Hematopoietic Protein-1 Regulates the Actin Membrane Skeleton and Membrane Stability in Murine Erythrocytes

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Abstract

Hematopoietic protein-1 (Hem-1) is a hematopoietic cell specific member of the WAVE (Wiskott-Aldrich syndrome verprolin-homologous protein) complex, which regulates filamentous actin (F-actin) polymerization in many cell types including immune cells. However, the roles of Hem-1 and the WAVE complex in erythrocyte biology are not known. In this study, we utilized mice lacking Hem-1 expression due to a non-coding point mutation in the Hem-1 gene to show that absence of Hem-1 results in microcytic, hypochromic anemia characterized by abnormally shaped erythrocytes with aberrant F-actin foci and decreased lifespan. We find that Hem-1 and members of the associated WAVE complex are normally expressed in wildtype erythrocyte progenitors and mature erythrocytes. Using mass spectrometry and global proteomics, Coomasie staining, and immunoblotting, we find that the absence of Hem-1 results in decreased representation of essential erythrocyte membrane skeletal proteins including z- and b- spectrin, dematin, p55, adducin, ankyrin, tropomodulin 1, band 3, and band 4.1. Hem-1/- erythrocytes exhibit increased protein kinase C-dependent phosphorylation of adducin at Ser724, which targets adducin family members for dissociation from spectrin and actin, and subsequent proteolysis. Increased adducin Ser724 phosphorylation in Hem-1/- erythrocytes correlates with decreased protein expression of the regulatory subunit of protein phosphatase 2A (PP2A), which is required for PP2A-dependent dephosphorylation of PKC targets. These results reveal a novel, critical role for Hem-1 in the homeostasis of structural proteins required for formation and stability of the actin membrane skeleton in erythrocytes.

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Introduction

Erythrocytes are unique cells in that they have a biconcave shape, can repeatedly deform and regain their shape, and can withstand turbulence and shear forces exerted upon them by fine capillary vessel walls [1]. These complimentary features of strength and flexibility are intrinsic to the interplay between their fluid cell membrane and the resilient yet pliable lattice structure of the membrane skeleton [1,2]. The importance of maintaining a strong yet flexible erythrocyte membrane skeleton is underscored by the fact that the majority of erythrocyte disorders, which are collectively the most common inherited disorders in humans worldwide [1], are caused by mutations in genes encoding essential membrane structural proteins.

Numerous studies have resulted in the identification of a set of core components of the erythrocyte membrane skeleton that allow for their unique mechanical characteristics. The plasma membrane consists of a phospholipid and cholesterol bilayer interspersed with critical transmembrane proteins such as band 3 and glycoporphins A and C (see [3] for review). These transmembrane proteins form two major intracellular complexes called the “Ankyrin Complex” (which links membrane bound glycoporphin A, band 3, Rh, and CD47 to intracellular ankyrin and protein 4.2) and the “Junctional Complex” (which links transmembrane glycoprotein C, band 3, Rh, and Glut1 to intracellular dematin, P55, protein 4.1, tropomodulin, tropomyosin, and actin). The ankyrin and junctional complexes further form “vertical” attachments or bridges to the underlying membrane skeleton, which consists of a precise hexagonal lattice of complexes containing short filaments of 12–18 actin monomers connected by longer flexible helices of z- and b- spectrin tetramers [2,3,4,5]. The associations between spectrin and actin with the junctional and ankyrin complexes are critical for allowing erythrocytes to maintain their shape and to withstand physical forces associated with transport in circulation. Disruption of these intricate interactions can result in erythrocyte fragmentation, removal by the spleen, and hemolytic anemia [1].

Because of its central location and multiple interactions with membrane skeletal proteins, the appropriate polymerization and organization of actin is paramount to maintaining the strength and deformability of erythrocytes. The formation of actin filaments (F-actin) is regulated by the actin regulatory complex (ARP2/3), which stimulates monomeric globular actin (G-actin) to polymerize at both the fast growing barbed end and slower
Hem-1 Regulates the Erythrocyte Actin Skeleton

Materials and Methods

Mice

Hem1−/− mice were generated through ENU mutagenesis as previously described [16]. Hem1−/− mice are phenotypically identical to wildtype mice and hence were used as normal controls. Rag2−/− mice were obtained from Taconic Farms, Incorporated (Hudson, NY) and maintained in a breeding colony at the University of Washington. Mice were housed under Specific Pathogen Free conditions. All mouse procedures were performed in accordance with The Guide for the Care and Use of Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at the University of Washington, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International.

Peripheral Blood Smear Evaluation and Erythrocyte Counting

Peripheral blood smears were stained with differential stain (Dip-Quick, Jorgensen Laboratories, Loveland, CO) and examined at ×600 magnification on a Nikon Eclipse 80i microscope (Nikon, Incorporated, Melville, NY). Images were captured with a Digital Sight DS-U1 camera; adjustments to image size and settings were made using NIS Elements Basic Research 3.0 software (Laboratory Imaging, Limited; Prague, Czechoslovakia).

Flow Cytometry

Vybrant CFDA SE Cell Tracer Kit carboxyfluorescein succinimidyl ester (CFSE) and Alexa Fluor 488 phalloidin were from Invitrogen (Eugene, OR). Biotin anti-mouse TER-119/2/3 mouse cell was from Pharmingen (BD Biosciences, San Diego, CA); GR-1 and CD61 antibodies were from eBioscience (San Diego, CA). Erythroblasts (CD11b+CD61−TER119+) and megakaryocytes (CD11b+CD61+TER119+) were FACs-sorted from total bone marrow for real-time PCR analyses.

For erythrocyte development analyses, flow cytometry was performed on total bone marrow or splenocytes freshly harvested from mice, without lysis of red cells. Phycocyanin-conjugated rat anti-mouse CD44 was obtained from BD Biosciences (no. 553134) and biotinylated anti-mouse TER119 was obtained from Pharmingen (no. 090B2D). Cells were stained in DPBS/3% fetal bovine serum (FBS) for 30 min on ice, washed once in DPBS/3% FBS, stained with TRI-COLOR streptavidin (Invitrogen, no. SA1006) in DPBS/3% FBS for 30 min on ice, washed twice in DPBS/3% FBS and detected using a FACScan flow cytometer (BD Biosciences). Live Ter119+ cells were gated and separated into developmental stages based on cell size (FSC) and expression of CD44. Data were analyzed using FloJo v. 8.6.1 software (Treestar Inc, Ashland OR).

Purification of Erythrocytes from Whole Blood

Whole blood was obtained from mice immediately following euthanasia via CO2 asphyxiation. Red blood cells were purified from whole blood by removal of leukocytes using a cellulose column as previously described by Beutler et al [21]. Briefly, anticoagulated blood (0.5–0.8 mL) collected from CO2 euthanized mice was washed in PBS and allowed to pass through a 2-mL column of cellulose. Cellulose columns were composed of equal dry weights α-cellulose and microcrystalline cellulose in 0.134 M NaCl. PBS (10 mM NaCl, 155 mM KCl, 10 mM glucose, 1 mM Na2HPO4).

Growing pointed end [6]. The newly formed actin filaments are further stabilized into fixed lengths by a number of actin capping proteins including adducin, tropomyosin, tropomodulin, dematin, capZ, p55, and protein 4.1 [4,7], which act to prevent actin monomers from associating or dissociating. The importance of stabilizing actin filaments in erythrocytes is underscored by the observations that mutations or deletions in genes encoding adducin [8], dematin [2], tropomyosin [9], tropomodulin [10], or protein 4.1 [11] all lead to red blood cell diseases associated with increased erythrocyte fragility and anemia (see [1] for review).

In some cell types such as T cells, the signaling pathways that stimulate reorganization of the actin cytoskeleton are partially understood [12]. However, in erythrocytes there is very little known about the intracellular signaling pathways that control the formation and stability of the erythrocyte membrane skeleton. Recent studies suggest that some members of the Rho family of GTPases (which include Rac -1 and -2, Cdc42, and Rho -A, -H, and -G in hematopoietic cells) may be involved in actin polymerization and reorganization in erythrocytes [13]. In human erythrocytes, RhoA has been found to translocate from the cytosol to the membrane, potentially resulting in active signaling modules [14]. In mice, conditional disruption of Rac1 and Rac2 results in microcytic anemia characterized by gaps in the actin membrane skeleton, irregularity of the spectrin scaffold, decreased deformability, and decreased representation of essential skeletal proteins including α- and β- spectrin and adducin [3]. Rac GTPases are activated in response to extracellular signals, which stimulate guanine nucleotide exchange factors to catalyze the exchange of bound GDP for GTP on Rac. GTP-bound activated Rac then interacts with PAK (P21-activated kinase), DIAP3 (Diaphanous homolog 3), and an assembly of proteins involved in actin polymerization and reorganization in erythrocytes [15]. In human erythrocytes, RhoA has been found to translocate from the cytosol to the membrane, potentially resulting in active signaling modules [14]. In mice, conditional disruption of Rac1 and Rac2 results in microcytic anemia characterized by gaps in the actin membrane skeleton, irregularity of the spectrin scaffold, decreased deformability, and decreased representation of essential skeletal proteins including α- and β- spectrin and adducin [3]. Rac GTPases are activated in response to extracellular signals, which stimulate guanine nucleotide exchange factors to catalyze the exchange of bound GDP for GTP on Rac. GTP-bound activated Rac then interacts with PAK (P21-activated kinase), DIAP3 (Diaphanous homolog 3), and an assembly of proteins collectively known as the WAVE complex, which stimulate ARP2/3 to initiate F-actin polymerization [15,16]. The WAVE complex is a pentameric heterocomplex consisting of WAVE (WAVE-1,-2, or -3); Abi (Abl-son-interacting protein, Abi-1 or -2); Hem (hematopoietic protein Hem-2 [also known as Nap1 (Nck-associated protein 1, or NckAP1)] or Hem-1 [also known as NckAP-lik]); Sra1 (also known as CYFIP1); and HSPC300 (hematopoietic stem/progenitor cell protein 300) [16,17,18,19].

Whereas the majority of WAVE complex subunits are ubiquitously expressed, Hem-1 is found predominantly in hematopoietic cells [16,20], suggesting a specialized role for Hem-1 in regulating the biology of hematopoietic cells. We recently generated an N-ethyl-N-nitrosourea (ENU) mutant mouse that contains a single, non-coding point mutation in the Hem1 gene, which results in the absence of Hem-1 protein [16]. Hem-1 null mice were noted to have a number of defects in immune cell development and function due in part to destabilization and degradation of WAVE complex proteins, which resulted in impaired F-actin polymerization and formation of the actin cytoskeleton [16]. Here we utilized Hem-1 null mice to show that loss of Hem-1 also results in microcytic, hypochromic anemia characterized by abnormal F-actin condensation, altered representation and phosphorylation of essential junctional complex proteins, altered erythrocyte morphology, and significantly reduced erythrocyte lifespan. These results identify a novel role for Hem-1, and perhaps the WAVE complex, in controlling the formation and stability of the actin membrane skeleton in red blood cells.
MgCl₂, 2.5 mM KHPO₄, pH 7.4) was allowed to pass through the column after the blood. The first 15 mL of filtrate were spun at 000 g×20 min for a total of 3 washes, then lysed as described below.

Preparation of Erythrocyte Ghosts and Hemoglobin-depleted Cytosolic Fraction

Purified erythrocytes were processed for immunoblot analysis in the presence of MgCl₂ to protect actin filament capping, as described by Kuhlman and Fowler [4]. Washed erythrocytes were lysed for 30 minutes on ice as per Kalla et al [3] in NaPi buffer [5 mM sodium phosphate, pH 7.4, 10 mM NaCl, 1 mM EGTA, 2 mM MgCl₂, with phenylmethylsulfonyl fluoride (PMSF) and sodium orthovanadate [Na₃VO₄] added fresh at concentrations of 2 mM and 1 mM, respectively]. After centrifugation, the supernatant (cytosolic fraction) was aspirated and frozen (directly, or in ura-SDS buffer), or depleted of hemoglobin (see “Mass spectrometry [MS] and proteomics”) then frozen (directly, or in ura-SDS buffer). The pellets (membrane ghosts) were then washed at least 3 times in PBS or NaPi buffer and resuspended in ura-SDS sample buffer prior to freezing. All samples were stored at −80°C.

For immunoblotting, samples were solubilized in boiling ura-SDS sample buffer for 5 minutes and loaded according to one of three standards: 1) cell number, 2) total protein, or 3) volume required for equivalent actin loading, as determined by previous immunoblots of the samples for β-actin. Results were similar regardless of the method of standardization.

Electrophoresis and Western Blotting

SDS-PAGE was performed as previously described by Kalla et al [3] using a 5% stacking gel and 6–8% running gels. Gels were stained with Coomassie blue or transferred to polyvinylidene fluoride (PVDF) membranes (NEN Life Science Products, Inc., Boston, MA) for immunoblotting. The following antibodies were used: rabbit anti-β-spectrin (Biogenetic, San Diego, CA, 617002); rabbit anti-ankyrin (kind gift from Raymond Robledo); mouse anti-dematin (Pharmingen/BD Biosciences, San Diego, CA); mouse anti-β-spectrin (Abcam, Cambridge, MA); rabbit antitropomodulin 3 (Lifespan Biosciences, Seattle); mouse anti-phosphatidyl-inositol-3-phosphate kinase (Millipore Corporation, Temecula, CA); anti-WAVE1, goat anti-WAVE2, goat anti-Aibi2, and donkey anti-goat IgG HRP from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-Hem1 (kind gift from Oriron Weiner); goat anti-rabbit IgG HRP and goat anti-mouse IgG HRP from Bio-Rad Laboratories (Hercules, CA); mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, A5441); anti-GYPA, anti-MMP1, and anti-TMOD1 (Sigma-Aldrich, St. Louis Mo. 63103); anti-PP2Ar (EMD Chemicals, Gibbstown, NJ 08027).

Developed films were scanned into Adobe Photoshop CS version 8.0 (Adobe Systems Incorporated, San Jose, CA). Scanned images were imported into Canvas version 9.0.2. (ACD Systems International Incorporated, Seattle, WA) for figure preparation.

Real-time Polymerase Chain Reaction (RT-PCR)

Bone marrow was harvested from Hem−/− and age-matched WT or Hem−/− mice, and erythroblasts and megakaryocytes were FACs-sorted (FACSAria BD Biosciences, Franklin Lakes, NJ) as previously described [22]. RNA was extracted from erythroblasts and megakaryocytes with the RNAqueous-4PCR kit (Ambion/Applied Biosystems, Austin, TX). cDNA was generated using SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY). Samples were normalized using β-actin [β-actin forward (5′ – TCCCTGTGTTGCCGTTCAC –3′) and β-actin reverse (3′ – ACCAGGCGACGGATATGCT –5′)] primers. Hem1 levels were determined using Hem1 forward (5′ – AGGTTGGCCATGCG-TACCTCCTGATCT –3′) and reverse (5′ – AGAGGCGAACC-CAGAAACTCTTT –3′); Aibi levels were determined using Aibi forward (5′ – TGGCACATTTGGAGAAAGACCC –3′) and reverse (5′ – ACTGGTGTGGGTTAAGCGGCT –3′); Sra1 levels were determined using Sra1 forward (5′ – ATGTCTCTGGCAACCAAGAACAG –3′) and reverse (5′ – AGGTACAGGGCAATCCCTGATGCT –3′); WAVE2 levels were determined using WAVE2 forward (5′ – ACCACCGAA-GACCCAGAAGCTC –3′) and reverse (5′ – TCTGGACGATCTTCTCCTCCCAA). Expression of Hem1 in erythroblasts relative to β-actin was normalized to relative expression of Hem1 in murine renal cells, which has previously been shown to be very low [16]. Expression of the WAVE complex genes relative to β-actin was compared to Cₖ value of 38, which represents a low expressing tissue. Experiments were performed using a real-time system sequence detector (PCR GeneAmp PCR System 9700, PE Applied Biosystems, Austin, TX) and PCR system (Mx3005P, Stratagene/Agilent Technologies, Santa Clara, CA) to quantitate the expression of Hem1, Aibi, Aibi2, Sra1, and WAVE2 using the comparative Cₖ method [23].

Erythrocyte Transfusion and Survival

Erythrocytes collected via retro-orbital bleeding of Hem−/− and WT mice were purified, washed three times in PBS/0.1% FBS, and were labeled with CFSE per manufacturer’s instructions. 5×10⁸ cells were transfused via retro-orbital injection into Rag2−/−γc−/− host mice under isoflurane anesthesia (n=4 mice receiving WT erythrocytes; n=3 mice receiving Hem−/− erythrocytes). Host mice were bled via tail vein at 24 and 48 hours post-transfusion, and at days 5, 9, 16, 23, 28, 36, 42, and 49 post-transfusion. Host erythrocytes were sampled by flow cytometry and data analyzed using FlowJo version 8.6.1 software (Tree Star, Incorporated, Ashland, OR). The percentage of CFSE-labeled erythrocytes in the 24-hour post-transfusion sample was considered 100% labeling, and percentages of labeled erythrocytes at subsequent timepoints were calculated relative to this.

Immunofluorescence of Erythrocytes

Coverslips for mounting erythrocytes were coated with 50 μL of 0.01% poly-L-lysine and allowed to dry. Peripheral blood was processed as described by Kalla et al [3] with the following modifications: 8 μL of whole blood were fixed with 100–200 μL 0.5% acrolein in solution for 5 min. Washed, diluted erythrocytes were adhered to coverslips for 2 hr, then were permeabilized for 30 min in rinsing solution containing 1.0% Triton X-100. Slides were stained with 0.4 U/slide Alexa Fluor 488 phallolidin (0.2 U/μL solution in methanol, diluted 1:50 with PBS) and anti-β-spectrin (1:250 in 3% BSA in PBS) at 4°C overnight, then with goat-anti-mouse secondary antibody labeled with Alexa Fluor 568 (1:1000 in 3% BSA in PBS; Molecular Probes, Eugene, OR) for 1 hour. Coverslips were mounted with ProLong Gold Antifade (Molecular Probes, Eugene, OR). The spatial distribution of the fluorescent probes was analyzed via confocal laser-scanning microscope (Zeiss LSM 510 META, 1.45 numerical aperture, 1000× magnification).

Mass Spectrometry (MS) and Proteomics

Wildtype and Hem−/− erythrocytes were compared using a label free comparative proteomic approach [24,25]. Erythrocytes were purified from independent whole blood samples using cellulose chromatography as described above. All subsequent
sample processing was standardized so as to minimize pre-analytical variability in the erythrocyte proteome. Erythrocytes were lysed hypotonically and centrifuged at 1000 g, 4°C for 15 min. The soluble fraction was removed and the insoluble fraction pellet (erythrocyte ghosts) was washed 5 times in PBS. Samples of soluble fraction samples were hemoglobin-depleted using a modification of the method of Ringrose et al [26]. For each sample, 1.0 mL packed Ni-NTA agarose resin (Qiagen Incorporated, Valencia, CA) was equilibrated by washing three times with 50 mM NaH2PO4/5 mM imidazole in a 2.0 mL microfuge tube. Hemoglobin-rich supernatant was adjusted to 5 mM imidazole, mixed with the packed Ni-NTA, and incubated with rocking overnight at 4°C. The resin was pelleted by centrifugation for 1 minute at 12,000 rpm at 4°C. The supernatant was combined with 1.0 mL fresh packed Ni-NTA for a second overnight incubation. The resulting clear supernatant was concentrated approximately 80% on a Speed Vac Concentrator (Savant). For all samples, protein concentrations were determined using the BioRad Protein Assay (BioRad Labs, Hercules, CA) and aliquots were stored at −80°C. Protein samples were prepared and profiled using 1 µg of protein per run on a hybrid linear ion trap FTICR mass spectrometer (LTQ-FIT Ultra, ThermoElectron, Waltham, MA) as previously described [25]. Peptide and protein identification and label-free analysis were as described previously [25]. Comparisons of the insoluble fraction were performed for retention time regions between 23 and 111 minutes, and utilized a data set of 5 WT technical replicates (derived from 2 biological replicates) and 8 Hem−1/− technical replicates (derived from 3 biological replicates). Soluble fraction comparisons were performed for retention time regions between 20 and 140 minutes, and utilized a data set of 4 WT technical replicates (derived from 2 biological replicates) and 4 Hem−1/− technical replicates (derived from 2 biological replicates).

Statistics
Student’s t-test and Fishers exact test were used to compare means and calculate significance.

Results

Hem1-null Erythrocytes Exhibit Altered Morphology and Condensed F-actin Foci

Our initial analysis of Hem−1/− mice indicated that loss of Hem1 results in microcytic, hypochromic anemia characterized by abnormal erythrocyte morphology and increased fragility [16]. Detailed characterization of the erythrocyte abnormalities in peripheral blood samples from Hem−1/− mice using differentially stained blood smears reveal abundant abnormal erythrocytes including acanthocytes, schistocytes, dacryocytes, and keratocytes. Hem−1/− erythrocytes also exhibit poikilocytosis, anisocytosis, hypochromia, and polychromasia (Figure 1A).

We next sought to determine whether the abnormal erythrocyte morphology in Hem−1/− mice is associated with aberrant actin polymerization. Using phalloidin and anti-spectrin staining followed by confocal microscopy, we found that whereas actin and spectrin co-localize in the cell membrane of WT erythrocytes, Hem−1/− erythrocytes contain less membrane-associated actin and spectrin, and increased cytoplasmic actin. Hem−1/− erythrocytes also contain abnormal foci of brightly staining condensed actin (yellow arrows) relative to WT erythrocytes, which lack condensed actin foci (Figure 1B). Using DeltaVision® microscopy, analyses of multiple cross-sections indicate that abnormal actin foci are present in the majority of Hem−1/− erythrocytes relative to WT erythrocytes (p<3×10−6) and co-localize with spectrin to the periphery in apparent association with the cell membrane. These studies indicate that disruption of Hem1 results in altered erythrocyte morphology, which correlates with disrupted organization of the actin membrane skeleton.

We next sought to determine whether the anemia in Hem−1/− mice is the result of abnormal erythropoiesis and/or the removal of abnormally shaped mature erythrocytes as they circulate in peripheral blood. To assess erythropoiesis in Hem−1/− and WT mice, we identified the percentage and number (not shown) of erythrocytes in different stages of erythrocyte development based on the expression of the CD44 adhesion molecule on Ter119+ erythroid lineage cells, in combination with forward light scatter characteristics [27]. We found that while there is less erythropoiesis in Hem−1/− bone marrow due to increased myelopoiesis and changes in the microenvironment as previously shown [16], splenic erythropoiesis is significantly increased in Hem−1/− mice relative to WT mice based on increased percentage and number (not shown) of total Ter119+ erythroid lineage cells (Figure 2A, left), and increased percentage of polychromatic erythroblasts (Stage III), orthochromatic erythroblasts (Stage IV-A), and reticulocytes (Stage IV-B) (Figure 2B, left) ([16]). Hem−1/− erythroblasts also have normal membrane structure relative to WT erythroblasts (Figure 2B, right). In contrast, the morphology of mature erythrocytes is significantly altered (Figure 1A) and the percentage of mature Hem−1/− erythrocytes (Stage V) is selectively and significantly decreased indicating that anemia in Hem−1/− mice likely results from the loss of abnormally shaped mature erythrocytes and not from cell intrinsic impairment in erythrocyte development. These findings are also consistent with our previous demonstration that Hem−1/− erythrocytes are more fragile relative to WT erythrocytes in response to osmotic stress [16].

Hem-1 and WAVE Complex Components are Expressed in Erythrocytes and Erythrocyte Progenitors

We had previously shown that Hem1 is expressed predominantly in hematopoietic cells including B cells, T cells, macrophages, and neutrophils [16]. Since Hem−1/− mice also present with defects in erythrocyte biology, we examined whether Hem1 and WAVE complex mRNA and protein are also expressed in erythrocytes and erythrocyte progenitors. Since mature erythrocytes lack nuclei, we first examined the expression of Hem1 and WAVE complex mRNA by real-time PCR on samples derived from FACs-sorted erythroblasts from WT and Hem−1/− mice. WT erythroblasts expressed high levels of mRNA encoding components of the WAVE complex including Hem1, Abi1, Abi2, Sra1, and WAVE2 (Figure 3A, left). Hem−1/− erythroblasts expressed equivalent levels of Abi1, Sra1, and WAVE2 mRNA, whereas Hem−1 (not shown) and Abi2 were significantly decreased (Figure 3A, right). Immunoblots of purified mature erythrocyte lysates from WT and Hem−1/− mice also indicated that WAVE proteins including Abi-2, WAVE-1, and WAVE-2 are present in nearly equal amounts relative to β-actin, whereas Hem-1 protein (top band) is completely absent (Figure 3B). This is in contrast to Hem−1 null lymphocytes and neutrophils, where WAVE complex proteins were significantly less abundant relative to WT cells [16]. These results suggest that Hem-1 and members of the WAVE complex are expressed in erythrocytes and that individual protein components of the WAVE complex are relatively stable in erythrocytes, even in the absence of Hem-1.
Global Alterations in Protein Expression in *Hem1*−/− Erythrocytes

We next utilized an unbiased global proteomics approach to investigate why loss of Hem-1 results in anemia and defects in the actin cytoskeleton. Specifically, we compared the abundance of a broad range of proteins present in WT and *Hem1*−/− erythrocytes using the label-free CRAWDAD (Chromatographic Retention Time Alignment and Warping for Differential Analysis of LC-MS Data) mass spectrometry approach [24], which uses information in LC-MS/MS spectra to identify the peptides present in a protease digest to estimate differences in abundance of peptides from their intensities in LC-MS spectra. Using this approach, we identified statistically significant differences in the abundance of 14 proteins in the insoluble (membrane bound) fraction (Figures 4A, 4B and Table 1) and 74 proteins in the soluble (cytosolic) fraction (Table 2) between *Hem1*−/− and WT erythrocytes. A complete list of proteins identified in these samples is presented in supplementary data (Tables S1–S4), and a complete list of peptide differences detected in the insoluble and soluble fractions is presented in Tables S5 and S6. Examples of peptide differences detected between WT and *Hem1*−/− insoluble fraction samples by CRAWDAD are illustrated in Figure 4B. The majority of the significant changes were associated with decreased protein levels in *Hem1*−/− erythrocytes relative to WT erythrocytes. Notably, important cytoskeletal proteins including...
4.1, tropomodulin 1, dematin, band 3, β-actin, p55, α- and β-spectrin, and ankyrin were significantly decreased in the insoluble fractions from Hem1−/− erythrocytes relative to WT erythrocytes (Figure 4B and Table 1), consistent with the profound changes in erythrocyte morphology and actin foci noted by microscopy. Interestingly, the soluble cytosolic fractions from Hem1−/− erythrocytes contained significantly decreased representation of a number of essential metabolic enzymes associated with glycolysis [such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase, and β- and γ-enolase] and other biochemical pathways (including malate dehydrogenase, phosphoglycerate phosphatase, phosphoglucomutase-2, fumarate hydratase, and 6-phosphogluconolactonase) relative to WT erythrocytes (Table 2). We also found significantly decreased levels of enzymes directly or indirectly responsible for inactivation of reactive oxygen species (ROS), such as phospholipid hydroperoxide glutathione peroxidase, and enzymes involved in protein repair (including ketosamine-3-kinase) and oxygen exchange, such as bisphosphoglycerate mutase. Importantly, and as further discussed below, Hem-1 null erythrocytes contained significantly decreased amounts of the regulatory A subunit (~7.06 fold) and regulatory B subunit (~10.16 fold) of the serine-threonine protein phosphatase 2A (PP2A) [28], which dephosphorylates proteins that are typically phosphorylated by protein kinase C (PKC) [29], including adducin.

**Figure 2. Loss of Hem1 does not impair erythropoiesis.** Flow cytometric analyses of splenocytes from Hem1−/− vs. wildtype (wt) littermate control mice. (A) Live-gated, Ter119+ cells (left panels) are shown plotted by cell-surface display of CD44 vs. Ter119+ (middle panels) or CD44 vs. forward scatter (right panels). Gates defining different erythroid progenitor populations (I-V) are shown. (B) (left) Erythroid progenitor populations are shown as percent of Ter119+ cells. Bars represent mean ±/− SEM for three animals per genotype. The asterisks indicate statistical significance: (*), p<0.01; (**) p<0.001. (right) Hem1−/− erythroblasts have normal morphology. Shown are representative photos of WT and Hem1−/− erythroblasts. doi:10.1371/journal.pone.0054902.g002

**Figure 3. Expression of WAVE complex mRNA and protein in wildtype (WT) and Hem1−/− mouse erythrocytes.** (A) (Left) Real-time PCR of FAC-sorted erythroblasts from WT mice shows relative expression of Hem1 and WAVE complex (Abi1, Abi2, Sra1, and Wave2) mRNAs normalized to β-actin. Expression of Hem1 mRNA is shown relative to kidney mRNA. Expression of WAVE component mRNAs are shown relative to a Cβ value of 38, which represents an arbitrary low expressing tissue. (*), p<0.0007; (**) p<0.0004; (***) p<0.0001. n = 3 mice per genotype. (Right) Expression of WAVE complex mRNAs are shown in Hem1 erythroblasts versus WT erythroblasts. Abi2 p<0.0004 (B) Immunoblots of purified erythrocyte ghosts from WT and Hem1−/− mice, loaded according to equivalent levels of β-actin. Each lane is representative of at least 3 individuals per genotype. Hem-1 protein (upper band, 110 kDa) is not detectable in erythrocyte ghosts from Hem1−/− mice. Although Abi2 mRNA levels are reduced in Hem1−/− erythrocytes, Abi2 protein levels are relatively normal, likely due to post-transcriptional and/or post-translational regulation. Numbers below each scan represent protein expression levels in Hem1−/− relative to WT samples. Samples were loaded based on equivalent total protein. doi:10.1371/journal.pone.0054902.g003

**Hem1−/− Erythrocytes Exhibit Altered Representation of Junctional and Actin-binding Membrane Skeletal Proteins**

Since mass spectrometric analyses indicated that multiple critical membrane skeletal proteins were decreased in Hem1−/− erythrocytes, we further examined the representation of prominent membrane skeletal proteins in Hem1−/− erythrocytes by SDS-PAGE and immunoblotting. We first utilized Coomassie-stained gels to analyze the representation of the most abundant non-hemoglobin erythrocyte proteins. We found that Hem1−/− erythrocytes had decreased levels of band 4.1, band 3, and α- and β-spectrin compared to WT erythrocytes (Figure 5A), which was highly consistent with our proteomics results. Hem1−/− erythrocyte membrane ghosts (membrane fractions) also exhibited decreased levels of adducin, dematin, β-spectrin, ankyrin, tropomodulin1, and p55 (MPPI) by immunoblot (Figure 5B). Interestingly, both phospho-adducin (which targets adducin for dissociation from actin and degradation) and tropomodulin 3 were increased in Hem1−/− erythrocytes relative to WT erythrocytes.

In neurons and platelets, adducin is serine-phosphorylated by protein kinase C (PKC) [30,31] and PKC-mediated phosphorylation is typically opposed by protein phosphatase 2A (PP2A) mediated dephosphorylation. To determine whether adducin is also phosphorylated by PKC in erythrocytes, we treated WT and Hem1−/− erythrocytes with the PKC-activator phorbol 12-myristate 13-acetate (PMA) for increasing amounts of time. We found that both WT and Hem1−/− erythrocytes are phosphorylated by PKC on serine 724 and that “basal” serine 724 phosphorylation is typically opposed by protein phosphatase 2A (PP2A). Interestingly, the soluble cytosolic fractions from Hem1−/− erythrocytes contained significantly decreased representation of a number of essential metabolic enzymes associated with glycolysis [such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase, and β- and γ-enolase] and other biochemical pathways (including malate dehydrogenase, phosphoglycerate phosphatase, phosphoglucomutase-2, fumarate hydratase, and 6-phosphogluconolactonase) relative to WT erythrocytes (Table 2). We also found significantly decreased levels of enzymes directly or indirectly responsible for inactivation of reactive oxygen species (ROS), such as phospholipid hydroperoxide glutathione peroxidase, and enzymes involved in protein repair (including ketosamine-3-kinase) and oxygen exchange, such as bisphosphoglycerate mutase. Importantly, and as further discussed below, Hem-1 null erythrocytes contained significantly decreased amounts of the regulatory A subunit (~7.06 fold) and regulatory B subunit (~10.16 fold) of the serine-threonine protein phosphatase 2A (PP2A) [28], which dephosphorylates proteins that are typically phosphorylated by protein kinase C (PKC) [29], including adducin.

**Figure 3. Expression of WAVE complex mRNA and protein in wildtype (WT) and Hem1−/− mouse erythrocytes.** (A) (Left) Real-time PCR of FAC-sorted erythroblasts from WT mice shows relative expression of Hem1 and WAVE complex (Abi1, Abi2, Sra1, and Wave2) mRNAs normalized to β-actin. Expression of Hem1 mRNA is shown relative to kidney mRNA. Expression of WAVE component mRNAs are shown relative to a Cβ value of 38, which represents an arbitrary low expressing tissue. (*), p<0.0007; (**) p<0.0004; (***) p<0.0001. n = 3 mice per genotype. (Right) Expression of WAVE complex mRNAs are shown in Hem1 erythroblasts versus WT erythroblasts. Abi2 p<0.0004 (B) Immunoblots of purified erythrocyte ghosts from WT and Hem1−/− mice, loaded according to equivalent levels of β-actin. Each lane is representative of at least 3 individuals per genotype. Hem-1 protein (upper band, 110 kDa) is not detectable in erythrocyte ghosts from Hem1−/− mice. Although Abi2 mRNA levels are reduced in Hem1−/− erythrocytes, Abi2 protein levels are relatively normal, likely due to post-transcriptional and/or post-translational regulation. Numbers below each scan represent protein expression levels in Hem1−/− relative to WT samples. Samples were loaded based on equivalent total protein. doi:10.1371/journal.pone.0054902.g003
Figure 4. WT and Hem1^-/- erythrocytes show differential expression of key membrane and structural proteins as revealed by proteomics. (A) Chromatographic alignments of replicate runs for WT and Hem1^-/- erythrocytes. Alignments of 5 WT (positive inflection line) and 8 Hem1^-/- (negative inflection line) erythrocyte technical replicate LC-MS runs are shown in a base peak plot (ion intensity vs. retention time). Individual replicates are shown in distinct colors for each genotype. (B) CRAWDAD detection of chromatographic difference regions for 55 kDa
 increased expression of the PP2Ac significantly reduced expression of PP2Ar α and β subunits and proteomics data which also indicated that loss of WT erythrocytes. These results are highly consistent with our results indicated that loss of Hem1 results in significantly reduced expression of PP2Ar A and B subunits and increased expression of the PP2Ac α and β subunits (Table S1).

**Hem1**/^−^ Erythrocytes have Decreased Lifespan

Defects in primary structural proteins have been shown to result in abbreviated erythrocyte survival due to decreased deformability, increased splenic trapping, and hemolysis [7]. To determine whether Hem-1 null mice have reduced numbers of erythrocytes due to shortened erythrocyte survival, we measured the lifespan of Hem1/^−^ erythrocytes relative to WT erythrocytes following transplantation into identical host environments. Erythrocytes were labeled with CFSE dye and adoptively transferred into immunodeficient Rag2/^−^/γc/^−^ mice [32], which lack T, B, and NK lymphocytes and thus eliminate any potential differences in the susceptibility to immune rejection. The percentage of CFSE-positive erythrocytes present in the hosts was then measured at approximately weekly intervals until 50 days post-transfusion. We found that the percentage of Hem1/^−^ erythrocytes began declining at a faster rate than WT erythrocytes by Day 5 post-transfusion (Figure 6). From day 15 post-transfusion until the end of the study 50 days post-transfusion, the percentage of circulating Hem1/^−^ erythrocytes remained less than half that of WT erythrocytes. These results indicate that Hem1/^−^ erythrocytes have significantly reduced lifespans relative to WT erythrocytes, and that the defects causing the abbreviated Hem1/^−^ erythrocyte survival are cell-autonomous.

### Discussion

The majority of erythrocyte disorders in humans are associated with mutations in genes encoding important structural proteins, which result in disruption of the erythrocyte actin membrane skeleton, decreased deformability, and subsequent removal of erythrocytes from circulation [1]. In this study, we show for the first time that the actin-regulatory protein Hem-1 and the associated WAVE complex are expressed in erythrocytes and erythrocyte progenitors. In addition, we find that the deletion of Hem1 in mice results in cell-autonomous microcytic, hypochromic anemia characterized by malformed erythrocytes with abnormal condensed F-actin foci, decreased representation and altered stoichiometry of essential membrane cytoskeletal proteins, and disrupted “metabolon”. These results collectively indicate that Hem-1 has previously unrecognized biochemical role(s) in regulating the formation and/or stability of the actin membrane skeleton in erythrocytes.

Orthologs of Hem-1 have been shown to be components of the WAVE complex, which signals downstream of Rac to stimulate F-actin polymerization (see [10] for review). In immune cells such as lymphocytes and neutrophils, disruption of Hem-1 results in destabilization and degradation of the WAVE complex [16,33], which prevents WAVE from stimulating ARP2/3-mediated nucleation of F-actin [19,34]. Other studies on WAVE orthologs have also shown that knockdown or ablation of any protein in the WAVE complex results in disruption of the structured F-actin cytoskeleton [35,36]. Furthermore, studies using mouse Hem1 knockdown cells have shown that these cells exhibit increased epithelial permeability [37] and microvesicle shedding [38], which is reminiscent of the vascular leakage and hemolysis associated with Hem1/^−^ erythrocytes. Collectively, these data support a role for Hem-1 in stabilizing the actin cytoskeleton and the actin containing organelles in the host cell.

### Table 1. Protein differences identified in insoluble fraction via proteomics in WT and Hem1^−/−^ erythrocytes.

| Protein                                      | Intensity Difference* | No. of Peptides | ID             |
|----------------------------------------------|-----------------------|-----------------|----------------|
| 1 Hemoglobin, alpha                          | -27.38                | 7               | IPI00845802.1  |
| 2 β-Spectrin                                  | -15.99                | 7               | IPI00131376.5  |
| 3 Ankyrin 2                                   | -14.96                | 2               | IPI00227235.4  |
| 4 Ankyrin 3                                   | -14.96                | 2               | IPI00173248.2  |
| 5 α-Spectrin                                   | -11.46                | 2               | IPI00896567.1  |
| 6 Protein band 4.1                           | -11.41                | 5               | IPI00649005.1  |
| 7 Tropomodulin 1                              | -11.01                | 2               | IPI00655150.1  |
| 8 Glycophorin A                               | -8.81                 | 3               | IPI00123673.1  |
| 9 Actin, beta                                 | -5.28                 | 5               | IPI00110850.1  |
| 10 Dematin                                     | -5.09                 | 3               | IPI00125328.3  |
| 11 55 kDa erythrocyte membrane protein        | -4.87                 | 2               | IPI00137706.1  |
| 12 Ankyrin 1                                  | -4.85                 | 15              | IPI00119871.3  |
| 13 Protein band 3 anion transport protein     | -3.30                 | 16              | IPI00120761.3  |
| 14 Erythrocyte protein band 4.2               | 2.62                  | 2               | IPI00421166.3  |

*n = 2 WT mice (5 technical replicates) and 3 Hem1^−/−^ mice (8 technical replicates).

*Fold difference in Hem1^−/−^ versus WT erythrocytes. p≤0.05.

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| Protein                                                                 | Intensity Difference* | No. of Peptides | ID          |
|------------------------------------------------------------------------|------------------------|-----------------|-------------|
| Parkinson disease (Autosomal recessive, early onset)                   | −155.1                 | 3               | IP00125923.1|
| Osteoclast-stimulating factor 1                                         | −86.32                 | 2               | IP00336324.1|
| Malate dehydrogenase, cytoplasmic                                       | −32.12                 | 5               | IP00137758.5|
| Secernin-3                                                             | −31.47                 | 3               | IP00649794.1|
| 100042424 Programmed cell death protein 5                               | −26.07                 | 2               | IP00116120.3|
| Vinculin                                                               | −25.24                 | 2               | IP00405227.3|
| Glia maturation factor, β                                              | −21.53                 | 2               | IP00467495.4|
| β-enolase                                                              | −20.98                 | 2               | IP00379245.2|
| Secernin 3, isoform CRA_b                                              | −20.32                 | 2               | IP00756194.1|
| Eukaryotic translation initiation factor 6                              | −16.62                 | 3               | IP00857575.2|
| 14-3-3 protein epsilon                                                 | −16.41                 | 2               | IP00118384.1|
| Dipeptidyl-peptidase 3                                                 | −11.52                 | 2               | IP00116134.1|
| γ-enolase                                                              | −11.17                 | 2               | IP00331704.7|
| Serine/threonine-protein phosphatase 2A regulatory subunit B'          | −10.16                 | 4               | IP00118723.3|
| Nucleosome assembly protein 1-like 4                                    | −9.71                  | 2               | IP00876202.1|
| UMP-CMP kinase                                                         | −9.59                  | 2               | IP00331165.1|
| Spectrin α chain, erythrocyte                                           | −9.58                  | 5               | IP00323230.5|
| Phosphoglycerate kinase 1                                              | −9.23                  | 8               | IP00555069.3|
| Phosphoglycolate phosphatase                                           | −8.65                  | 2               | IP00380195.1|
| Ubiquitin-conjugating enzyme E2 N                                      | −7.86                  | 2               | IP00165854.3|
| Adenosylhomocysteinease                                                 | −7.7                   | 2               | IP00230404.6|
| Phospholipid hydroperoxide glutathione peroxidase                      | −7.6                   | 2               | IP00463220.2|
| Erythrocyte membrane protein band 4                                    | −7.47                  | 2               | IP00421166.3|
| Serine/threonine-prot. phosphatase 2A 65 kDa regulatory, subunit A α isoform | −7.06                  | 2               | IP00310091.8|
| 26S protease regulatory subunit 6A                                      | −7.03                  | 4               | IP00133206.1|
| Proteasome subunit β type-4                                            | −7                     | 3               | IP00129512.3|
| Heat shock protein HSP 90-α                                            | −6.92                  | 2               | IP00330804.4|
| Putative uncharacterized protein                                       | −6.76                  | 2               | IP00458922.1|
| Ketosamine-3-kinase                                                    | −6.68                  | 2               | IP00321994.1|
| Myotrophin                                                             | −6.65                  | 2               | IP00228583.5|
| Heat shock protein 2                                                    | −6.35                  | 2               | IP00387494.1|
| Ribonuclease inhibitor                                                 | −6.35                  | 3               | IP00313296.3|
| OTTMUSG000000014946 hypothetical protein                               | −6.22                  | 6               | IP00762304.1|
| V-type proton ATPase subunit B, brain isoform                           | −6.03                  | 3               | IP00119113.3|
| Moesin                                                                 | −5.96                  | 3               | IP00105884.8|
| Bisphosphoglycerate mutase                                             | −5.96                  | 3               | IP00221663.5|
| Glutamate–cysteine ligase regulatory subunit                           | −5.77                  | 2               | IP00114329.1|
| Peroxisiredoxin 6, related sequence 1                                  | −5.71                  | 2               | IP00221454.1|
| LOC100048075 similar to nonselenium glutathione peroxidase            | −5.38                  | 4               | IP00756385.1|
| Peroxisiredoxin-6                                                      | −5.57                  | 5               | IP00555059.2|
| 36 kDa protein                                                         | −5.56                  | 8               | IP00399459.2|
| Glyceraldehyde-3-phosphate dehydrogenase                               | −5.55                  | 10              | IP00273646.9|
| Inorganic pyrophosphatase                                              | −5.54                  | 2               | IP00110684.1|
| 14-3-3 protein γ/δ                                                     | −5.31                  | 2               | IP00116498.1|
| Proteasome subunit alpha type-7/Psmα8 Proteasome subunit a-type-7-like | −5.3                   | 2               | IP00131406.1|
| Similar to Proteasome 265 subunit, non-ATPase, 2 isoform               | −5.25                  | 2               | IP00675436.2|
| 26S proteasome non-ATPase regulatory subunit                            | −5.25                  | 2               | IP00123494.3|
actin polymerization in erythrocytes is constitutive. In contrast, erythrocytes may not have the activation of adhesion) for situations requiring protection from actin-based structures (such as podosomes and phagosomes, and PAMPs), which assists in limiting the generation of autoimmunity [12,35,36].

WAVE complex proteins in immune cells and erythrocytes may compensate for the absence of Hem-1 in erythrocytes (data not shown). These results suggest that differences in the stability of the WAVE complex (including Hem-1/2) induces instability and degradation of the remaining WAVE complex protein subunits [19,34]. However, despite abnormal F-actin organization in Hem1−/− erythrocytes, we found nearly equal amounts of WAVE-1, WAVE-2, and Abi-2 proteins in lysates from mature Hem1−/− erythrocytes relative to WT erythrocytes, indicating that loss of Hem-1 does not significantly impair stability of the WAVE complex in erythrocytes. We also did not find upregulation of Hem2 in Hem1−/− erythroblasts, suggesting that Hem-2 does not compensate for the absence of Hem-1 in erythrocytes (data not shown). These results suggest that differences in the stability of WAVE complex proteins in immune cells and erythrocytes may reflect differences in active inducible actin polymerization in immune cells versus constitutive actin polymerization in erythrocytes. In immune cells, actin polymerization is inducible in response to activation by antigens or pathogen-associated molecular patterns (PAMPs), which assists in limiting the generation of actin-based structures (such as podosomes and phagosomes, and the activation of adhesion) for situations requiring protection from pathogens, thus protecting against excessive inflammation and autoimmunity [12,35,36]. In contrast, erythrocytes may not have evolved mechanisms to degrade the WAVE complex acutely since actin polymerization in erythrocytes is constitutive.

To investigate how loss of Hem-1 results in disruption of the actin membrane skeleton in erythrocytes, we utilized an unbiased global proteomics approach to compare the relative representation of specific proteins in erythrocytes from Hem1−/− and WT mice. We noted statistically significant decreases in the representation of essential membrane and membrane cytoskeletal proteins including α- and β- spectrin, ankyrin, dematin, tropomodulin 1, protein 4.1, and band 3 in Hem1−/− erythrocytes. We further confirmed by Coomassie staining that Hem1−/− erythrocytes contain significantly decreased levels of α-spectrin, β-spectrin, band 4.1, and band 3 relative to WT erythrocytes. Immunoblot analyses of erythrocyte ghosts confirmed that β-spectrin, spectrin, tropomodulin, dematin, ankyrin, tropomodulin 1, and MPP1 (p55) were decreased relative to actin in Hem1−/− erythrocytes. These results collectively suggest that the absence of Hem-1 in erythrocytes results in altered homocytosis of essential membrane and membrane skeletal proteins, which results in defects in cytoskeletal structure, stability, deformability, and likely the localization of key biochemical events such as glycolysis [37].

Interestingly, we also found via immunoblot that phosphorylation of α-adducin at Ser724 was increased in Hem1−/− erythrocytes as were the relative levels of tropomodulin 3. In erythrocytes, adducin caps (stabilizes) actin protofilaments at the rapidly-growing barbed ends, while tropomodulin caps actin at the slow-growing pointed ends. In human platelets, phosphorylation of adducin on Ser726 (corresponding to Ser724 on mouse α-adducin) causes dissociation of adducin from spectrin and actin [31], resulting in proteolysis of Table 2. Cont.

| Protein | Intensity Difference* | No. of Peptides | ID |
|---|---|---|---|
| 50 Isform Erythrocyte of Band 3 anion transport protein | −5.23 | 5 | IP00120761.3 |
| 51 GTP-binding nuclear protein Ran, testis-specific isoform | −4.99 | 2 | IP00126133.1 |
| 52 Phosphoglucomutase-2 | −4.96 | 2 | IP00338302.1 |
| 53 Isoform 1 of Cytosolic 5′-nucleotidase 3 | −4.96 | 2 | IP00648235.1 |
| 54 Spectrin β1 | −4.72 | 4 | IP00131765.5 |
| 55 Fumarate hydratase, mitochondrial | −4.71 | 2 | IP00129928.2 |
| 56 Lactoylglutathione lyase | −4.56 | 6 | IP00321734.7 |
| 57 Proteosome subunit α type-4 | −4.56 | 2 | IP00277001.4 |
| 58 LOC100046081 Ubiquitin thioesterase OTUB1 | −4.52 | 2 | IP00154004.1 |
| 59 Cofilin-1 | −4.48 | 4 | IP00890171.1 |
| 60 6-phosphogluconolactonase | −3.97 | 2 | IP00132080.1 |
| 61 Nuclear transport factor 2 | −3.92 | 3 | IP00124149.1 |
| 62 Aldose reductase | −3.89 | 2 | IP00223757.4 |
| 63 36 kDa protein | −3.39 | 6 | IP00625893.3 |
| 64 Nans Putative uncharacterized protein | −3.17 | 2 | IP00114925.1 |
| 65 Eukaryotic translation initiation factor 5A-2 | −3.17 | 2 | IP00331514.6 |
| 66 1700009N14Rik RIKEN cDNA 1700009N14 gene | −2.69 | 2 | IP00127109.4 |
| 67 Actin, cytoplasmic | 34.35 | 3 | IP00110850.1 |
| 68 Serine/threonine-protein phosphatase 2A catalytic subunit β isoform | 27.16 | 2 | IP0011556.1 |
| 69 Serine/threonine-protein phosphatase 2A catalytic subunit α isoform | 25.19 | 2 | IP00120374.1 |
| 70 Gart phosphoribosylglycinamide formyltransferase | 8.32 | 2 | IP00230612.3 |
| 71 Sepiapterin reductase | 7.83 | 2 | IP00129641.1 |
| 72 Isoform 1 of Glyoxalas domain-containing protein 4 | 6.47 | 2 | IP00110721.5 |
| 73 Putative uncharacterized protein | 4.53 | 2 | IP00830250.1 |
| 74 22 kDa protein | 2.96 | 2 | IP00605090.5 |

n = 2 WT mice (4 technical replicates) and 2 Hem1−/− mice (4 technical replicates). *Fold difference in Hem1−/− vs WT erythrocytes, p = 0.05. doi:10.1371/journal.pone.0054902.t002
Hem-1 Regulates the Erythrocyte Actin Skeleton

A

MW (kDa)  WT  Hem1⁻/⁻

α-Spectrin
β-Spectrin

Band 3
Band 4.1
Band 4.2

Actin

β-actin

B

MW (kDa)  WT  Hem1⁻/⁻

Total-Adducin
p-Adducin
Dematin
β-Spectrin
Ankyrin
Tropomodulin1
MPP1 (p55)
Tropomodulin3

C

WT  Hem1⁻/⁻
PMA 0'  1'  5'  10'  0'  1'  5'  10'

p-Adducin

Ratio (phospho-to-total adducin)

D

WT  Hem1⁻/⁻

PP2Ac  1  2.0

PP2Ar  1  0.5

β-Actin  1  0.8

E

WT  Hem1⁻/⁻

OA  0'  1'  5'  10'  0'  1'  5'  10'

p-Adducin

Ratio (phospho-to-total adducin)

Okadaic Acid
aducin by calpain [38]. Similarly, in renal epithelial cells phosphorylated α-adducin is cleaved by caspase, which results in its dissociation from spectrin-actin [39]. In neurons and platelets, aducin is serine-phosphorylated by protein kinase C (PKC) [30,31] resulting in decreased F-actin capping. In Hem1−/− erythrocytes, we found increased phospho-adducin (Ser724), which correlated with altered representation of membrane skeletal proteins and abnormal condensation of F-actin into discrete foci. We also found that aducin is phosphorylated at Ser724 by PKC in erythrocytes, and that aducin was hyper-phosphorylated in the absence of Hem1. We further determined by mass spectrometry that Hem1 null erythrocytes contain significantly reduced levels of the protein phosphatase 2A (PP2A) regulatory subunits A (−7.06 fold) and B (−9.58 fold) which are required for PP2A function, and increased levels of PP2A catalytic subunit isoforms α (25.19 fold) and β (27.16 fold), which are known to be upregulated in response to reduced PP2A function [40]. In addition, we confirmed by immunoblot that PP2Ar is reduced and PP2Ac is increased in Hem1−/− erythrocytes. PP2A is the major phosphatase that dephosphorylates and opposes PKC-mediated serine/threonine phosphorylation. Hence, our studies suggest that disruption of Hem-1 in erythrocytes results in an imbalance between PP2A regulatory and catalytic subunits (which is known to result in reduced PP2A function [41]) and increased phosphorylation of aducin on Ser724. Increased phosphorylation of aducin leads to further dissociation of actin from spectrin and aducin, and overall alterations in membrane skeletal organization and stability. The mechanism of how disruption of Hem-1 results in reduced expression of PP2A regulatory subunits requires further investigation. However, Hem-1 [33], PKC [42], and PP2A [43] have all been shown to interact with Rac, suggesting that disruption of Hem-1 could alter the stoichiometry of these enzymes and shift the balance towards increased phospho-adducin (Figure 7).

In addition to the spectrins, actin, and adducin, we observed decreased levels of other erythrocyte junctional complex proteins including dematin [44], tropomodulin 1 (Tmod1) [45], p55 [46], and protein 4.1 in lysates from Hem1−/− erythrocytes relative to WT erythrocytes. The junctional complex creates a bridge between the membrane phospholipid bilayer and the underlying cytoskeleton, and many previous studies have shown that this complex is essential for the maintenance of erythrocyte shape, membrane stability, and mechanical properties [44]. For example, Tmod1-null mice exhibit anemia associated with increased osmotic fragility and reduced red blood cell deformability [10]. Expression of tropomodulin 3 (which does not normally occur in wildtype erythrocytes) is upregulated in Tmod1-null erythrocytes perhaps as a compensatory mechanism to aid in capping actin protofilaments. We also observed increased tropomodulin 3 expression in Hem1−/− erythrocytes. Similarly, mice deficient in...
the junctional complex proteins dematin and β-adducin (dematin/β-adducin double knockout mice) exhibit anisocytosis, poikilocytosis, polychromasia, and abnormal and fragmented erythrocytes with decreased lifespan [2]. Dematin/β-adducin double knockout erythrocytes also showed some membrane skeletal protein changes in common with Hem1−/− erythrocytes, including decreased

**Figure 7. Model of the erythrocyte membrane cytoskeleton in wildtype and Hem-1 null mice.** (top) The red cell membrane in WT mice consists of a lipid bilayer embedded with two main complexes of structural proteins: The ankyrin complex and the junctional complex (also known as the 4.1R complex), which are connected by horizontal flexible helices of α- and β- spectrin heterodimers and tetramers. Stability of the complexes is regulated in part by phosphorylation of adducin (on Serine 724 in mice) by protein kinase C (PKC), which leads to decreased F-actin capping and dissociation of spectrin from actin. Since PKC-mediated serine phosphorylation is typically opposed by protein phosphatase 2A (PP2A), we propose that PP2A dephosphorylates adducin. PP2A, PKC (bottom), and Hem-1 (top) have all been shown to associate with Rac1. Loss of Hem-1 results in decreased PP2A regulatory subunit B (PP2Ar) and structural subunit A protein expression and increased PKC-mediated phosphorylation of Ser724 on adducin (bottom). Phospho-adducin is then degraded, resulting in the dissociation of spectrin from actin and decreased stability of junctional complex proteins and the membrane cytoskeleton. GPA (Glycophorin A), GPC (Glycophorin C), PP2Ac (protein phosphatase 2A catalytic subunit), PP2Ar (protein phosphatase 2A regulatory subunit).

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spectrin, actin, and protein 4.1. Mice deficient in the junctional complex protein 4.1R also exhibit hemolytic anemia characterized by abnormal erythrocyte morphology, lowered membrane stability, and reduced levels of spectrin [47]. Our results collectively suggest that disruption of Hem-1 preferentially affects components of the junctional complex (such as spectrin, p55, dematin, tropomodulin, and protein 4.1), whereas components of the ankyrin complex are affected to a lesser extent (Figure 7).

Kalfa and colleagues previously found that mice deficient in Rac1 and Rac2 (Rac1−/−Rac2−/− mice) exhibited mild microcytic anemia with significant anisocytosis, poikilocytosis, polychromasia, and increased hypochromic cells, target cells, and fragmented erythrocytes [3]. Rac-deficient erythrocytes also exhibited irregular aggregation of membrane skeletal actin and large gaps in the membrane skeleton when compared to the more uniform lattice-like structure in WT erythrocytes [3]. Rac-null erythrocyte morphology is strikingly similar to Hem1−/− erythrocytes, which is consistent with the observation that Rac1 and Rac2 act upstream of Hem-1 and the WAVE complex in other systems and cell types (see [18] for review). Rac1−/−Rac2−/− erythrocytes also contained increased phospho-adducin and decreased total adducin, similar to Hem1−/− erythrocytes. These collective results suggest a working model whereby Rac acts through Hem-1 to regulate F-actin polymerization and/or stability in erythrocytes. Both Rac and Hem-1 appear to inhibit phosphorylation of α-adducin at Ser724 and β-adducin at Ser713, perhaps by recruiting or stabilizing the PP2A enzyme subunits, thus strengthening the interactions between F-actin and spectrin and preventing degradation of phospho-adducin [48]. This model explains the observed increase in erythrocyte fragility and subsequent abnormal erythrocyte morphology, decreased lifespan, and anemia in both Rac1−/−Rac2−/− and Hem1−/− erythrocytes.

Our proteomic studies also revealed significant alterations in the representation of important metabolic enzymes within the cytosolic fractions of Hem1−/− versus WT erythrocytes. Mass spectrometry indicated statistically significant differences in the expression of 74 proteins in Hem1−/− versus WT erythrocytes, including enzymes involved in key biochemical events such as glycolysis. For example, Hem-1 null erythrocytes contain significantly reduced levels of phosphoglycerate kinase 1 (PGK-1), a glycolytic enzyme which is found deficient in a subset of patients afflicted with nonspherocytic haemolytic anemia [49]. In addition, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is known to bind band 3 and localize to the cytoplasmic surface of the erythrocyte membrane, is also reduced in the cytosolic fraction of Hem-1 null erythrocytes relative to WT erythrocytes [50]. We speculate that disruption of the actin membrane skeleton and reduction in membrane proteins such as band 3 in Hem-1 null erythrocytes results in the release and degradation of key metabolic enzymes, leading to altered energy homeostasis. Additional investigation of the interrelationships between F-actin, membrane skeletal proteins, and key metabolic enzymes in WT and Hem1−/− erythrocytes is needed to better understand the dynamics of the erythrocyte membrane skeleton in the context of normal and abnormal actin protofilaments.

**Supporting Information**

**Table S1 Proteins identified in wildtype erythrocyte insoluble fraction on LTQ-FT.** (XLS)

**Table S2 Proteins identified in Hem1−/− erythrocyte insoluble fraction on LTQ-FT.** (XLS)

**Table S3 Proteins detected in wildtype erythrocyte soluble fraction on LTQ-FT.** (XLS)

**Table S4 Proteins identified in Hem1−/− erythrocyte soluble fraction on LTQ-FT.** (XLS)

**Table S5 Peptide differences in WT and Hem1−/− erythrocyte insoluble fraction detected by CrawDad.** (XLS)

**Table S6 Peptide differences in WT and Hem1−/− erythrocyte soluble fraction detected by CrawDad.** (XLS)

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**Author Contributions**

Conceived and designed the experiments: BMI MMC JMW GM DMG DKH-S JAR. Performed the experiments: BMI MMC JMW MT HP JAR. Analyzed the data: JMW MT HP GM ER JAR. Contributed reagents/materials/analysis tools: BMI MMC JMW GM ER.

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**Supporting Information**

**Table S1 Proteins identified in wildtype erythrocyte insoluble fraction on LTQ-FT.** (XLS)

**Table S2 Proteins identified in Hem1−/− erythrocyte insoluble fraction on LTQ-FT.** (XLS)

**Table S3 Proteins detected in wildtype erythrocyte soluble fraction on LTQ-FT.** (XLS)

**Table S4 Proteins identified in Hem1−/− erythrocyte soluble fraction on LTQ-FT.** (XLS)

**Table S5 Peptide differences in WT and Hem1−/− erythrocyte insoluble fraction detected by CrawDad.** (XLS)

**Table S6 Peptide differences in WT and Hem1−/− erythrocyte soluble fraction detected by CrawDad.** (XLS)

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**Author Contributions**

Conceived and designed the experiments: BMI MMC JMW GM DMG DKH-S JAR. Performed the experiments: BMI MMC JMW MT HP JAR. Analyzed the data: JMW MT HP GM ER JAR. Contributed reagents/materials/analysis tools: BMI MMC JMW GM ER.

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