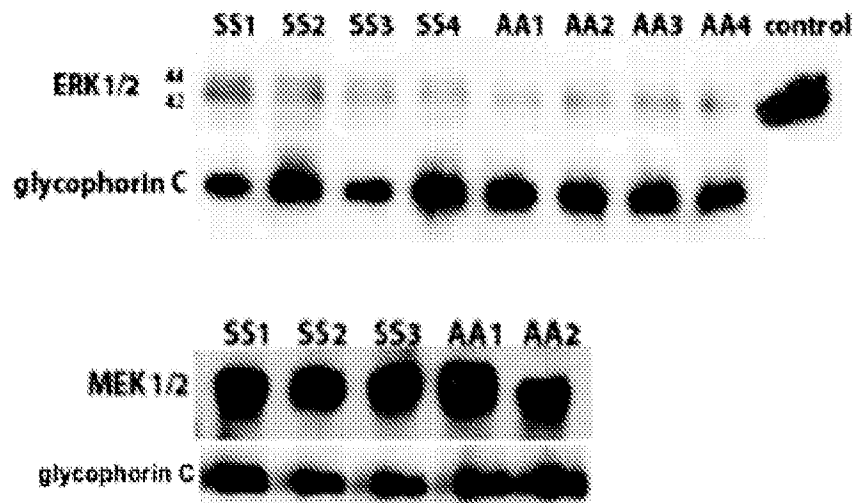


Fig. 1A

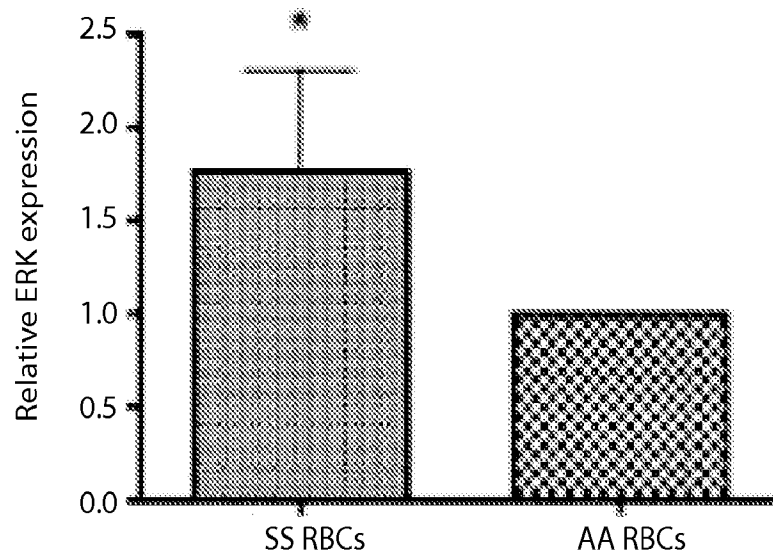


Fig. 1B

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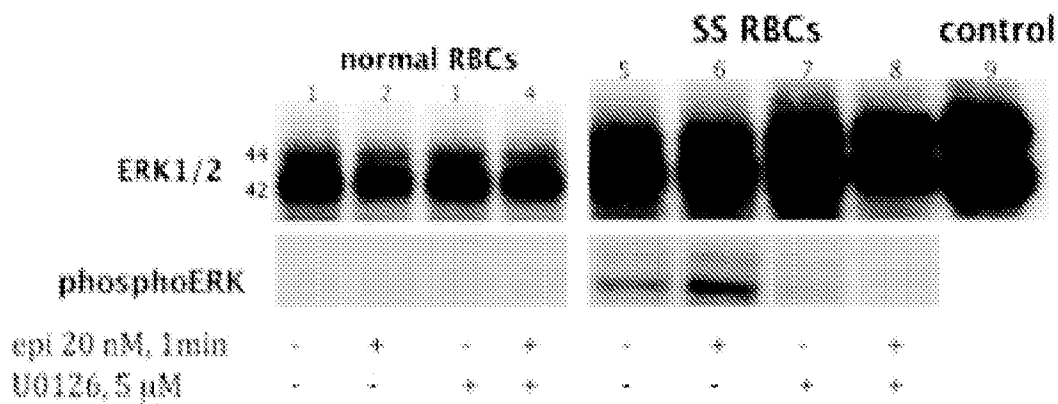


Fig. 1C

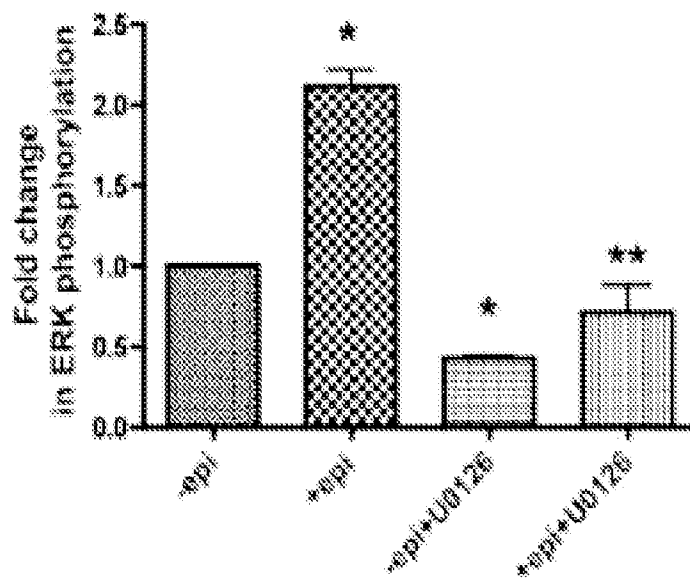


Fig. 1D

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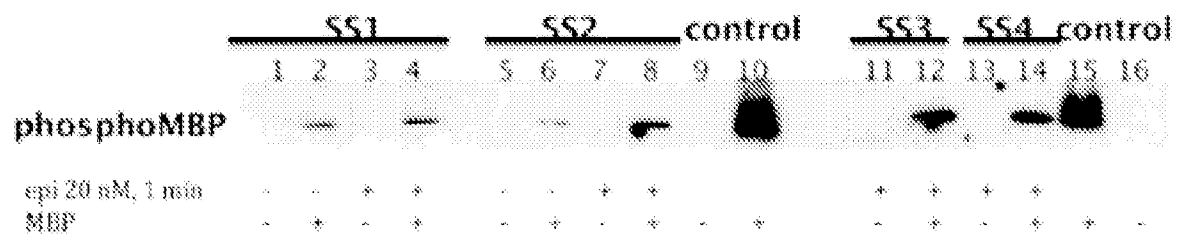


Fig. 1E

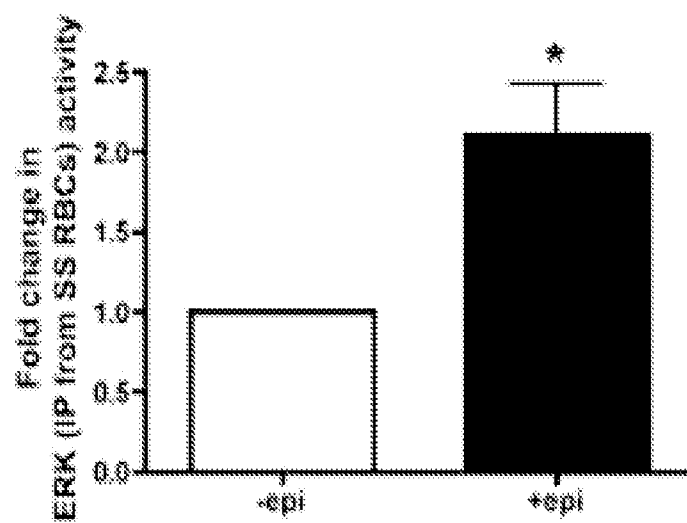


Fig. 1F

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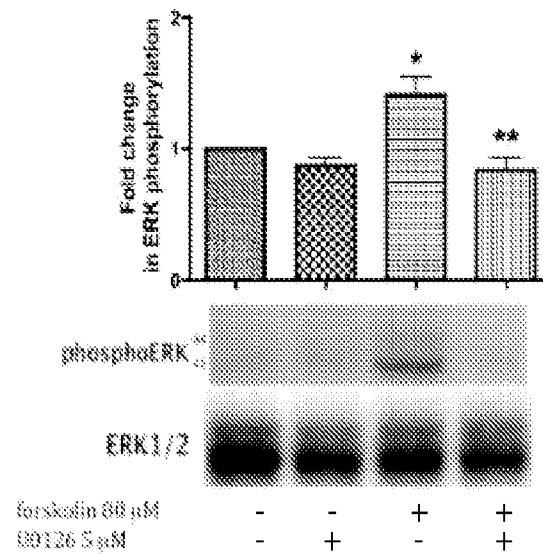


Fig. 2A

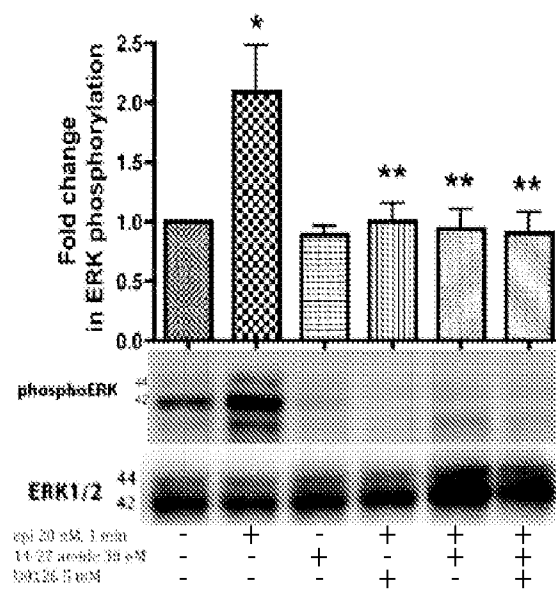
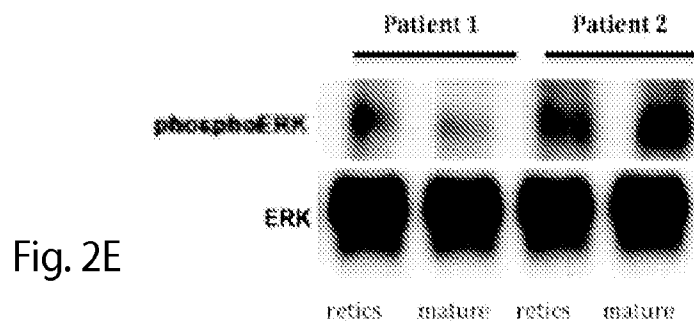
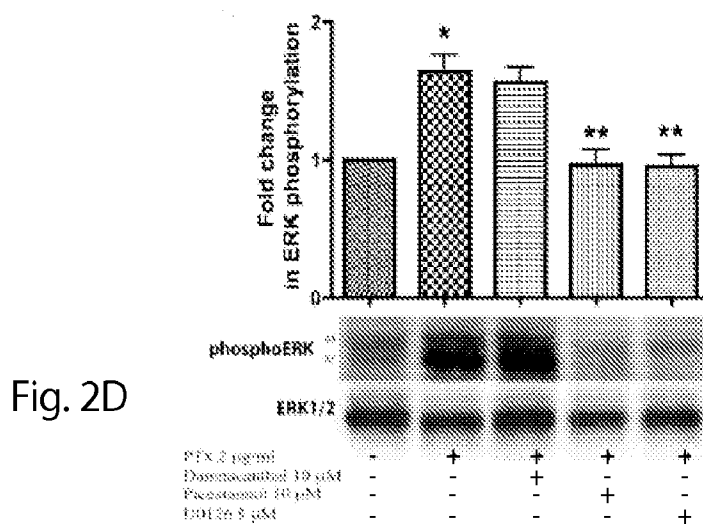
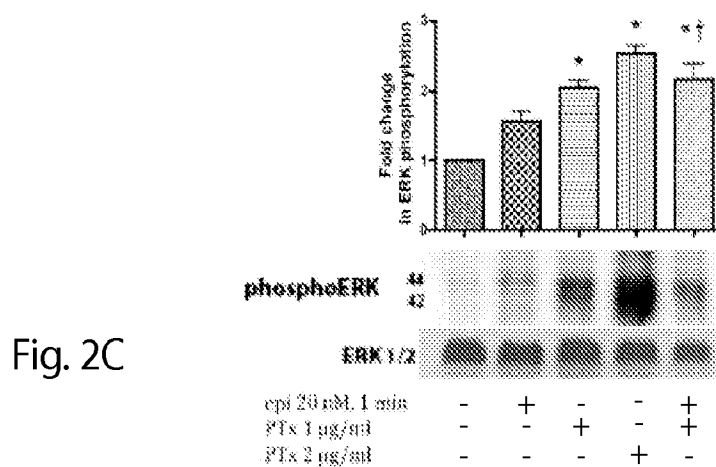


Fig. 2B

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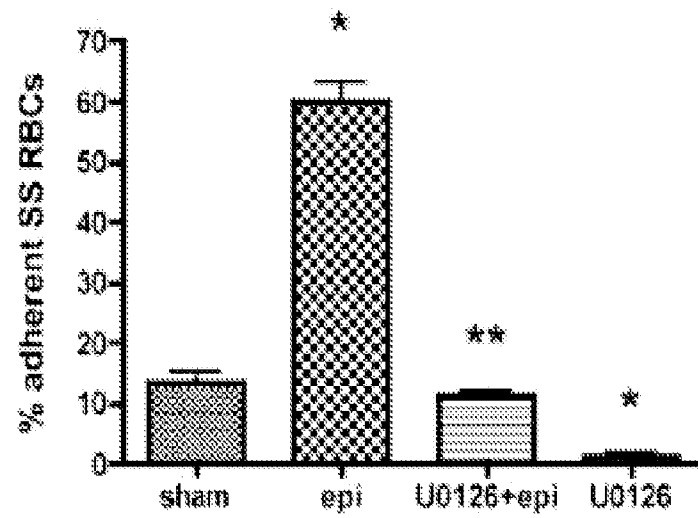


Fig. 3A

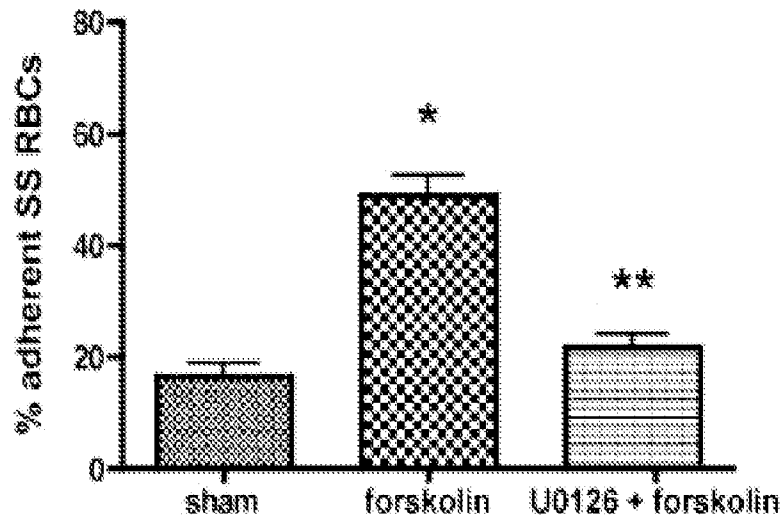


Fig. 3B

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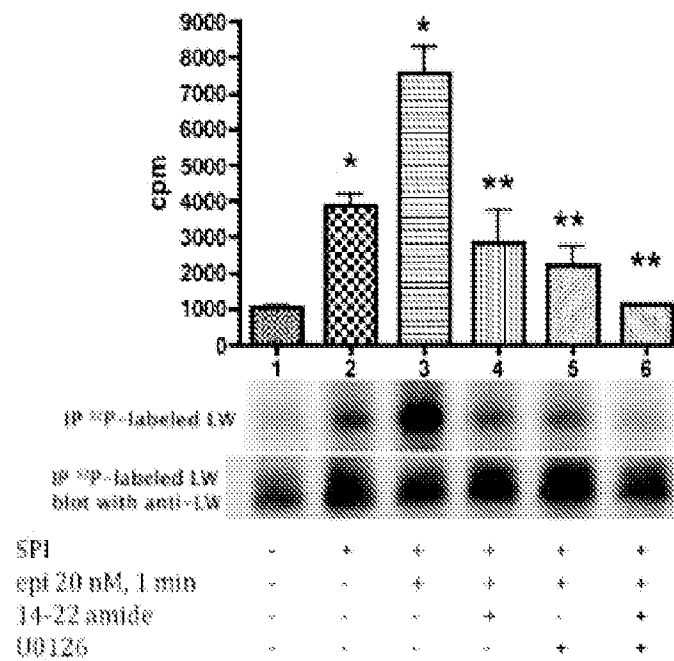


Fig 3C

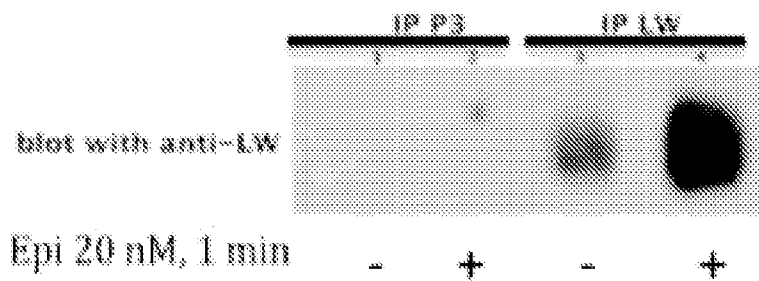


Fig. 3D

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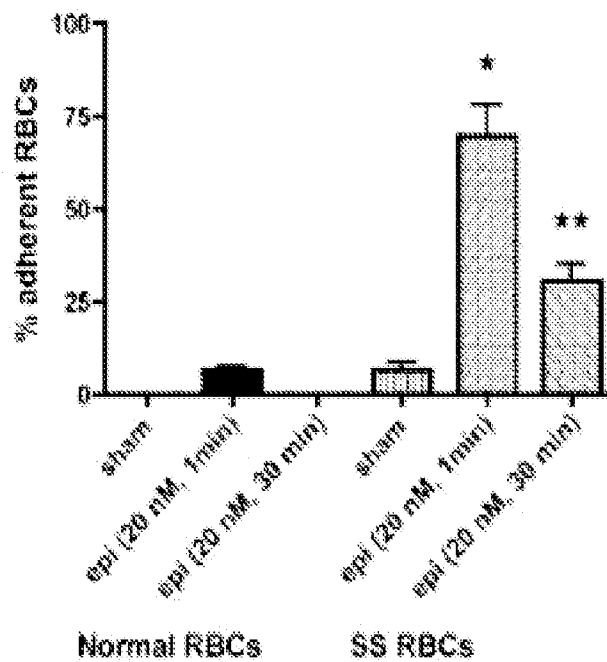


Fig. 4A

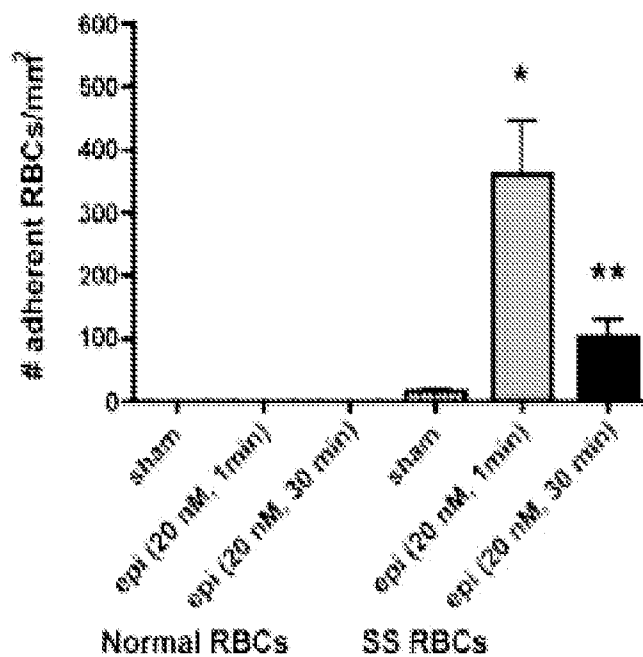


Fig. 4B

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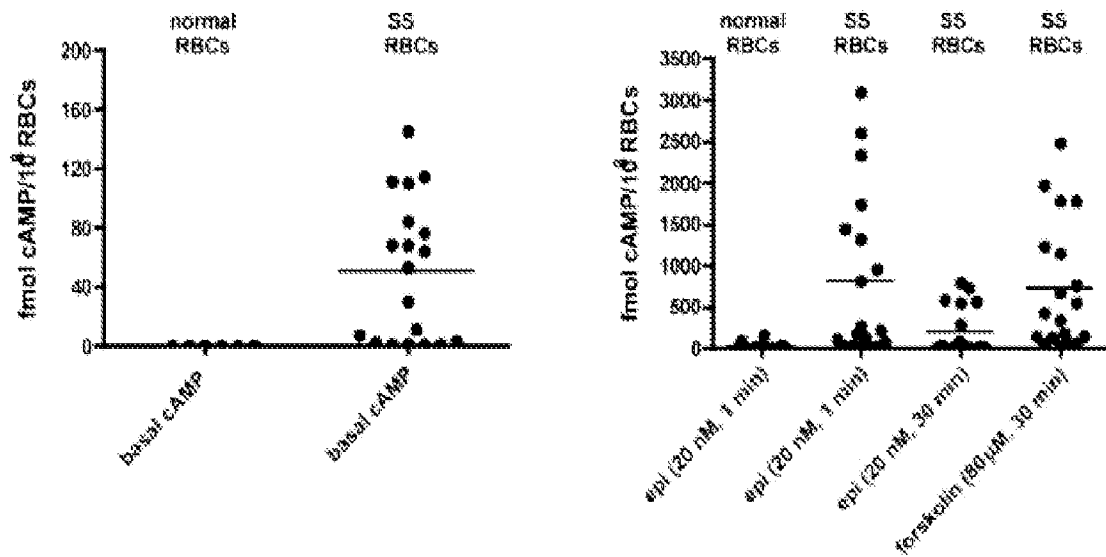


Fig. 4C

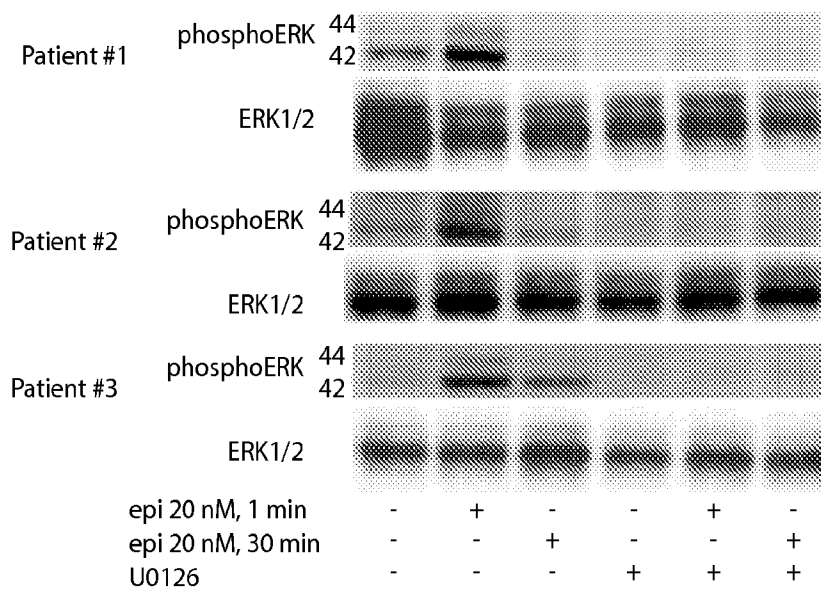


Fig. 4D

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Fig. 4E

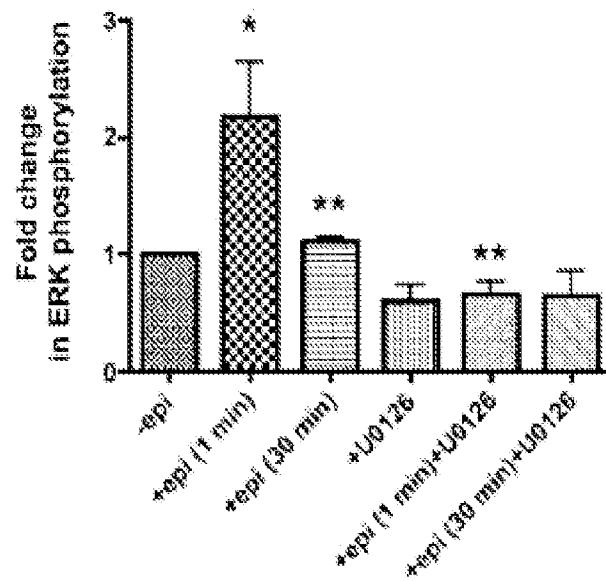


Fig. 4F

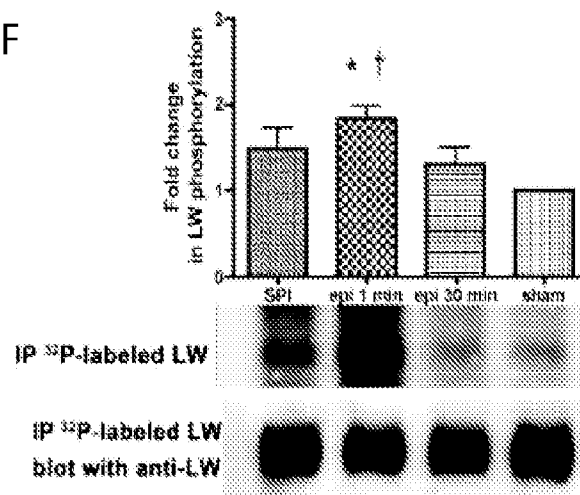
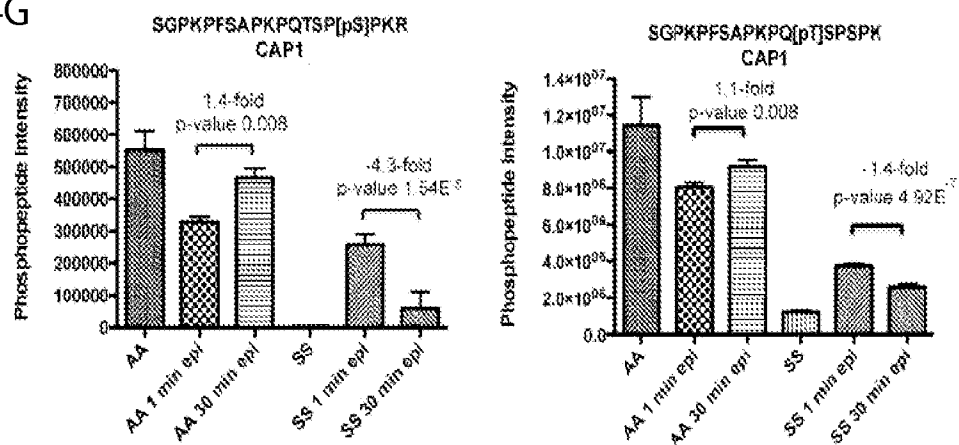


Fig. 4G



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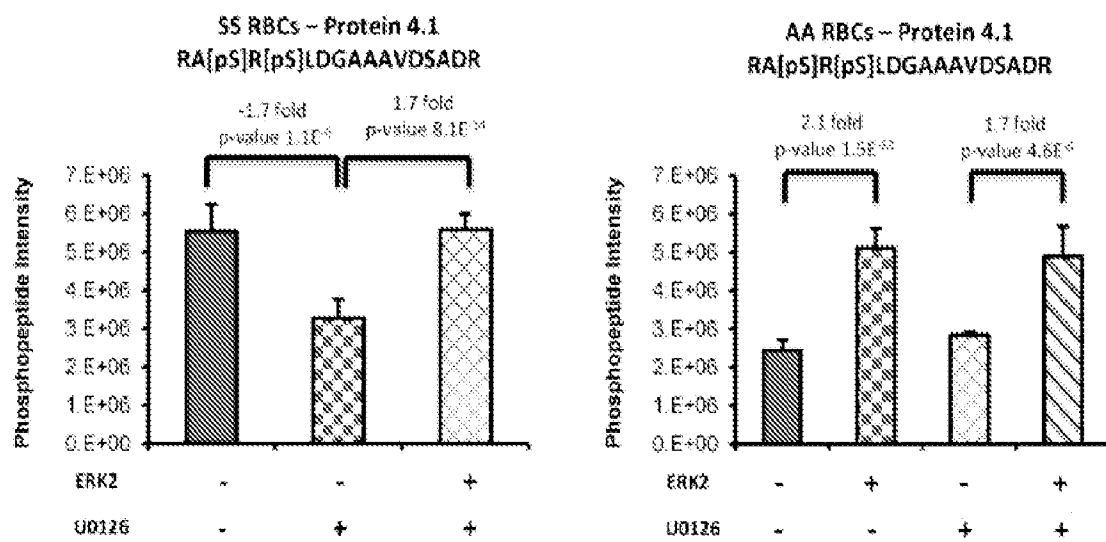


Fig. 5

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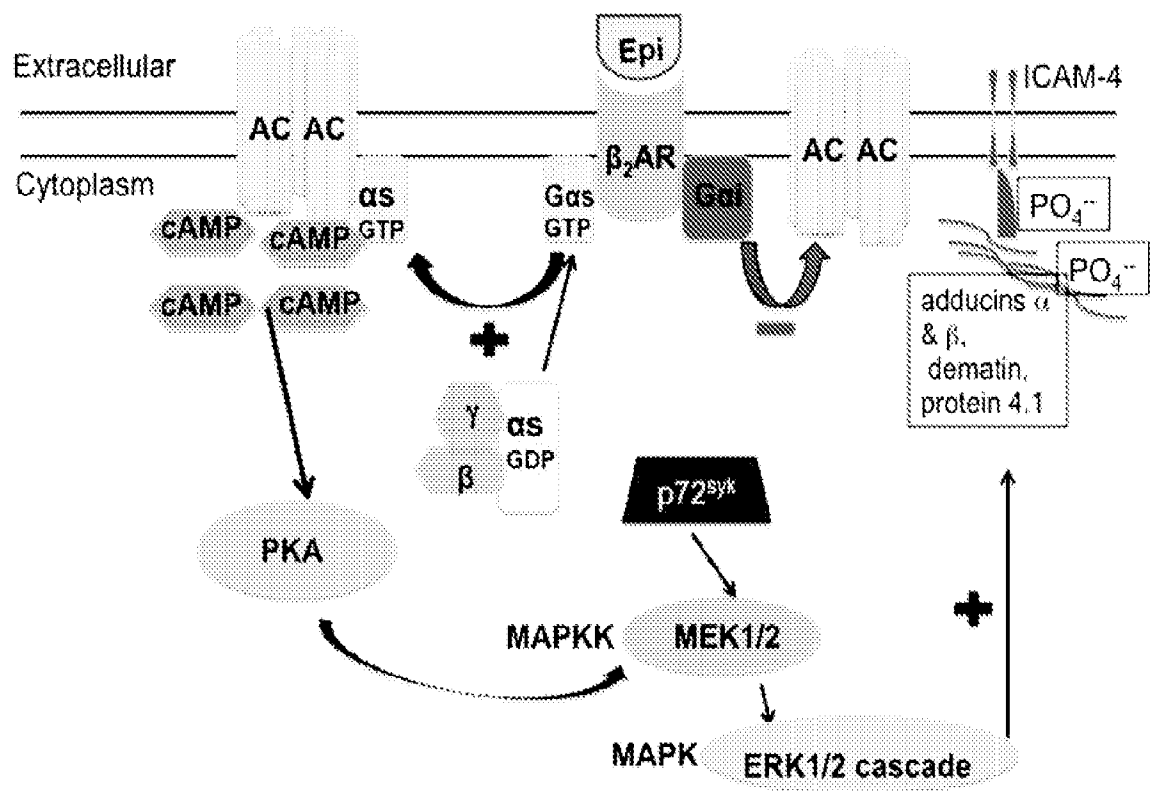
Activation of ERK1/2 Signaling Pathway in SS RBCs by Epinephrine

Fig. 6

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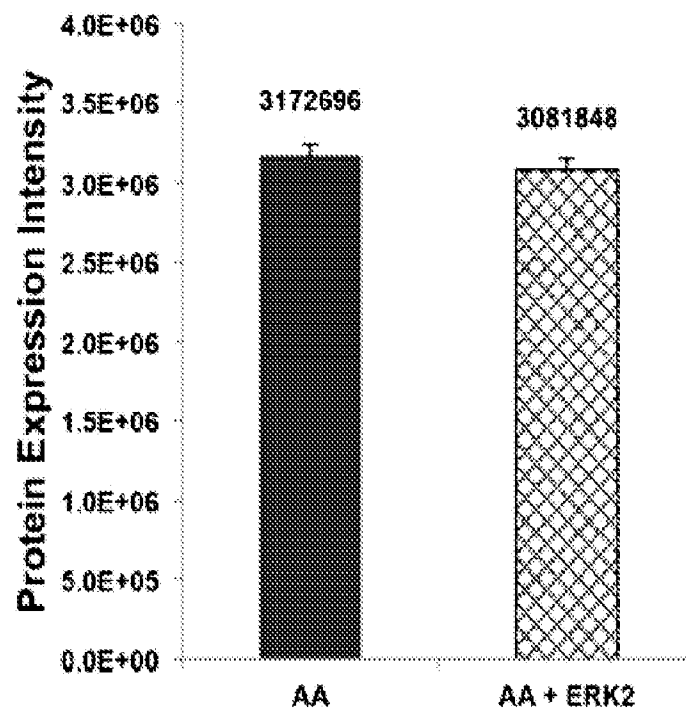


Fig. 7A

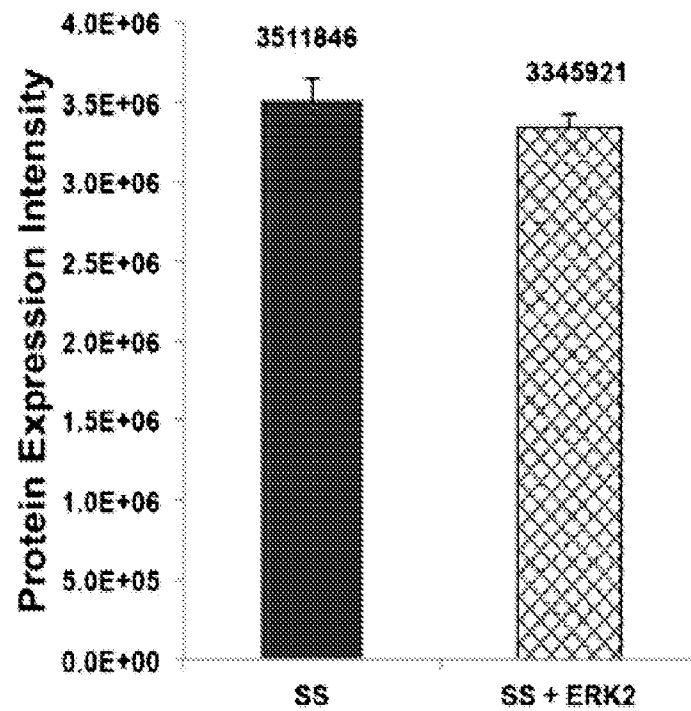


Fig. 7B

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Fig. 8A

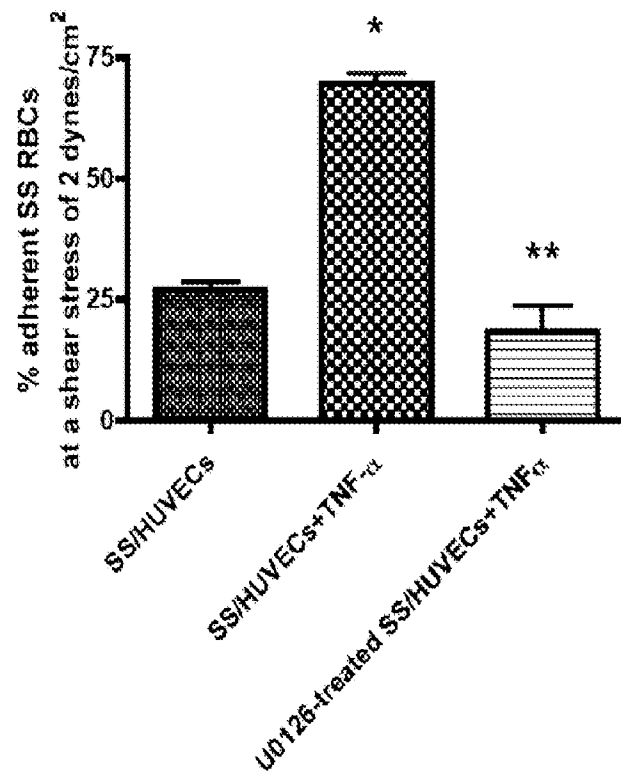
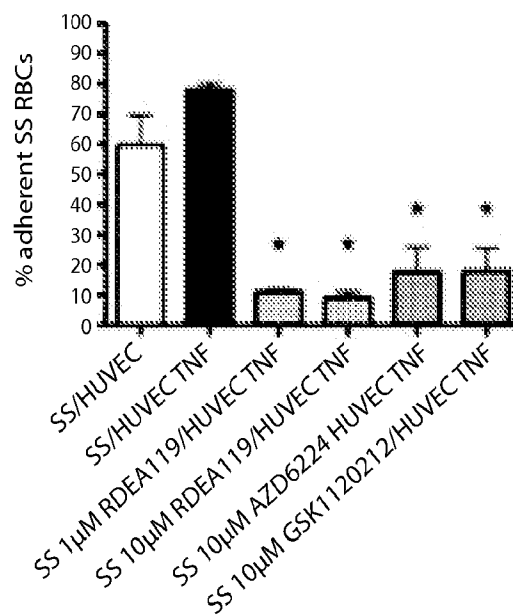


Fig. 8B



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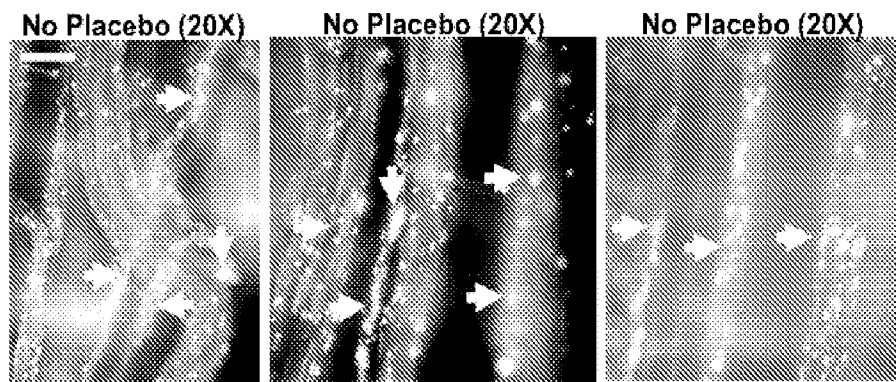


Fig. 9A

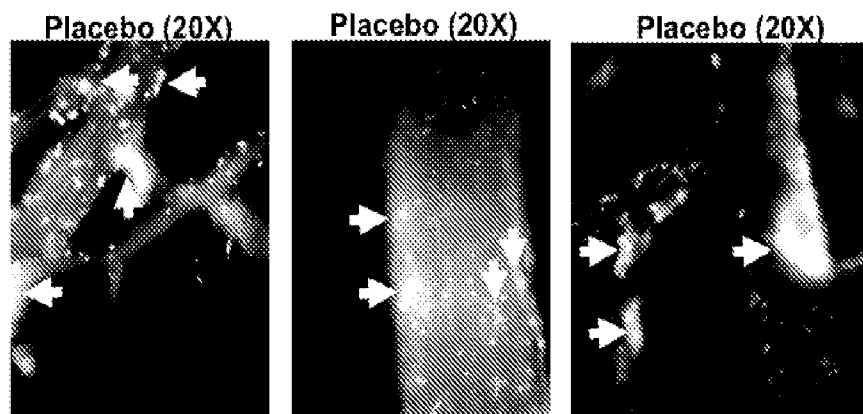


Fig. 9B

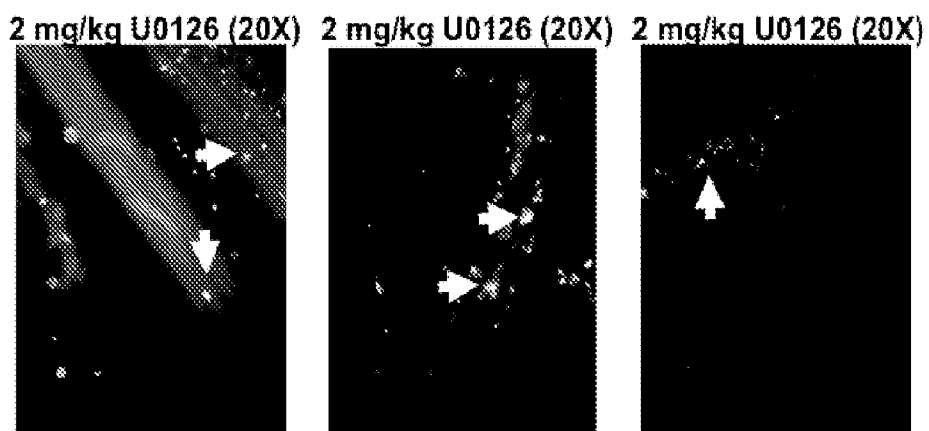


Fig. 9C

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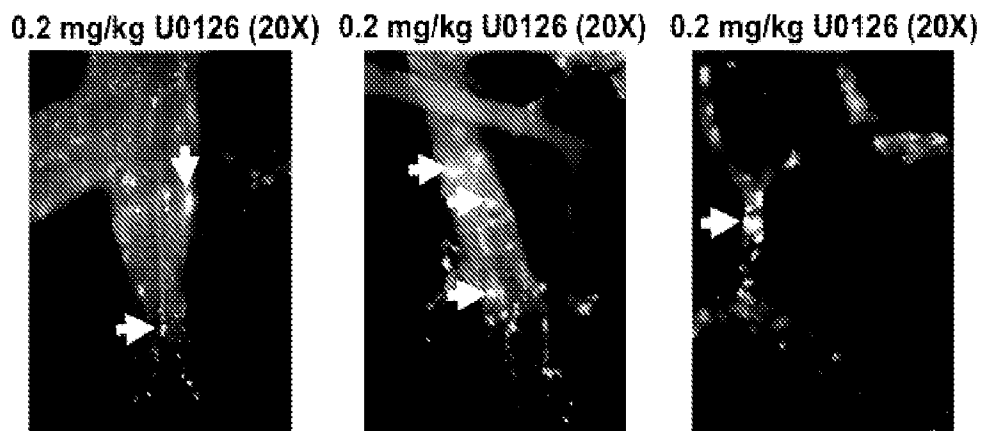


Fig. 9D

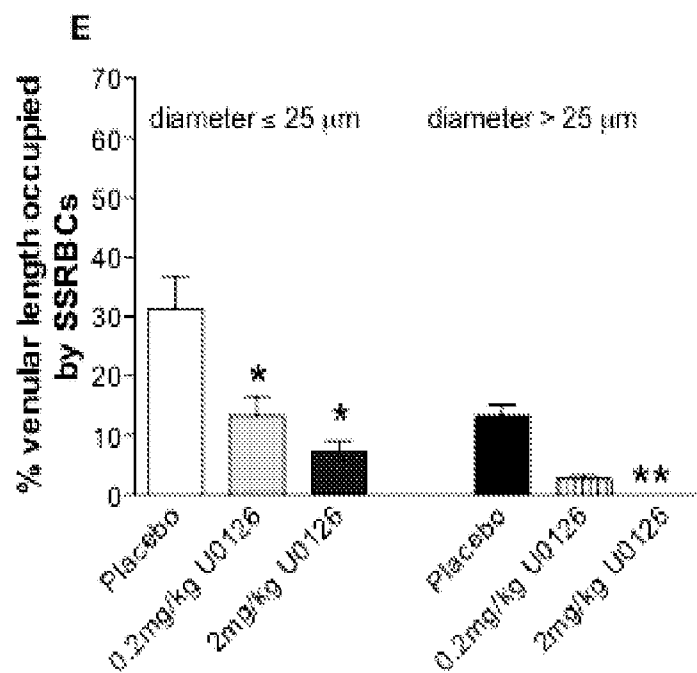


Fig. 9E

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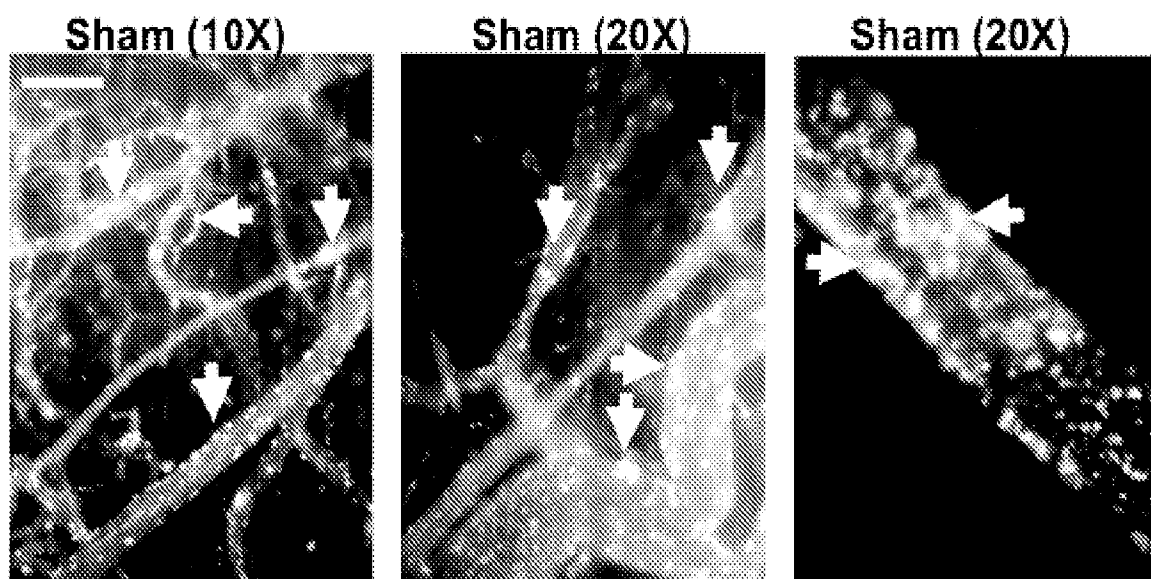


Fig. 10A

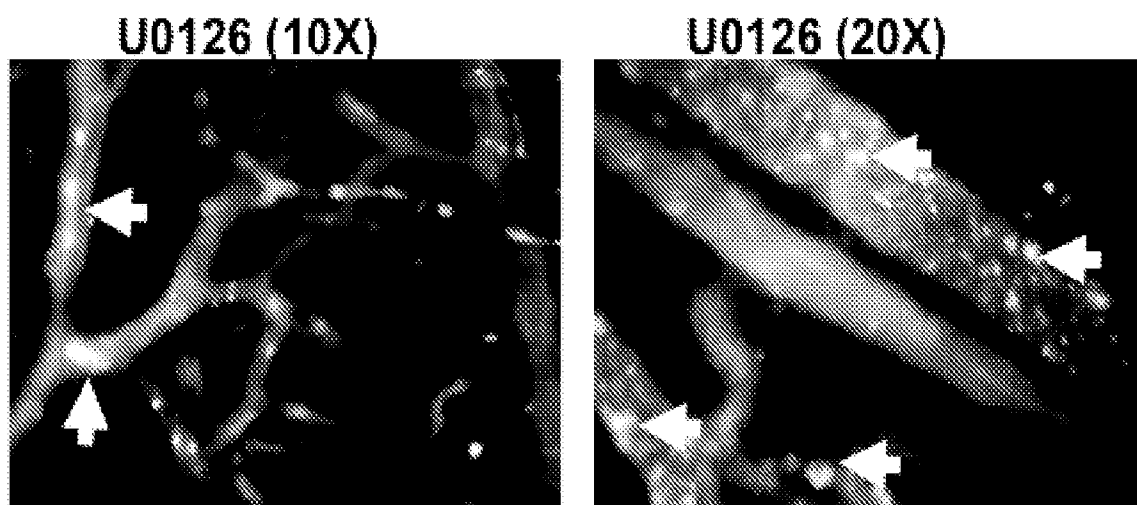


Fig. 10B

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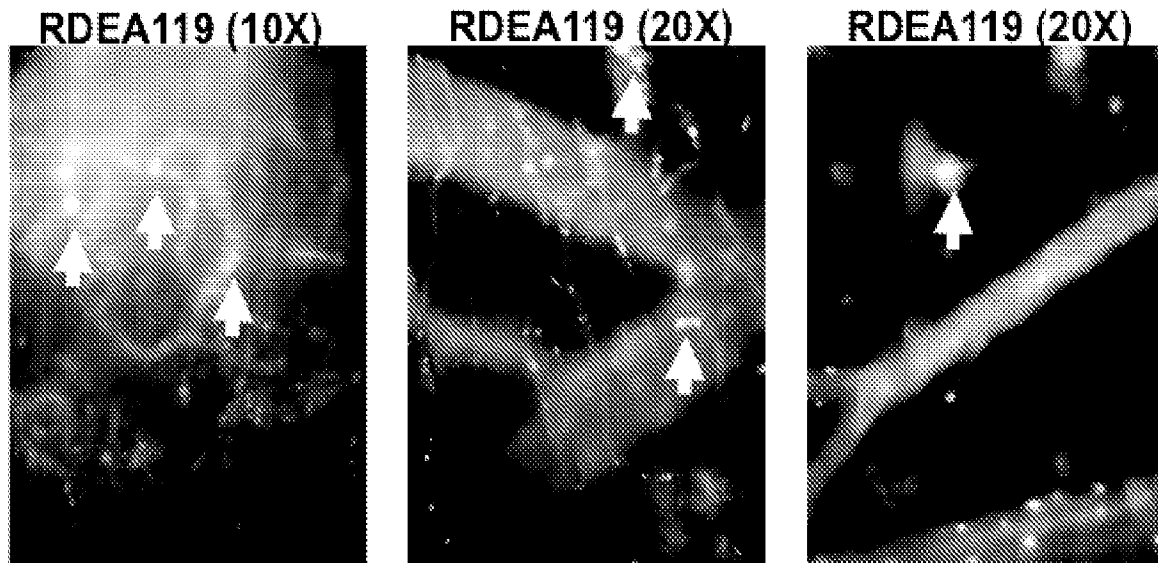


Fig. 10C

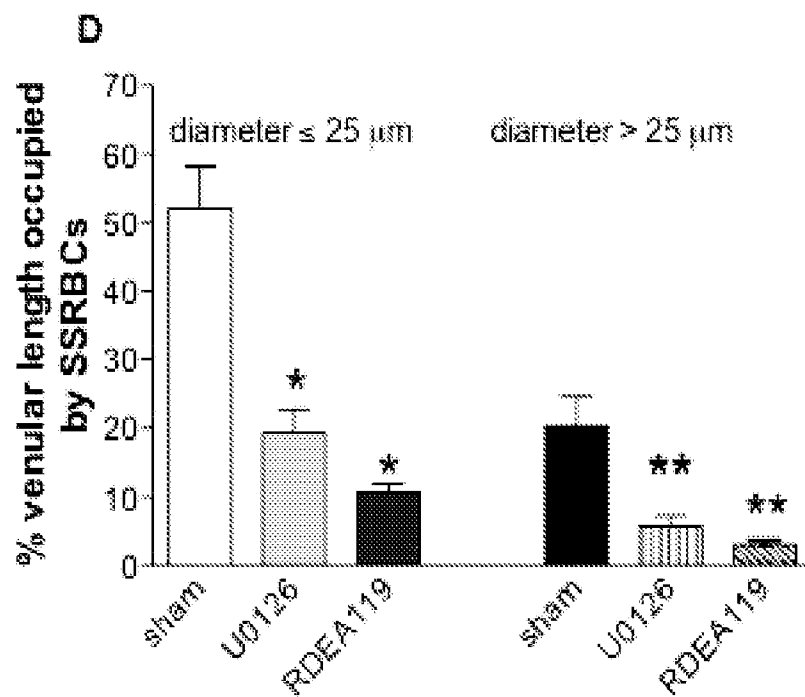


Fig. 10D

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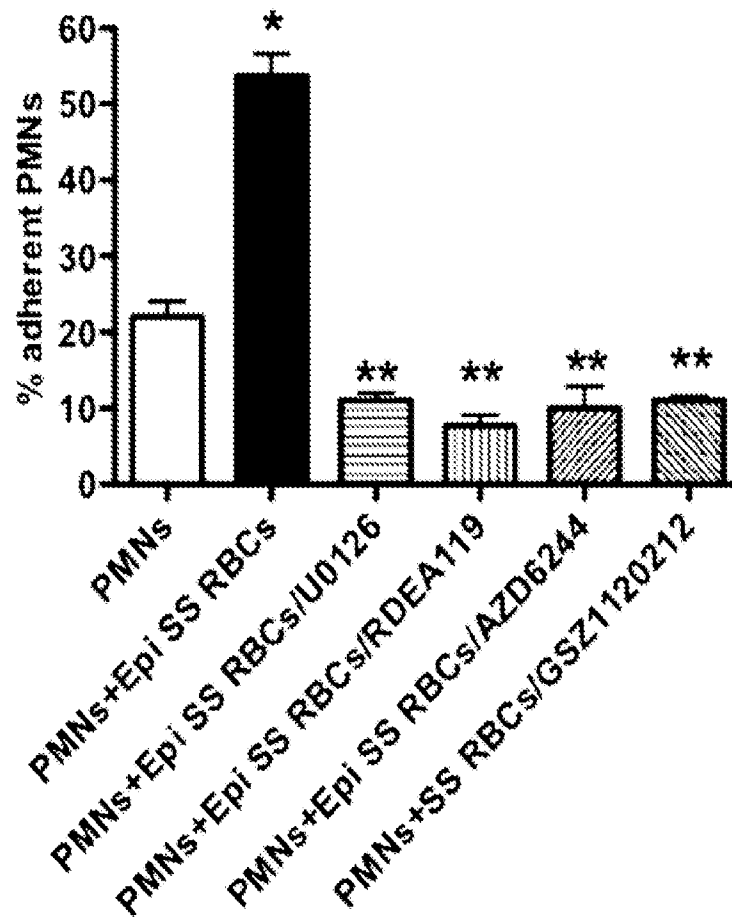


Fig. 11

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Fig. 12A

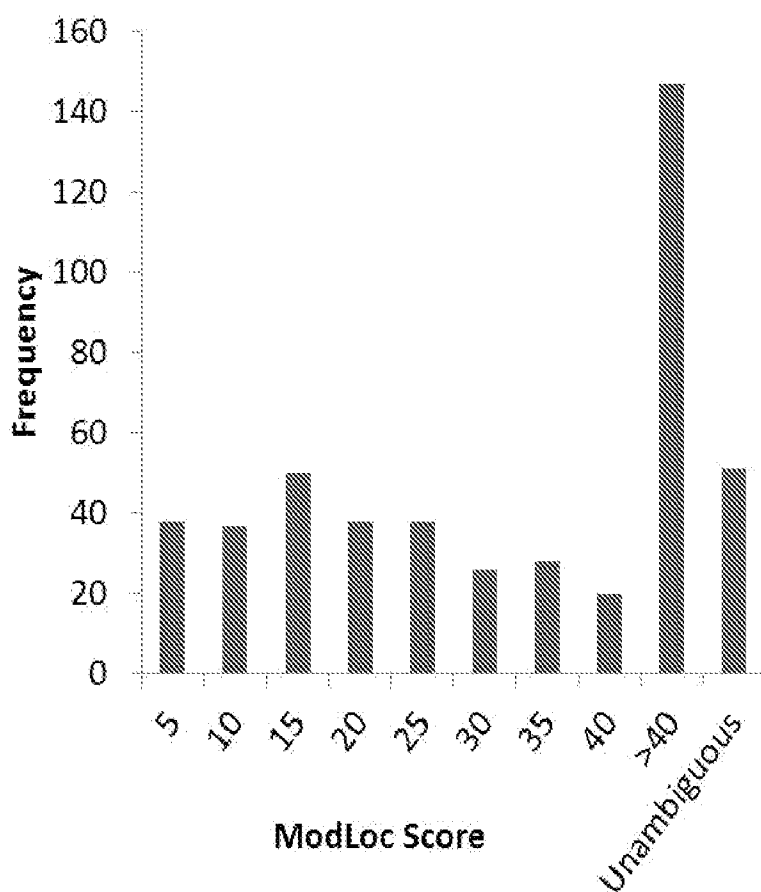
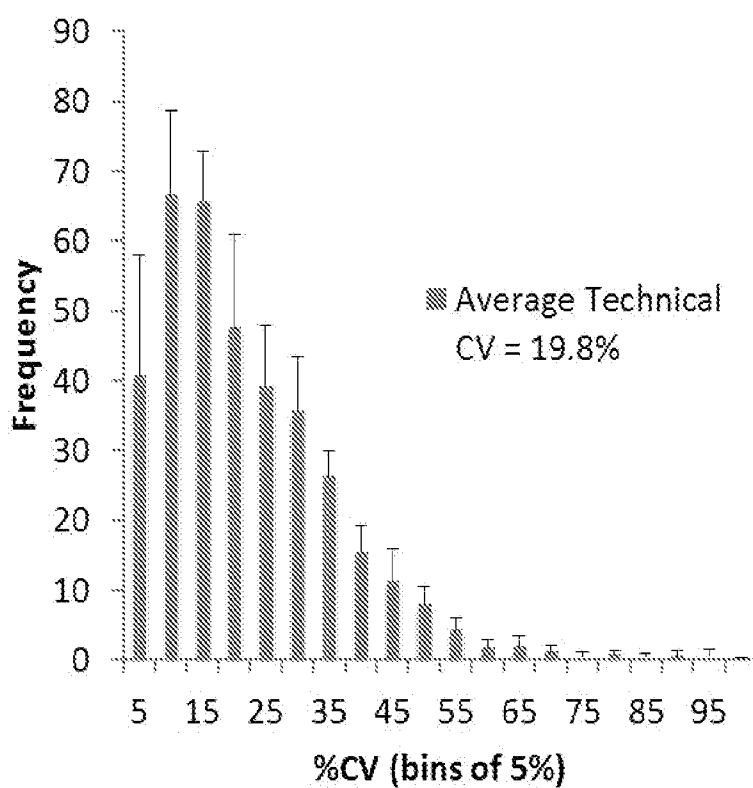


Fig. 12B



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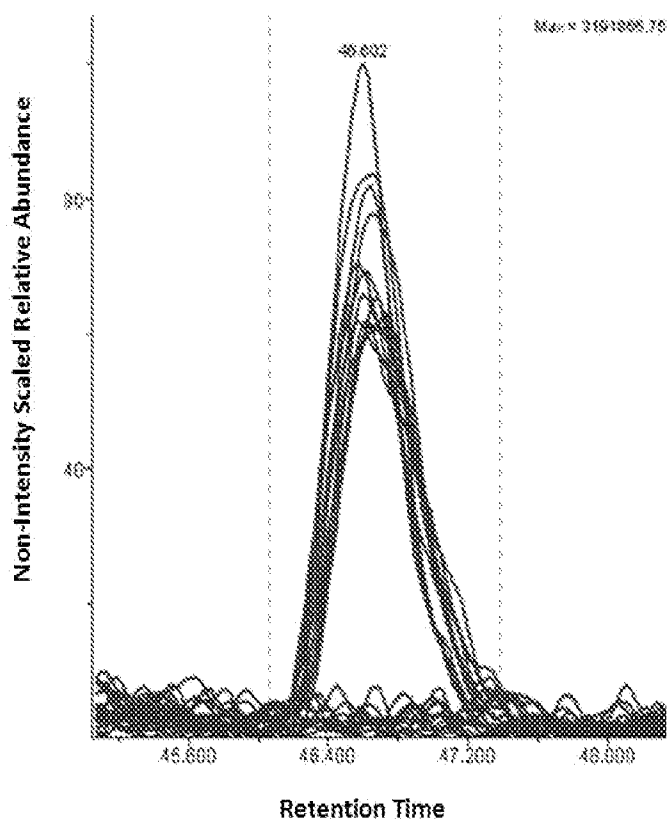


Fig. 13A

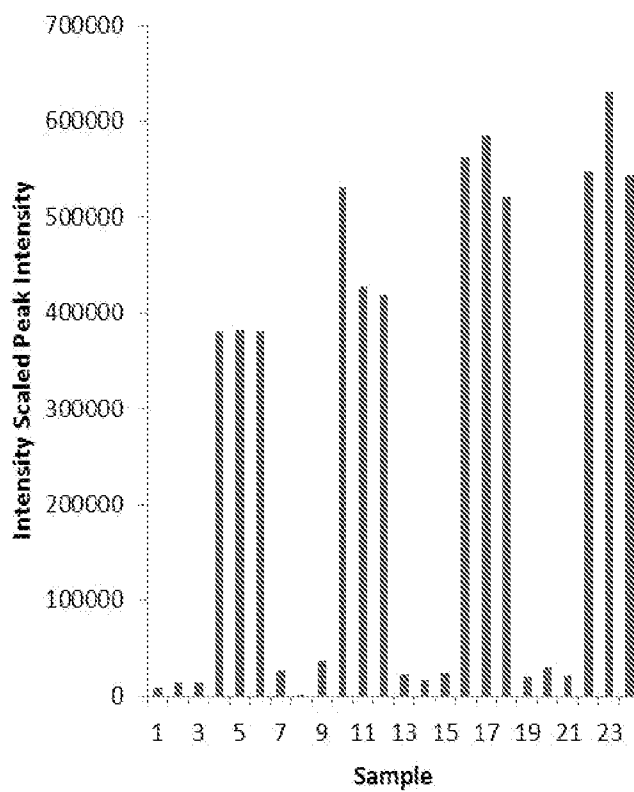


Fig. 13B

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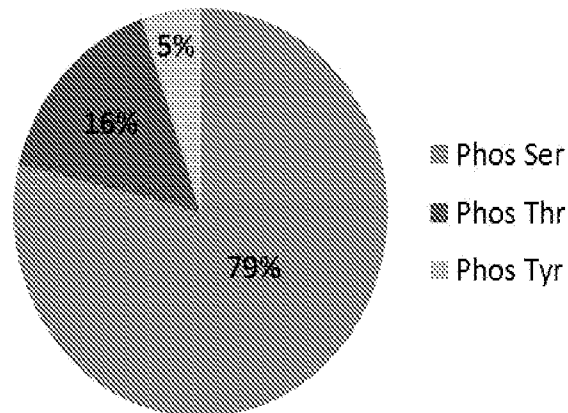
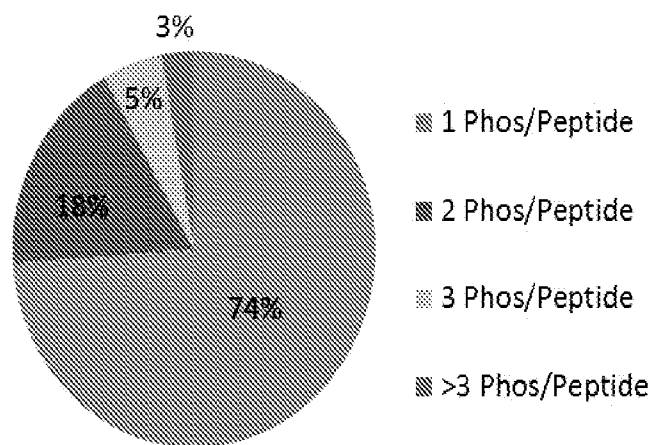
Phosphorylated Residue Distribution**Phosphorylated residues per Peptide**

Fig. 14A

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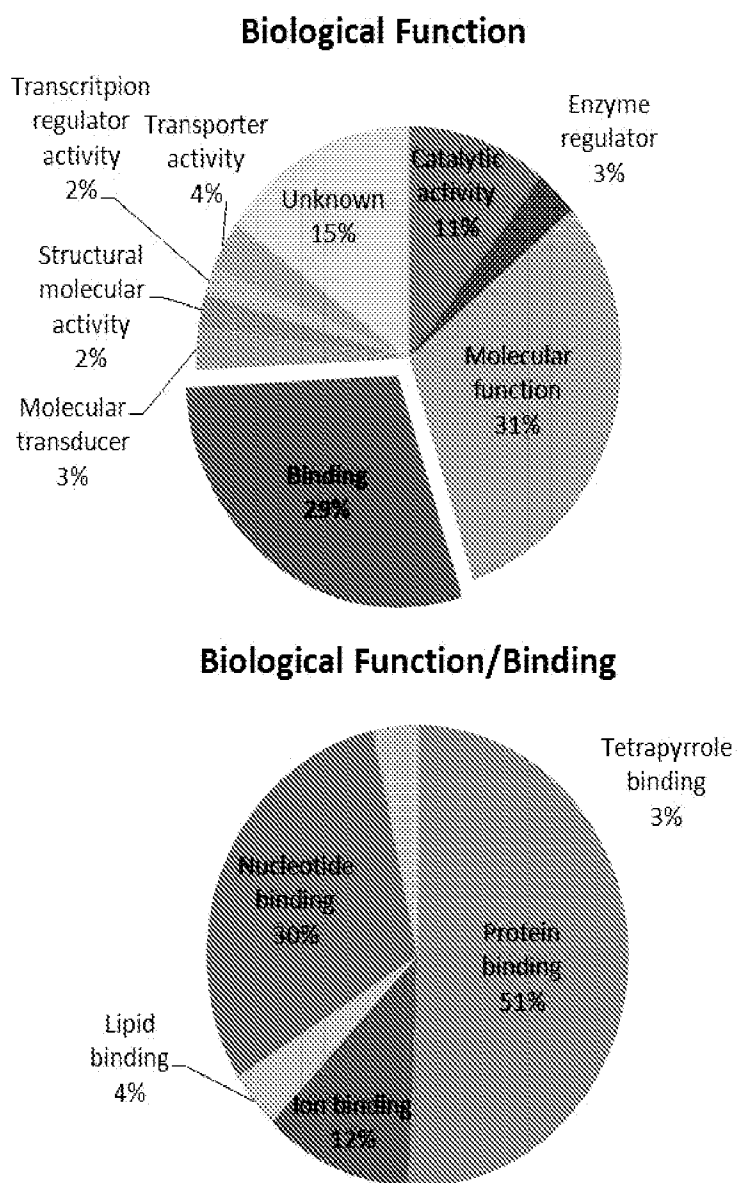


Fig. 14B

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Fig. 15A

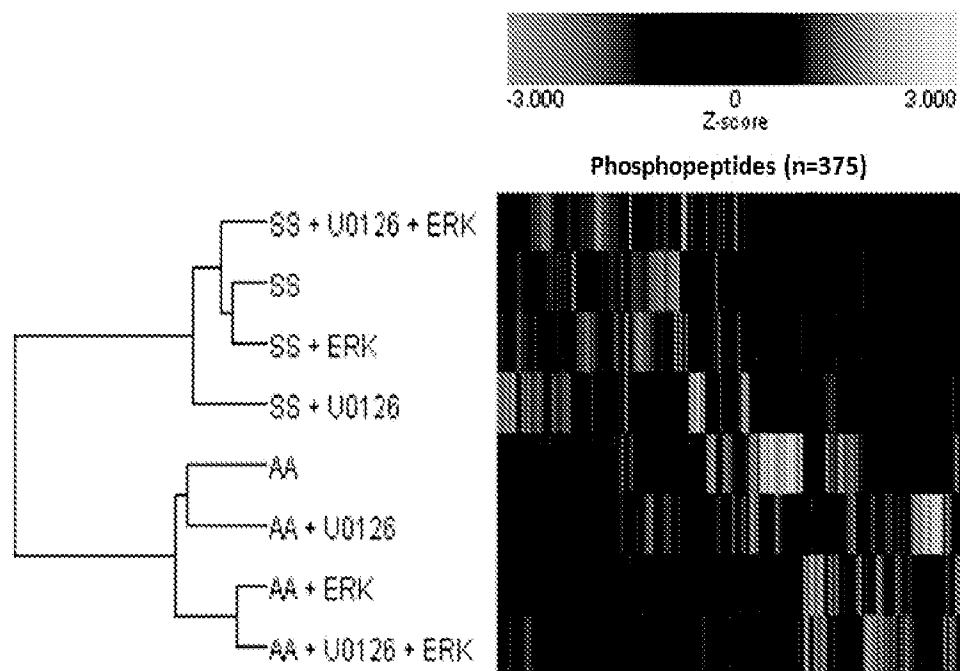
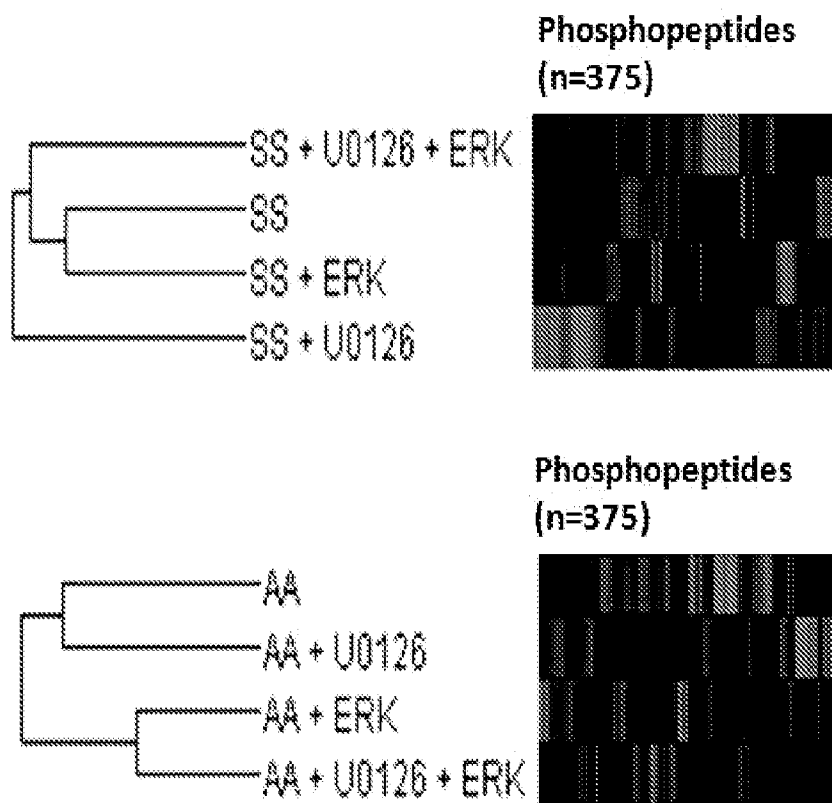


Fig. 15B



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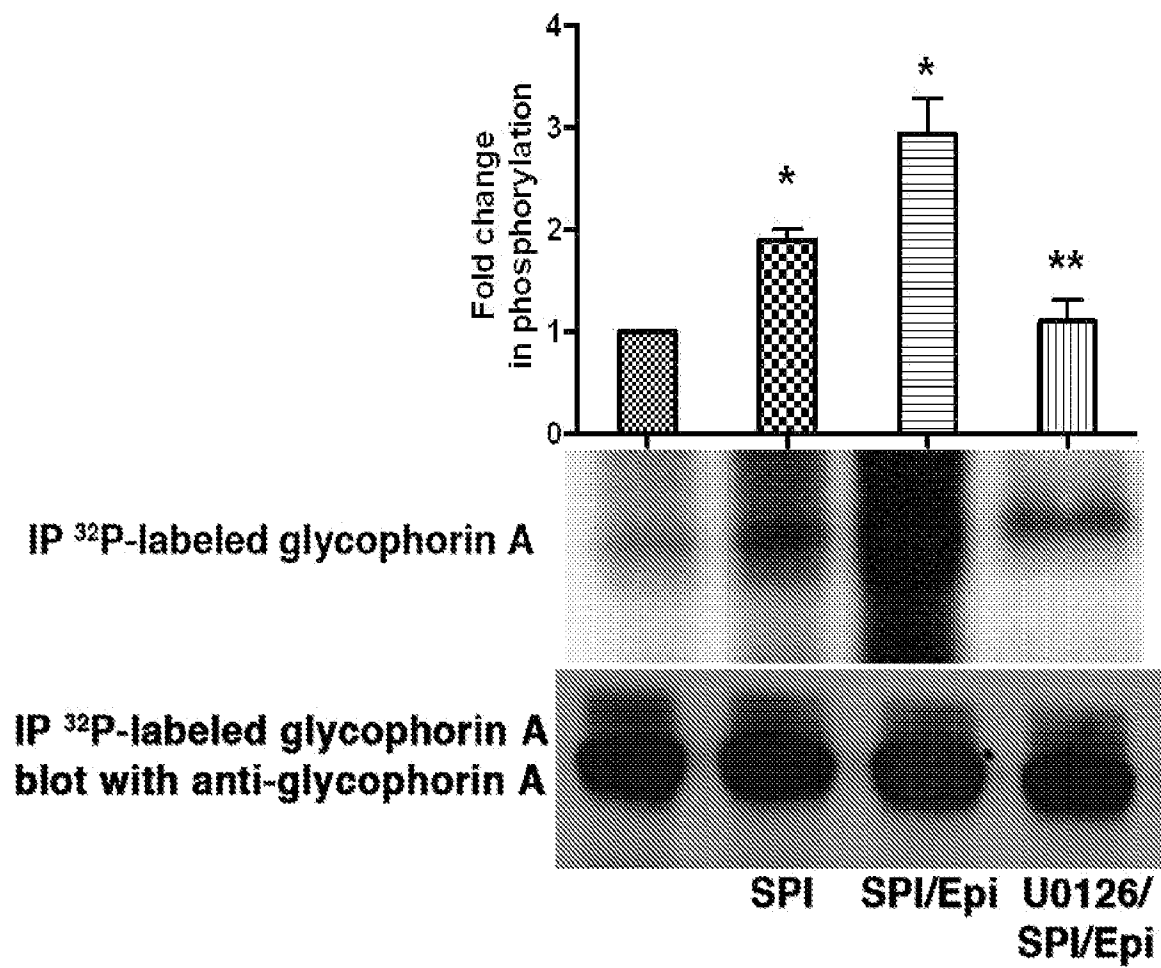


Fig. 16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/35837

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/18, A61P 7/00 (2012.01)

USPC - 514/341

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 514/341

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/349, 514/601.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST -- PGPB,USPT,USOC,EPAB,JPAB; Dialog Classic Files -- 654, 652, 349, 348, 340, 35, 65, 155; USPTO Web Page; Entrez Pubmed; Google Scholar; Search terms -- sickle cell RBCs, sickle cell anemia, symptoms, pain alleviation, MEK kinase, ERK, Raf kinase, inhibitors, cell adhesion, leukocytes, endothelial cells, aggregation, vaso-occlusive disease

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0286178 A1 (IBRAHIM et al.) 11 November 2010 (11.11.2010) para [[0013], [0015], [0018], [0046], [0048]-[0054]	1-4, 9, 14, 15/14
Y	US 2007/0049591 A1 (PINKERTON et al.) 01 March 2007 (01.03.2007) para [0066]-[0068], [0537], [0578]	5-8, 10-13, 15/(10-13)
Y	SELVARAJ et al., Mechanism of monocyte activation and expression of proinflammatory cytokines by placenta growth factor. Blood, 10 April 2003, Vol.102, No 4, pages 1515-1524. Especially pg 1515, abstract, col 2, para 1; pg 1521, col 1, para 2 -- col 2, para 1; pg 1523, col 2, para 2, 3	5-8, 10-13, 15/(10-13)
Y	POLANOWSKA-GRABOWSKA et al., P-Selectin-Mediated Platelet-Neutrophil Aggregate Formation Activates Neutrophils in Mouse and Human Sickle Cell Disease. Arteriosclerosis, Thrombosis and Vascular Biology, 10 November 2010, Vol. 30, No 12, pages 2392-2399. Especially pg 2395, col 2, para 3; pg 2397, col 2, para 1; pg 2398, col 1, para 1, 2	7, 8, 12, 13, 15/12,13
X, P	US 2011/0293558 A1 (SURESH et al.) 01 December 2011 (01.12.2011) para [0016], [0073], [0189]	5-15

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 July 2012 (23.07.2012)

Date of mailing of the international search report

16 AUG 2012

Name and mailing address of the ISA/US

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Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/35837

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 16-21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.