Supplement 1

Additional Methods:

Isoelectric focusing analyses. Isoelectric focusing (IEF) analysis of different Townes mice blood samples were carried out using RBC lysates on a precast IEF agarose gel (pH 3-10 IEF Gel, Thermo Scientific). The gel was electrofocused using Novex® power supply at 100 V for 1 hr, 200 V for 1 hr, and 500 V for 30 minutes.

Preparation of microparticles from mouse blood. For the majority of experiments, MPs were generated by shear stress from RBCs (18). Heparinized whole blood from either individual animals or from a group of 3 animals was pooled for the preparation of RBC-MPs. Briefly, RBCs were separated from the whole blood on the next day following blood draw and were washed 3 times with Ca2+/Mg2+ free PBS by centrifugation at 500Xg for 10 min. In each wash, the top $1/4^{th}$ layer was carefully discarded using a glass Pasteur pipette to remove any residual WBCs and platelets. Washed RBCs were then passed rapidly through 25G needle (length 2 inch) using a syringe for a total of 15 times. RBCs were washed with PBS by centrifugation at 2,500Xg for 10 min and the supernatant, rich in RBC derived MPs was collected to a new tube. MP-rich supernatant was again centrifuged at 2,500Xg for 10 min to remove any residual RBCs. Cell-free supernatant was then centrifuged for 1hr at 25,000Xg to isolate RBC derived MPs. The MP pellets obtained were washed two times by centrifugation at 25,000Xg with Ca2+/Mg2+ free PBS. MP suspensions were aliquoted and snap-frozen immediately in liquid nitrogen and kept at -80°C for further use.

For some proteomic experiments, plasma derived (circulating) MPs were also collected by a procedure involving differential centrifugation. Circulating MP-rich supernatant was obtained from plasma pooled from blood obtained from a group of 5 mice. First, plasma samples were

centrifuged at 2,500Xg for 10 min at 4°C. The supernatant was centrifuged for 1hr at 25,000Xg to isolate circulating MPs. MP pellets obtained were washed by centrifugation at 25,000Xg with Ca2+/Mg2+ free PBS. Finally, MP suspensions were aliquoted and snap-frozen immediately in liquid nitrogen and kept at -80°C for further use.

Flow cytometric and electron microscopic analysis of RBC microparticles. RBC-derived MPs (ranging in size between 100-300nm in diameter) bearing phosphatidylserine (PS+) on the surface were isolated from Townes mice blood and counted by flow cytometry using FITC-labelled Annexin V. Isolated MPs were also visualized through transmission electron microscopy (TEM). Cryo-TEM was performed using a cryo-transmission electron microscope (Jeol 1400 TEM/STEM) equipped with a LaB6 gun. The temperature of the liquid ethane was maintained at −175 °C. Samples were transferred to a Cryo-TEM holder (Gatan 914) and observed in a pre-cooled cryo-TEM at 120 kV using minimum dose system mode. Images were recorded with a digital CCD Camera (Gatan ORIUS™ SC1000).

Reverse phase HPLC analyses of hemoglobin oxidative changes within microparticle oxidation. RP-HPLC analyses were performed on a Zorbax 300 SB C3 column (4.6x250 mm) using a Waters HPLC system consisting of a Waters 626 pump, 2487 dual-wavelength detector and a 600-controller installed with Empower 2 software (Waters Corp, Milford, MA). Microparticles were mixed with water to induce lysis and the lysates were centrifuged at 13,000 rpm for 5 minutes, and then the supernatant (Hb ~20 μg) in 25 μL of water was loaded on the C3 column equilibrated with 35% acetonitrile containing 0.1% TFA. Globin chains were eluted with a gradient of 35-50% ACN within 100 min at a flow rate of 1 mL/min. The eluate was monitored at 280nm for globin chains and at 405 nm for the heme components (13).

Autoxidation experiments. Quantification and determination of the oxidation state within MPs was achieved by using Nanodrop 1000 spectrophotometer together with use of conventional spectrophotometers. Spectral changes were measured using a highly specialized double beam spectrophotometer (Nanodrop 2000c, Thermo Scientific), which measures light intensity ratios (ideally suited for MP solutions) and is therefore not as sensitive to fluctuations in the light source or detector. Absorbance changes in the range of 350–700 nm due to the spontaneous oxidation of Hbs were recorded every 10 minutes in a photodiode array spectrophotometer (HP 8453). Autoxidation experiments were carried out by incubating 40 μM Hb (heme) samples in PBS, pH 7.4 under air in sealed at 37 °C for 24h. Multicomponent analysis was used to calculate, the ferrous (oxy), met and ferryl based on extinction coefficients of each species (22).

Biochemical analysis of RBC microparticles. For proteomic, biochemical and spectroscopic analysis, MPs were compared following measurement of protein concentration by the standard BCA method (Thermo Scientific). Protein and lipid contents of the MPs were analyzed as markers of oxidative modifications. Protein carbonylation was monitored spectrophotometrically following derivatization with DNPH (2,4-dinitrophenylhydrazine) using a kit purchased from Cayman Chemical (Ann Arbor, Michigan). Lipid hydroperoxides were extracted from the lipid fractions of isolated MPs using chloroform and were measured by a commercial kit purchased from Cayman Chemical.

Preparation of ferric and ferryl hemoglobins. Ferric (met) Hb was generated as previously reported (22) by incubating 2 mM ferrous (oxy) Hb with 3 mM potassium ferricyanide in PBS at room temperature for 15 mins. Excess ferro- and ferricyanide were removed by passing the reaction solution through a Sephadex G-25 column equilibrated with PBS at 4°C. Ferryl Hb solutions were prepared as previously reported by mixing metHb (1 mM) with H_2O_2 (20 mM) in

PBS buffer at room temperature for 1 minute. Excess H_2O_2 was rapidly removed by passing the reaction solution through two G25 desalting columns (HitrapTM, 5 mL, GE Healthcare) connected in series (13). Ferryl Hb was also verified by derivatization of freshly prepared ferryl Hb with sodium sulfide (Na_2S) to generate more stable sulfHb (22).

Hydrogen peroxide-mediated oxidation of hemoglobin and the effects of hydroxyurea. H_2O_2 induced ferryl Hb species formation was evaluated following previously-described methods (22). HU (240 μM) was mixed with HbS (60 μM) in a cuvette and incubated for 15 min at 37 °C. The mixture was then brought to room temperature and incubated with increasing concentrations of H_2O_2 (0, 60 μM, 150 μM, 300 μM, or 600 μM) for 5 min at 25°C. Catalase (200 units/mL) was added to the solution to terminate the oxidation reactions. Absorbance spectra between 350 and 700 nm were measured to monitor the reactions. For verification of the ferryl intermediate, 2 mM Na_2S was added immediately to derivatize the ferryl heme into sulfHb. SulfHb concentration was monitored by the appearance of an absorbance band at 620 nm. The concentration of sulfHb was calculated using an extinction coefficient of $\varepsilon 620nm = 24.0\ mM^{-1}cm^{-1}$ (66).

Western blotting and co-immunoprecipitation. The primary antibodies used were in 1:2500 dilutions. The proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). The expression of HO-1, ubiquitin, band 3, α and β subunit of Hb were detected with specific monoclonal antibodies. Monoclonal mouse anti HO-1 antibody (Catalog No ab13248) and rabbit polyclonal anti-actin antibody (Catalog No ab1801) were purchased from Abcam. Monoclonal rabbit antibody against α- subunit of Hb (Catalog No MABF745) were purchased from EMD Millipore. Polyclonal rabbit anti-ubiquitin antibody (Catalog No 3933S) and rabbit monoclonal hemoglobin β antibody (Catalog No 84934) were purchased from Cell Signaling Technology. Mouse anti-band 3 antibody was purchased from Sigma Aldrich (Catalog

No. B9277-.2ML). For co-immunoprecipitation of mouse band 3 protein, a polyclonal rabbit anti-band 3 antibody was purchased from United States Biological (Catalog No 303147-100ug). For detection of the protein bands appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma Aldrich. For quantification, the density of HO-1 was normalized with density of β-actin as previously reported (62).

Co-immunoprecipitation assays of ubiquitin or band 3 were done using a commercial protein G immunoprecipitation kit purchased from Sigma Aldrich. Briefly, the samples were pre-cleared using protein G agarose beads for 2hr at 4°C and then incubated overnight at 4°C with appropriate bait antibodies (band 3 or ubiquitin) in 0.5X IP buffer supplied in the protein G immunoprecipitation kit (Sigma-Aldrich, MO). The beads were then washed 4 times with 1X IP buffer and finally 2 times with PBS. Proteins were eluted from the protein G agarose beads using 100 µl NuPage LDS sample buffer containing reducing agent (Thermo Scientific). Eluted immunoprecipitates were resolved by SDS–PAGE using 4-12% bis-tris gels and analyzed for Hb protein binding by western blotting as described earlier. For ubiquitin Co-IP experiments, whole washed RBCs were incubated for 4 hr at room temperature with proteasome inhibitor MG 132 (10µM) and deubiquitinating enzyme inhibitor N-ethylmalemide (NEM, 1 mM). Following incubation, RBCs were washed two times with PBS before lysis.

LCMS analysis of band 3 co-immunoprecipitation. Proteins in band 3 immunoprecipitates resolved by SDS–PAGE were subjected to mass spectrometric analysis using LCMS. Gel slices were excised and digested in gel with proteomics grade trypsin (Roche). The tryptic peptides were extracted from the gel (www.rockefeller.ede/ms_inGelDigestion). Salts and other contaminants that might interfere with mass spectrometric analysis were removed using C18 ZipTips. The purified tryptic digests were analyzed using A Waters Xevo QTOF mass spectrometer coupled to

a NanoAcquity UPLC system. Peptides were separated using a 75µm x 100 mm, 130 Å, 1.7 µm nanoAcquity BEH C18 columns. LCMS data files were converted to .mgf file format using Mascot Distiller and searched against an IPI database that was appended with amino acid sequences from Hemoglobin variants (69,025 sequences). Search parameters were set o 0.1 Da for peptides and 50 ppm for MSMS data. Variable modifications included oxidation of methionine and carbamidomethylation at cysteine. One missed tryspin cleavage was allowed. Protein identifications were considered positive identifications if the contained at least two peptides with scores above the Mascot identity threshold.in at least one equivalent gel band.

Vascular endothelial cell culture. Cryopreserved human umbilical vein endothelial cells (HUVEC) were obtained from Thermo Fisher Scientific and cultured in M200 media supplemented with Low Serum Growth Supplement (LSGS, Thermo Fisher Scientific) without any antibiotics. The cells were used between passages 5-10 after thawing. The media was changed every 48 hr. For some experiments serum- free media was used where M200 media was supplemented with all components of the LSGS kit (Thermo Fisher Scientific) except the FBS.

Exposure of HUVEC cells to red cell derived microparticles. HUVEC cells were grown to 80-90% confluency in complete media. Before exposures, the cells were serum starved for 6 hr. The cells were then exposed to different RBC derived MPs isolated from Townes mice blood (1000 MP/ µl) for varying periods of time up to 12hr.

Assessment of endothelial damage by transwell assay and apoptotic activation. Loss of endothelial monolayer integrity is considered as an important marker of endothelial functional impairment. Endothelial permeability change was studied by monitoring passage of FITC-labelled high molecular weight dextran (40 KD) through an HUVEC monolayer grown on a specialized

collagen coated PTFE-Transwell membrane inserts (Corning Inc. Corning, NY) placed over a 24-well culture plate as described before (60). Passage of FITC-dextran across the Transwell membrane was monitored after 12hr incubation with Townes-SS or Townes-AA RBC-MPs by measuring the FITC fluorescence in the lower chamber media using Synergy HTX Multi-Mode Reader (Biotek Instruments, Inc., Winooski, VT). In a similar set of experiments HUVECs were also grown on glass coverslips for 5-6 days to form tight monolayers. Cells were incubated with equal numbers of shear stress MPs for 12 hr. Following MP exposure, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% tritonX-100 and then endothelial membrane integrity was observed using an antibody against endothelium specific VE-cadherin (Cell Signaling Technology, Catalog No 2158) by the immunofluorescence technique described earlier (13). Loss of endothelial membrane integrity was visualized under EVOS fluorescence microscope (Thermo Fisher Scientific). Percentage of apoptotic cell population in MP challenged HUVEC was counted by Nexcelom Automated Cell Counter using annexin V and propidium iodide staining as per manufacturer recommended protocol (Nexcelom Bioscience).

Mitochondrial bioenergetic and glycolytic flux measurements with Seahorse Analyzer. Endothelial bioenergetic function and glycolytic flux were monitored in intact HUVEC cells by measuring mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) respectively using a XF24 extracellular flux analyzer (Agilent-Seahorse Bioscience, Billerica, MA). Briefly, HUVEC cells were seeded (50,000cells/well) and cultured for 24hr in a specialized 24-well XF cell culture plate (V7) obtained from Seahorse Bioscience. Prior to the exposure, cells were cultured in serum-free media overnight. The cells were then exposed to different MPs for 12hr. Assay for measuring mitochondrial OCR was done using XF-assay media (Seahorse Bioscience, Billerica, MA) supplemented with 10mM glucose, 5mM pyruvate and 2mM

glutamate. The assay media used for measuring the extracellular acidification rate (ECAR) was free of glucose. Complete OCR profile was obtained through a sequential automated injection of oligomycin (1μM), carbonyl cyanide-p-trifluoro-methoxyphenylhydrazone (FCCP, 1μM) and a combination of rotenone and antimycin A (1μM each) to the assay media. Similarly, glucose (10mM), oligomycin (1μM) and glycolytic inhibitor 2-deoxyglucose (2-DG, 100mM) were sequentially added to the wells to obtain the ECAR profile. Endothelial OCR and ECAR from individual wells were recorded and plotted using Seahorse XF24 software, version 1.8 as per manufacturer's instructions and as described before (13, 60). Various cellular bioenergetic parameters were calculated from average rate data obtained from individual wells as described before (67, 68). Briefly, basal OCR was calculated by subtracting rotenone/antimycin inhibited non-mitochondrial OCR from the initial OCR recorded before addition of oligomycin. Mitochondrial reserve capacity was calculated as the difference of OCR before oligomycin addition and FCCP induced maximum OCR. Glycolytic capacity was measured as the maximum ECAR obtained by the addition of oligomycin which effectively shuts down oxidative phosphorylation and drives the cell to use glycolysis to its maximum capacity.

Proteomic analysis of microparticles. Prior to MS analysis, the total protein concentration of all RBC derived MPs was determined using a standard BCA assay. Afterward, samples were tryptically digested, desalted, and analyzed by mass spectrometry as previously described (69). Briefly, 1 ug of tryptic peptides representing each sample were analyzed (three technical replicates for each sample) by reverse phase liquid chromatography mass spectrometry (RP LC/MS/MS) using an Easy nLC II Proxeon nanoflow HPLC system coupled online to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Data were acquired using a top10 method (for 120 minutes), dynamically choosing the most abundant precursors (scanned at 400-2000 m/z) from the

survey scans for HCD fragmentation. All quantitative data processing and visualization was performed using Protalizer DDA software (Vulcan Analytical, Birmingham, AL) version 1.1.2418 (70). Peptide and protein identifications were made using the X! Tandem Piledriver search engine version 2015.04.01.1 (71) against the forward and reversed human Swiss-Prot database containing 20,202 sequences (not including decoys) downloaded July 10th 2016 with a 20 ppm parent and fragment ion mass tolerance. MS1 and MS2 peaks were calibrated by the median error values for all peptide precursor and fragment ions within each 25 *m/z* mass range bin across the entire scan range. The calibrated spectra were then researched with a more stringent tolerance of 10 ppm parent and 15 ppm fragment ion mass tolerance. Global modifications searched included oxidation of M residues, deamidation of Q and N residues, pyro-glutamic acid at N-terminal E and Q residues, N-terminal acetylation, tri-oxidation to cysteic acid at C residues, ubiquitination at K residues and phosphorylation at S, T and Y residues. Carbamidomethylation of cysteine residues was searched as a static modification. Peptides with up to 1 trypsin miscleavages were included in the analysis. Only peptides detected at a 1% protein false discovery rate (FDR) were reported by the algorithm based on a target-decoy search strategy comparing the number of decoy reversed identifications to those made in the actual human database. Quantification by MS1 precursor AUC intensities was performed as described previously (70). For calculating protein-level relative abundance across the biological conditions compared, peptides detected in each sample were used whenever possible. Median peptide relative abundance and P-values from an unpaired t-test were reported for all the peptides used to quantify a target protein in the protein quant summary.

Statistical analysis. All values are expressed in the bar diagrams as mean ± SEM or as box plots where the upper horizontal line represents 75th percentile, lower horizontal line represents 25th percentile, horizontal line within the box represent mean value. Vertical error bars represent

95% confidence interval. Statistical calculations were done using Student's t-test, two-tailed, where $P<0.05$ was considered significant between groups. All calculations and data plotting were done using Graph Pad Prism 7 software. For the proteome analysis represented by volcano plots, all peptide-spectrum-matches were obtained using a strict 1% protein false discovery rate (FDR).