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Authors

Nishimura, Katherine
Kim, Edward
Hsu, Ann
et al.

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Genetic disruption of KCC cotransporters in a mouse model of thalassemia intermedia.

Boris E. Shmukler[1,2,*], **Alicia Rivera**[3,4,*], **Parul Bhargava**[5], **Katherine Nishimura**[1], **Edward H. Kim**[1], **Ann Hsu**[1], **Jay G. Wohlgemuth**[6], **James Morton**[6], **L. Michael Snyder**[7], **Lucia De Franceschi**[8], **Marco B. Rust**[9], **Christian A. Hubner**[10], **Carlo Brugnara**[2,3], **Seth L. Alper**[1,4]

[1]Renal Division and Vascular Biology Research Center, Beth Israel Deaconess Medical Center, Boston, MA 02215

[2]Department of Medicine, Harvard Medical School, Boston, MA 02215

[3]Department of Laboratory Medicine, Boston Children's Hospital, Boston, MA 02115

[4]Department of Pathology, Harvard Medical School, Boston, MA 02115

[5]Department of Laboratory Medicine, UCSF, San Francisco, CA

[6]Quest Diagnostics, San Juan Capistrano, CA

[7]Quest Diagnostics, Marlborough, MA

[8]Dept. of Medicine, Universita Verona and Azienda Ospedaliera Universitaria Verona, Policlinico GB Rossi, Verona, Italy

[9]institute of Physiological Chemistry, Philipps-Universität Marburg, Marburg, Germany

[10]Institute of Human Genetics, Universitatsklinikum Jena, Jena Germany

Abstract

β-thalassemia [β-Thal] is caused by defective β-globin production leading to globin chain imbalance, aggregation of free alpha chain in developing erythroblasts, reticulocytes, and mature circulating red blood cells. The hypochromic thalassemic red cells exhibit increased cell dehydration in association with elevated K^+ leak and increased K-Cl cotransport activity, each of which has been linked to globin chain imbalance and related oxidative stress.

We therefore tested the effect of genetic inactivation of K-Cl cotransporters *KCC1* and KCC3 in a mouse model of β-thalassemia intermedia. In the absence of these transporters, the anemia of β-Thal mice was ameliorated, in association with increased MCV and reductions in CHCM and hyperdense cells, as well as in spleen size. The resting K content of β-Thal red cells was greatly increased, and Thal-associated splenomegaly slightly decreased. Lack of KCC1 and KCC3 activity in Thal red cells reduced red cell density and improved β-Thal-associated osmotic fragility. We conclude that genetic inactivation of K-Cl cotransport can reverse red cell dehydration and partially attenuate the hematologic phenotype in a mouse model of β-thalassemia.

Keywords

K^+ efflux; osmotic fragility; red cell density; spleen

INTRODUCTION

β-globin gene mutations associated with β-thalassemia carrier status affect >1-5% of the world population concentrated in the Mediterranean basin and the equatorial belt, with local allele frequencies as high as 30%. These mutations include missense, nonsense and variably sized indel mutations in both coding and noncoding regulatory regions of the β-globin gene, causing decreased abundance of structurally intact β-globin polypeptide, and leading to α/β globin chain imbalance and consequent precipitation of unassembled or misfolded globin chains that cannot assemble into normal hemoglobin $\alpha_2\beta_2$ heterotetramers (19, 37, 38).

β-thalassemia intermedia manifests as chronic hemolysis, ineffective erythropoiesis, and iron overload (19, 37, 38). Long-term treatment has until recently been limited to blood transfusion and iron chelation. Genetic or pharmacological derepression of γ-globin expression, or controlled genetic or pharmacological suppression of α-globin expression remain early stage approaches with challenging safety profiles (19, 37, 38). Although hematopoietic stem cell transplantation from a fully matched donor can be curative, even the few such matches available are accompanied by risks of graft vs. host disease or rejection (4).

Recent therapeutic options to have entered clinical trial include activin receptor trap ligands such as sotatercept and luspatercept, and JAK2 inhibitors such as ruxolitinib. Iron restriction strategies such as minihepcidins and TMPRSS6 inhibitors are designed to reduce transfusion requirement and the need for chelation therapy (19, 37, 38). Perhaps the most exciting long-term therapeutic development is *ex vivo* complementation (39) or gene-editing correction (40) of β-globin mutations in patient hematopoietic stem cells. However, small molecule therapies of lower cost and wider availability will remain needed for treatment of the vast majority of thalassemia patients, especially those in less developed countries.

In this context, we have readdressed the possible therapeutic utility of targeting pathological red cell dehydration in thalassemia intermedia. β-Thalassemic [β-Thal] red cells in humans (9, 31) and in mice (11) exhibit increased cell dehydration in association with elevated K^+ leak and elevated K-Cl cotransport activity. Higher mean corpuscular hemoglobin concentration [MCHC] and lower mean corpuscular volume [MCV] were found to correlate

with greater clinical severity of β/+ thalassemia (29). Indeed the increased K^+ leak in thalassemia has been associated with the abundance of intracellular globin aggregates arising from globin chain imbalance and the resulting oxidative stress (25). Increasing red cell Mg content through dietary supplementation can reduce red cell dehydration in thalassemic mice (11), but subsequent attempts to confirm this result in humans have not shown efficacy (12). Currently available small molecule inhibitors of the elevated K-Cl cotransporters of thalassemic red cells are not of adequately high affinity and specificity to allow clinical use.

To evaluate the possible utility of such K-Cl cotransport inhibitors for thalassemia, we generated mouse models of thalassemia intermedia genetically deficient in the erythroid K-Cl cotransporters *Kcc1* and *Kcc3*. We characterized the hematological indices and K^+ transport properties of the red cells of these mice. We found that genetic and functional inactivation of the two K-Cl cotransporters KCC1 and KCC3 led to near-complete rescue of hematocrit, in parallel with increased MCV, decreased CHCM and increased osmotic fragility. Loss of KCC activity also decreased red cell density and increased red cell K content. However, cellular heterogeneity (RDW) was not decreased, while splenomegaly was only slightly decreased. Moreover, reticulocyte count was increased, rather than decreased. Thus, the pathological cell dehydration of circulating thalassemic red cells was substantially reversed by genetic inactivation of the K-Cl cotransporters KCC1 and KCC3.

MATERIALS AND METHODS

Materials:

Salts, reagents and solvents were from Sigma-Aldrich (St. Louis, MO). Staurosporine, wortmannin, PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine), D4476 (4-[4-(2,3-Dihydro-1,4-benzodioxin-6-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide), and DMAT [2-(Dimethylamino)-4,5,6,7-tetrabromo-1H-benzimidazole] were from Calbiochem (San Diego, CA).

Mice:

Mice were housed in humidity- and temperature-controlled rooms in the AAALAC-approved Animal Research Facility of Beth Israel Deaconess Medical Center under IACUC-approved protocols. Hbb$^{th3/+}$ (β-Thal] transgenic mice originally on the C57BL/6Jx129Ola background (42) had been bred onto a C57BL6 background. K-Cl cotransporter-deficient mice $Kcc1^{+/-}$, $Kcc3^{+/-}$ and $Kcc1^{-/-};Kcc3^{+/-}$ (obtained from Prof. Thomas J. Jentsch) were created and genotyped as described (30). Thal;$Kcc1^{-/-}$ mice were created by crossing β-Thal mice with $Kcc1^{-/-}$ mice, then back-crossing Thal;$Kcc1^{+/-}$ mice with $Kcc1^{-/-}$ mice and selecting the desired genotype. Thal;$Kcc1^{-/-};Kcc3^{+/-}$ and Thal;$Kcc1^{-/-};Kcc3^{-/-}$ (Thal2KO) mice were generated by crossing Thal;$Kcc1^{-/-}$ mice with $Kcc1^{-/-};Kcc3^{+/-}$ mice, with intercrossing of progeny to select the desired genotypes. In every mating to generate β-Thal progeny, the β-Thal allele was provided by a single parent. Wild type mice used for comparison with knockout mice were wild type progeny of β-Thal x WT C57BL6/J crosses [WT^{Thal}] and of further generations of β-Thal x WT^{Thal} crosses.

As previously observed (30, 33), $Kcc3^{-/-}$ mice reached weaning at 22% of predicted Mendelian frequency, and of these 50% survived to age 6 weeks. Thal;$Kcc3^{-/-}$ mice reached weaning at a frequency 34% of predicted, but none survived to age 6 weeks. Interestingly, although Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ mice reached weaning at a similar 30% of predicted frequency, 93% of weaned mice survived to age 6 weeks. Thus, further studies were conducted with Thal;$Kcc1^{-/-}$ and with Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ mice.

Genotyping:

Hbb$^{th3/+}$ [β-Thal] transgenic mice were generated by "plug and socket" targeted deletion of both β-major and β-minor genes (42) with targeted replacement by a construct including human hypoxanthine phosphoribosyltransferase 1 (HPRT). We therefore followed the Thal allele with oligos selective for human but not mouse HPRT. Forward oligo HPRT.F1 (5'-TTCAAGTTGAGTTTGGAAACATCTG-3'] and reverse oligo HPRT.R2 (5'-TTCAATCACTTCTATTCTTTCCTGC-3'] were annealed at 60°C and produced a 369 bp PCR product after 35 cycles of PCR. We included as a PCR reaction positive control the primer pair of KCC1.E20F (5'-ACCTCCGCCTGGAAGCTGAAG-3'] and KCC1.CtM (5'-CTCAGGARTAGATGGTGATGACTTC-3'], which yielded a 1,297-bp mouse KCC1 genomic amplification product (30).

Histology:

After terminal exsanguination, spleens were excised and immersion-fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS, then stored at 4°C in PBS containing 0.02% sodium azide. Paraffin-embedded tissue was sectioned and stained with hematoxylin-eosin for microscopic examination.

Preparation of erythrocytes for flux studies:

Blood was collected in heparinized syringes by cardiac puncture of Avertin-anesthetized mice according to protocols approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center. Blood was centrifuged at 2,500 rpm in 50 ml Falcon tubes for 5 min at 4°C. After careful removal of the buffy coat by aspiration, packed cells were washed 5 times at 4°C in ~20 volumes of wash solution (in mM): 172 Choline Cl, 1 $MgCl_2$, 10 Tris MOPS, pH 7.40 at 4°C. Cells were suspended to 30–50% cytocrit in wash solution and kept at 4°C for same-day use in flux studies. Red blood cell indices were measured with the ADVIA 120™ hematology analyzer, using mouse software (Siemens Diagnostic Solutions, Tarrytown, NY) (16, 32). Total erythrocyte contents of Na^+, K^+, and Mg^{2+} were determined in freshly isolated mouse erythrocytes by atomic absorption spectrophotometry (AAnalyst 800; Perkin Elmer, Norwalk, CT) (27).

Erythrocyte K^+ fluxes:

K-Cl cotransport (KCC) activity was determined as bath chloride-dependent K^+ efflux in erythrocytes exposed to hypotonic swelling in the presence of 1 mM ouabain and 10 μM bumetanide (27, 30, 32, 33). Freshly isolated erythrocytes were incubated in either isotonic or hypotonic solutions containing (in mM) 115 NaCl or Na Sulfamate, 1 $MgCl_2$, 10 glucose, 10 Tris-MOPS pH 7.4 at 37°C, 1 ouabain, and 0.01 bumetanide (255–265 mOsm) or into

isotonic NaCl or Na sulfamate solutions containing (in mM) 160 NaCl, 1 $MgCl_2$, 10 glucose, and 10 Tris-MOPS pH 7.4 at 37°C.

Aliquots were removed after 5 min and 25 min incubation at 37°C and immediately transferred to precooled 4 ml plastic tubes. Post-centrifugation supernatant $[K^+]$ was calculated from K content measured by atomic absorption. K^+ efflux was calculated from the slope of the linear regression of K^+ content vs. time. Chloride-dependent KCC activity was estimated by subtracting K^+ efflux in the presence of sulfamate from that in the presence of chloride. Chloride-dependent KCC activity measured in isotonic medium was compared with that measured in hypotonic medium, or with isotonic medium containing added staurosporine (1 μM) or added urea (500 mM).

Phthalate density profile:

Erythrocyte density distribution profiles of freshly obtained whole blood were measured at 23°C in microhematocrit tubes containing phthalate esters of density between 1.076 and 1.108 g/ml (16). The % cells of density >X was calculated from the total cell content sedimented below the phthalate oil density boundary in the lower oil zone divided by the total cell volume. The phthalate oil D_{50} value is the 50th %ile of density of the red cell population, and describes alterations in RBC density profiles (8, 10).

Osmotic fragility measurement:

Osmotic fragility of erythrocytes was measured on freshly collected heparinized blood. RBCs washed 3x with choline wash solution and once in isotonic saline were resuspended at 10% cytocrit. Aliquots were suspended in varying concentrations of NaCl and centrifuged 5 min at 3,000 RPM at 22°C. A_{540} was measured in the resultant supernatants and appropriate controls to calculate values of % RBC lysis. Osmotic fragility curves were generated by plotting salt concentration vs. % hemolysis.

Statistical analysis

All results are expressed as means ± SEM. As the data were non-parametric, they were analyzed by ANOVA with Mann-Whitney or Kruskall-Wallis non-paired tests using Prism 8 software (GraphPad). Significance was set at $p < 0.05$.

Results

Genetic inactivation of *Kcc1* and *Kcc3* increases hematocrit and MCV and reduces dehydration in β-Thal mouse red cells.

Genetic inactivation of *Kcc1* and *Kcc3* resulted in amelioration of murine β-Thal anemia, whereas the effect of *Kcc1* inactivation alone was much more modest (Table 1). The near-normal hematocrit of Thal;$Kcc1^{-/-};Kcc3^{-/-}$ mice was accompanied by increased red cell mean corpuscular volume (MCV; 53.4 vs. 35.9 fL for Thal) and reduced red cell values of corpuscular hemoglobin concentration, mean (CHCM; 21.6 vs. 27.5 g/dL). Although β-Thal red cells were not on average dehydrated compared to WT^{Thal} erythrocytes (Table 1), the % of hyperdense red cells was elevated in β-Thal mice (3.7% vs 0.2% for WT). The proportion of hyperdense cells in Thal;$Kcc1^{-/-};Kcc1^{-/-}$ mice trended towards lower values as

compared to β-Thal mice, but the comparison did not achieve statistical significance (Table 1).

The elevated red cell volume distribution width (RDW) value of β-Thal red cells (33.7%) was decreased by combined inactivation of $Kcc1^{-/-}$ and $Kcc3^{-/-}$ (29.3%), even as accompanied by exacerbated reticulocytosis (27.8% vs. 18.6% for β-Thal red cells; (Table 1). Profiles of red cell volume and hemoglobin concentration from representative individual mice are shown in Figure 1. The left-shifted distribution of single cell volume in β-Thal vs. WT^{Thal} red cells changes was reversed and exceeded in red cells from a Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ mouse. In contrast, the left-shifted distribution of single cell hemoglobin concentrations in Thal red cells compared with WT^{Thal} red cells was further left-shifted in β-Thal red cells by combined inactivation of $Kcc1$ and $Kcc3$.

The changes induced by combined inactivation of $Kcc1^{-/-}$ and $Kcc3^{-/-}$ in circulating red cells were qualitatively paralleled by the smaller relative changes observed in MCV and CHCM values of ADVIA-defined reticulocyte populations. Thal reticulocyte MCV remained lower than that of WT^{Thal} reticulocytes, even while % hyperdense cells among ADVIA-defined reticulocytes was slightly increased. However, combined inactivation of $Kcc1^{-/-}$ and $Kcc3^{-/-}$ in β-Thal red cells completely rescued reticulocyte MCV to wild-type values (Table 2). The less dramatically elevated values of RDW and % hyperdense cells in reticulocytes were not statistically reduced in Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ mice (Table 2). Inactivation of either or both K-Cl cotransporter genes exacerbated reticulocytosis in β-Thal mice.

Genetic inactivation of *Kcc1* and *Kcc3* increases cation content of Thal red cells.

The greatly elevated MCV of Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ red cells was paralleled by elevated red cell K content (Table 3). Interestingly, the elevated Na content also present in Thal red cells was further elevated in the genetic absence of KCC1 and KCC3. Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ red cells also displayed elevated Mg content compared to β-Thal (Table 3).

Combined genetic inactivation of Kcc1 and Kcc3 does not exacerbate the splenomegaly of β-Thal mice.

Figure 2 demonstrates that the splenomegaly of thalassemic mice is not to a statistically significant degree further increased by genetic inactivation of *Kcc1*, or by incremental haploinsufficiency of *Kcc3*. However, combined inactivation of *Kcc1* and *Kcc3* slightly attenuates the splenomegaly of β-Thal mice. Histological examination of spleens from 2.5 month old Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ mice revealed congested red pulp (Fig. 3 A,B,D,E) with hemosiderin-laden macrophages, nucleated erythroid precursors and anucleate RBCs. The spleen from an older β-Thal mouse exhibited a more developed white pulp together with continued red pulp congestion (Fig. 2C,F) with more abundant hemosiderin deposits and trilineage hematopoiesis, including clusters of megakaryocytes, myeloid, and erythroid precursors.

Genetic inactivation of Kcc1 and Kcc3 further decreases Thal red cell density.

Although the phthalate density profile of Thal red cells was greatly left-shifted from that of WT^{Thal} red cells (Figure 4), the proportion of β-Thal red cells of density   1.1 g/ml was measurable, whereas that of WT cells was near-zero. β-Thal red cell density was minimally reduced by inactivation of *Kcc1* alone, or with incremental haploinsufficiency of *Kcc3*. However, combined inactivation of *Kcc1* and *Kcc3* led to a dramatic left shift of the β-Thal red cell density profile which, nonetheless, preserved some red cells of density   1.1 g/dL (Figure 4).

Genetic inactivation of Kcc1 and Kcc3 partially reverses the reduced osmotic fragility of β-Thal red cells.

β-Thal red cells displayed reduced osmotic fragility compared to WT^{Thal} red cells, with a greatly left-shifted osmotic fragility profile much flatter than the sigmoidal WT profile (Figure 5). Genetic inactivation of *Kcc1* alone or with incremental haploinsufficiency of *Kcc3* produced minimal change in β-Thal red cell osmotic fragility. However, combined inactivation of *Kcc1* and *Kcc3* partially restored β-Thal red cell osmotic fragility towards WT levels for the most osmotically resistant 65% of cells, while maintaining the flattened profile characteristic of β-Thal cells. The remaining 35% of Thal;*Kcc1$^{-/-}$;Kcc3$^{-/-}$* red cells exhibited osmotic fragility exceeding that of WT cells, and half of these more fragile Thal;*Kcc1$^{-/-}$;Kcc3$^{-/-}$* red cells also exceeded the osmotic fragility of the least fragile β-Thal cells (Figure 5). Thus, loss of erythroid KCC activity in β-Thal red cells led to a complex change in osmotic fragility phenotype.

K-Cl cotransport activity in β-Thal red cells without and with genetic inactivation of *Kcc1* and *Kcc3*.

Although basal KCC activity (defined as Cl$^-$-dependent K$^+$ efflux in isotonic medium) was indistinguishable between WT^{Thal} and β-Thal red cells, KCC activity stimulated by hypotonicity (HYPO) or by 1 μM staurosporine (Stauro) was higher in β-Thal than in WT red cells (Figure 6). In contrast, urea-stimulated KCC activity was not higher in β-Thal than in WT red cells. Genetic inactivation of Kcc1 in β-Thal red cells reduced staurosporine-stimulated KCC activity to WT levels, while further increasing urea-stimulated KCC activity above WT and β-Thal levels (Figure 6).

Red cells from Thal;*Kcc1$^{-/-}$;Kcc3$^{+/-}$* mice retained stimulation by hypotonicity and by staurosporine at lower levels than in Thal;*Kcc1$^{-/-}$* red cells, whereas stimulation by urea was only minimally reduced. However, combined inactivation of *Kcc1* and *Kcc3* abrogated stimulation of K-Cl cotransport by all three stimulatory conditions (Figure 6).

The measurements of KCC activity summarized in Figure 6 were limited to experiments in which each red cell sample was tested in the presence of Cl$^-$-containing media and SFA-containing media. Combined ion transport results from the entire study, including experiments in which red cells were tested in the presence of either Cl$^-$- or SFA-containing media, but not both (due to blood volume limitations or other reasons), are presented in Supplemental Figures 1 and 2.

Kinase inhibitor effects on KCC activity in β-Thal red cells.

Hypotonic stimulation of KCC activity in normal human red cells is mediated in large part by inhibition of KCC phosphorylation mediated by the WNK1-OSR1 kinase cascade (26) (34, 43). The wide-spectrum, ATP-competitive kinase inhibitor, staurosporine, has been shown directly to inhibit WNK1 (41), and as shown in Fig. 6 and Supplememental Fig. 3 also activates KCC activity in β-Thal red cells. PP1 and PP2, wide spectrum Src family tyrosine kinase inhibitors, also inhibit WNK1 directly (41), and PP2 activates KCC in β-Thal red cells, but to a lesser degree than staurosporine. Although evidence of wortmannin-sensitive Akt phosphorylation and NOS activation in red cells has been reported (22, 36), exposure of β-Thal red cells to the PI3-kinase inhibitor wortmannin failed to stimulate KCC activity in isotonic medium. In addition, CK1 inhibitor D4476, the CK2 inhibitor DMAT. The direct effects of these inhibitors in the presence or absence of bath Cl^- (with SFA substitution) is presented in Supplemental Figure 3.

Discussion

β-thalassemic red cells of humans (9, 29, 31) and mice (11) have been reported to be dehydrated, with decreased K content and increased K^+ leak mediated by elevated K-Cl cotransport activity (usually without elevation of KCNN4 activity). Elevated MCHC and lower MCV correlate with increased clinical severity of β/+ thalassemia (29), in association with increased intracellular globin chain aggregation.

Red cell dehydration predisposes to increased clinical severity in sickle cell disease by shortening the delay time for the polymerization of deoxyhemoglobin S that causes red cell sickling. Human sickle red cells exhibit elevated KCNN4 and KCC activities. Inhibition of red cell dehydration by the KCNN4 inhibitor senicapoc in Phase 2 and 3 trials increased blood hemoglobin by nearly 1 g/dL (5, 6). Inhibitors of red cell KCC are not of sufficiently high affinity and selectivity for clinical use, but genetic inactivation of KCC1 and KCC3 was shown to reduce red cell dehydration in a mouse model of sickle cell disease (30). Incremental inactivation of KCNN4 further reduced and reversed red cell dehydration in SAD mice (33).

We tested here the hypothesis that *Kcc1* and *Kcc3* are major determinants of the erythrocyte ion composition and volume characteristics in a mouse model of thalassemia. We found that the reduced red cell MCV of our β-Thal mouse strain was unaccompanied by dehydration of the cell population compared to WT^{Thal} red cells, but displayed increased % of hyperdense cells. However, the reduced hematocrit of β-Thal mice was substantially elevated by genetic inactivation of erythroid K-Cl cotransport, accompanied by greatly elevated MCV and decreased CHCM, reduction in % hyperdense cells, and greatly increased β-Thal red cell K content to normal or abovenormal levels. The reduced red cell density of thalassemic red cells (vs. WT) was little altered by absence of KCC1 or by incremental haploinsufficiency of KCC3, but further reduced by the combined absence of KCC1 and KCC3 (Figure 4). The dramatically left-shifted and flattened osmotic fragility curve of β-Thal red cells (vs. WT) was similarly little altered by lack of KCC1 or by incremental haploinsufficiency of KCC3. However, complete lack of KCC1 and KCC3 in Thal red cells right-shifted the osmotic fragility curve. This right shift partially restored osmotic fragility in the majority of cells

towards the WT condition, in a manner consistent with the corresponding increase in MCV. However, the small fraction of the most osmotically sensitive Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ red cells exceeded the osmotic fragility of intact Thal red cells (Figure 5), suggesting increased membrane fragility perhaps related to loss of KCC-associated membrane scaffolding/ signaling or other cytoskeletal functions, possibly leading to reduced surface area and/or increased membrane leakiness.

Splenomegaly and reticulocytosis:

The splenomegaly of β-Thal mice was not significantly affected by genetic inactivation of *Kcc1* or by additional heterozygosity for *Kcc3*, but was slightly improved by combined inactivation of Kcc1 and Kcc3. Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ spleens were remarkable for erythroid congestion, and numerous hemosiderin-laden erythroblasts, indicative of extramedullary hematopoiesis. The increased reticulocytosis of Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ mice could reflect attenuation of ineffective erythropoiesis, as perhaps suggested by the splenic erythroid hyperplasia. Consistent with this possibility is the increased calculated value for mean corpuscular hemoglobin (MCH) in Thal red cells lacking both KCC1 and KCC3 (not shown). However, such an interpretation might contrast with our previous report (14) that levels of KCC1 and KCC3 mRNAs and KCC polypeptides increased in cultured human CD34+ normal and thalassemic erythropoietic precursors during late-stage erythopoiesis. Moreover, treatment of thalassemic erythropoietic precursors with K-Cl cotransport inhibitor DIOA blocked *in vitro* differentiation at the orthochromatic normoblast stage and upregulated p21 and caspase 8, accompanied (in both cell models) by cyclin-D upregulation and reduction in S-phase cells (14). Further study will be required to test if these changes occur during murine erythroid development in the genetic absence of KCC1 and KCC3. Nonetheless, the failure of genetic inactivation of KCC1 and KCC3 to correct the reticulocytosis of this Thal mouse model suggests that monotherapy with small molecule KCC inhibitors will not likely reduce the hemolysis, erythroid turnover, and resultant iron overload underlying much of the chronic pathology of B-thalassemia intermedia.

Red cell ion content:

We found that β-Thal ($Hbb^{Th3/+}$) red cell ion contents differed from those previously reported, as compared to WT cell ion contents. Thus, in ENU-mutated $Hbb^{Thal/Thal}$DBA2/J mice (35) subsequently bred into the C57BL6/J background (11, 15, 28), the K content of β-Thal red cells from $Hbb^{Thal/Thal}$ on the C57BL/6J background was much lower than that of corresponding WT red cells. In a previous report on $Hbb^{Th3/+}$ mice 4-6 months of age, red cell K content was less dramatically decreased compared to that in C57B16/J red cells (17). However, we found that $Hbb^{Th3/+}$ red cell K content in our colony was equivalent to that of WT^{Thal} cells. As the average K content of the circulating red cell population includes both dehydrated cells with low K and young overhydrated cells with high K, these components of the population may vary across the various β-Thal mouse lines. Nevertheless, the K content of β-Thal red cells was dramatically increased by genetic inactivation of *Kcc1* and *Kcc3*, resembling the increase in sickle red cell K content produced by similar genetic ablation in the SAD mouse model of sickle cell disease (33).

The increased β-Thal red cell K content resulting from combined genetic inactivation of *Kcc1* and *Kcc3* was accompanied by increased intracellular contents of both Na and Mg. This result differed from the unchanged Na and Mg contents of red cells from $Hbb^{Thal/Thal}$ mice on the C57BL/6J background (11,15) or the previously reported statistically insignificant increase in Na content of $Hbb^{Th3/+}$ red cells (17). The increased Mg content in Thal;$Kcc1^{-/-};Kcc3^{-/-}$ red cells in our study is consistent with the reduced inward Na^+ gradient, which would decrease the inward Na^+ driving force driving red cell Mg^{2+} efflux mediated by Na^+/Mg^{2+} exchange activity.

The elevated Na content could reflect inhibition of Na^+,K^+-ATPase activity by elevated intracellular Mg^{2+}, as reported in normal human red cells (20). Indeed, phenylhydrazine treatment of normal human red cells to mimic the oxidative damage of thalassemia also decreased Na^+,K^+-ATPase activity (25). In contrast, red cell membranes (24) and intact red cells (25) from human *β*-thalassemia intermedia patients exhibited increased Na^+,K^+-ATPase activity. However, this increased Na^+,K^+-ATPase activity in intact cells was greatly reduced by dietary Mg supplementation, in parallel with increased cell Na content (12). The elevated Na content of mouse Thal;$Kcc1^{-/-};Kcc3^{-/-}$ red cells in the current study might reflect elevated oxidative stress (1, 23) in our β-Thal colony at some point during erythropoiesis (14) and/or increased mechanical stress experienced by circulating cells of larger volume (25).

KCC regulation in β-Thal red cells:

The stimulation of mouse β-Thal red cell KCC activity by hypotonicity and by staurosporine confirms previous reports of De Franceschi et al (17), which established a role for oxidative membrane damage in increasing both basal and stimulated KCC activity. Our current data further establish the partial dependence of staurosporine-stimulated KCC activity on the *Kcc1* gene product (Fig. 6). These data also demonstrate for the first time that urea also stimulates KCC activity in mouse β-Thal red cells, but (unlike for staurosporine or hypotonicity) not to a greater degree than in WT red cells. Curiously, urea-stimulated KCC activity in β-Thal red cells was potentiated by solitary genetic inactivation of *Kcc1*, whereas KCC stimulation by hypotonicity was unaffected by loss of *Kcc1* (Fig. 6)

Kinase inhibitor-mediated KCC activation in β-Thal red cells:

KCC activity in β-Thal red cells was stimulated by the nonspecific kinase inhibitor, staurosporine and, to a lesser extent, by the Src family kinase inhibitor, PP2 (Supplemental Fig. 3). PP2 stimulation of K-Cl cotransport was previously demonstrated in WT mouse red cells, but was shown not to further stimulate the already elevated level of red cell K-Cl cotransport in the SAD mouse model of sickle cell disease (18). Both staurosporine and PP2 can also inhibit WNK1 kinase (41), which is active in normal human red cells as a regulator of erythroid KCC proteins (26) and NKCC1 (43). PP2-mediated inhibition of Src family kinases also contributes to KCC activation by increasing activity and decreasing membrane localization of protein phosphatases −1 and −2 (7, 13).

The inability of D4476 and DMAT to stimulate β-Thal red cell KCC makes a stimulatory role for casein kinases unlikely. Demonstrated cross-inhibition of p38-MAPK by D4476 and

of Pim kinases by DMAT further suggests lack of a KCC stimulatory role for these erythroid kinases, both of which function during erythropoiesis, and (in the case of p38-MAPK) persist in circulating erythrocytes (3, 21). The inability of wortmannin to stimulate β-Thal red cell KCC activity makes a stimulatory role for phosphatidylinositol-3-kinase unlikely. Crossinhibition of PLK1 (polo-like kinase-1) by wortmannin additionally suggests lack of a KCC stimulatory role for PLK1, which (at least by pharmacological evidence) remains active in circulating normal human red cells (2).

In summary, genetic ablation of *Kcc1* and *Kcc3* in a mouse model of thalassemia increased indices of red cell hydration, but was accompanied by elevated reticulocytosis and complex effects on red cell osmotic fragility. We conclude that KCl cotransport activity encoded by the KCC polypeptide products of the *Kcc1* and *Kcc3* genes is a major determinant of red cell dehydration in the *Hbb^(Th3/+)* mouse model of β-thalassemia minor. Additional experiments will be required to explore the possibility, in light of the heterogeneous changes in Thal;*Kcc1^(−/−)*;*Kcc3^(−/−)* mouse red cell osmotic fragility, that the concomitant increase in reticulocytosis may in part reflect attenuation of ineffective erythropoiesis and/or prolongation of thalassemic erythrocyte circulating half-life for at least a fraction of these cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

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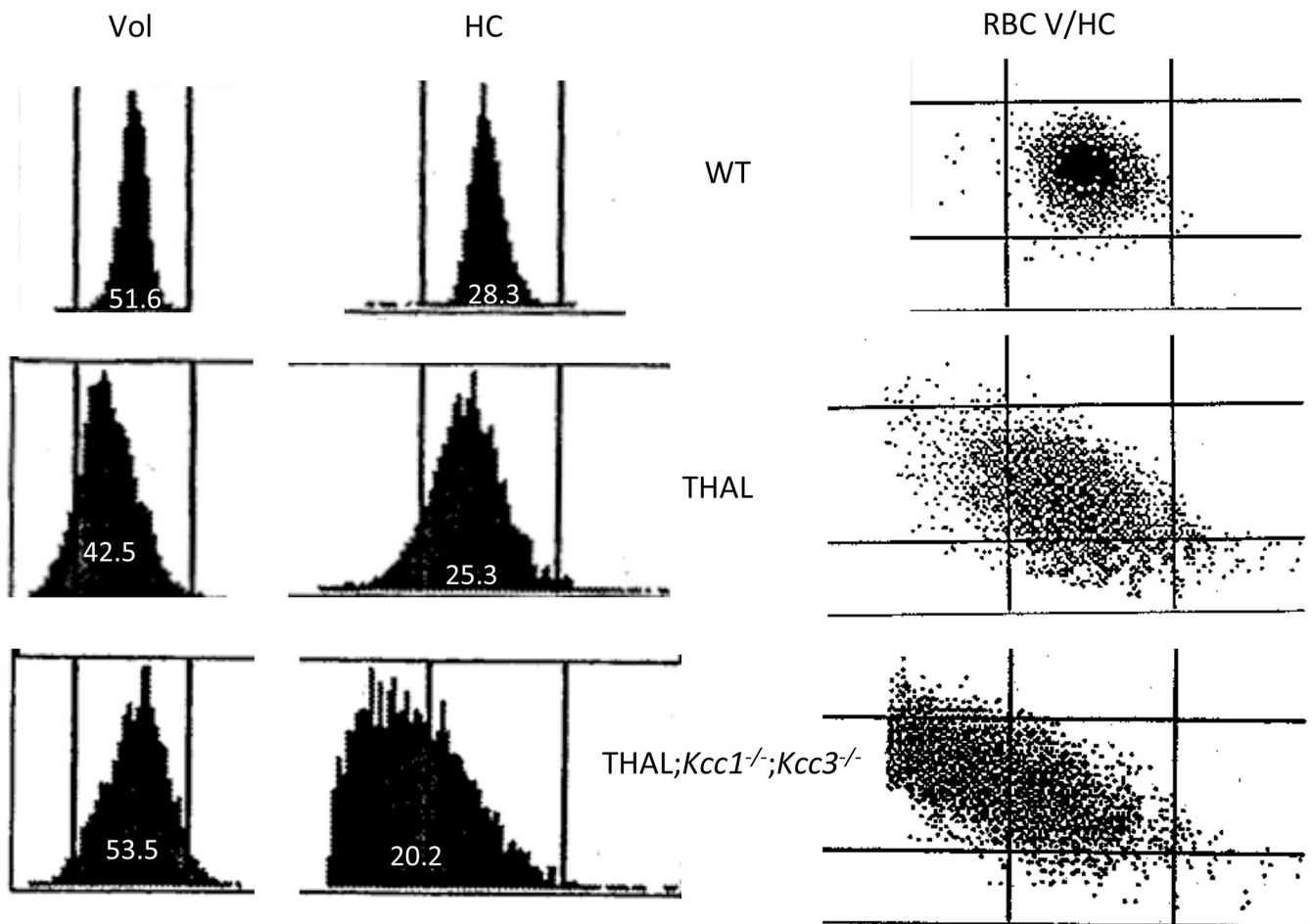
Figure 1. Distributions of single red cell volume and hemoglobin concentration from representative mice of the indicated genotypes.

At left, hemoglobin concentration distributions (HC) and volume distributions (Vol) among red cells from individual mice of the indicated genotypes. Inset values of MCV and of CHCM are in white. Rightmost graphs plot cell volume (Y axis) vs. cell hemoglobin concentration (X axis).
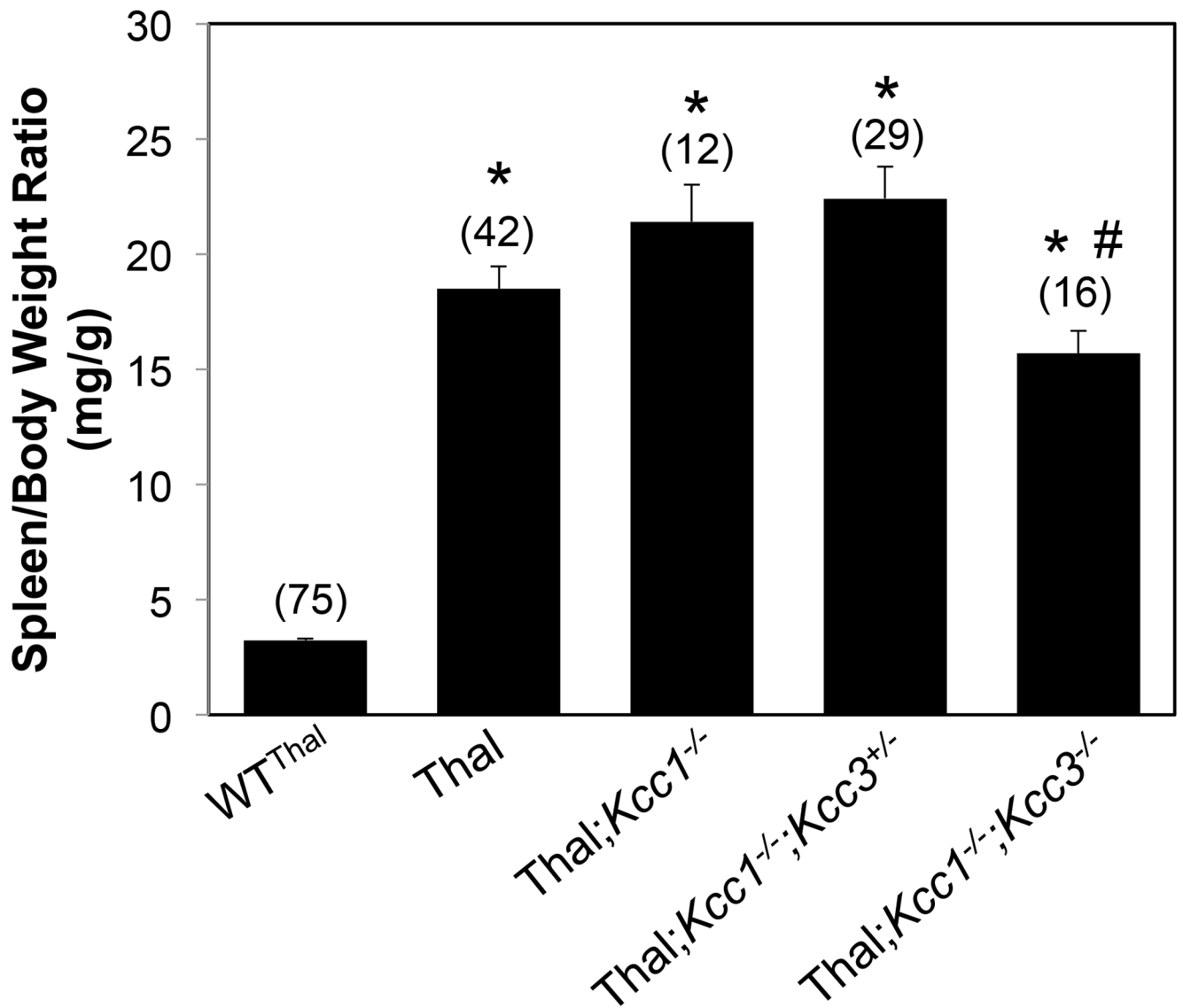
Figure 2. Spleen-body weight ratio of WT and β-Thal mice of indicated genotypes.
Values represent mean ± SEM for (n) mice. *, $p < 0.0001$ vs. WT[Thal] (ANOVA, non-parametric Kruskal-Wallis. Thal;$Kcc1^{-/-}$;$Kcc3^{+/-}$ vs Thal; $Kcc1^{-/-}$;$Kcc3^{+/-}$; #, $p < 0.001$ vs. Thal;$Kcc1^{-/-}$;$Kcc3^{+/-}$ (Mann-Whitney test, non-paired).
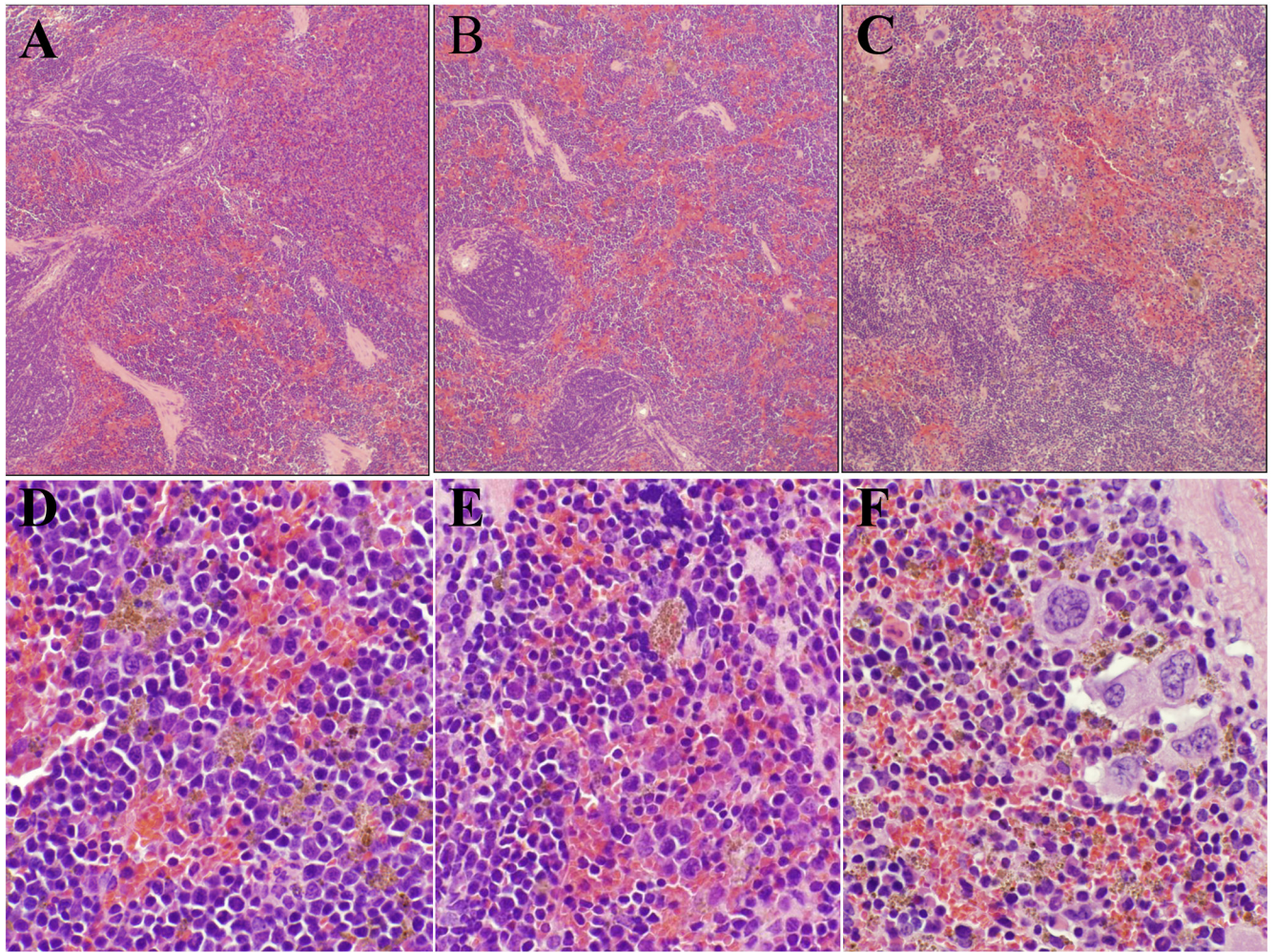
Figure 3. Spleen histology.
Hematoxylin-eosin-stained sections of spleens from two Thal;*Kcc1⁻/⁻;Kcc3⁻/⁻* mice, aged 2.5 months (A,B; 10x magnification) show congested red pulp. The 60x-magnified photomicrographs (D,E) reveal abundant hemosiderin-laden macrophages with golden-brown pigment, nucleated erythroid precursors and anucleate RBCs. The spleen of a β-Thal mouse aged 5.7 months (C, 10x) shows a more developed white pulp along with congested red pulp. At 60x magnification (F) the β-thal spleen reveals abundant hemosiderin deposits and trilineage hematopoiesis, including clusters of megakaryocytes, myeloid and erythroid precursors.
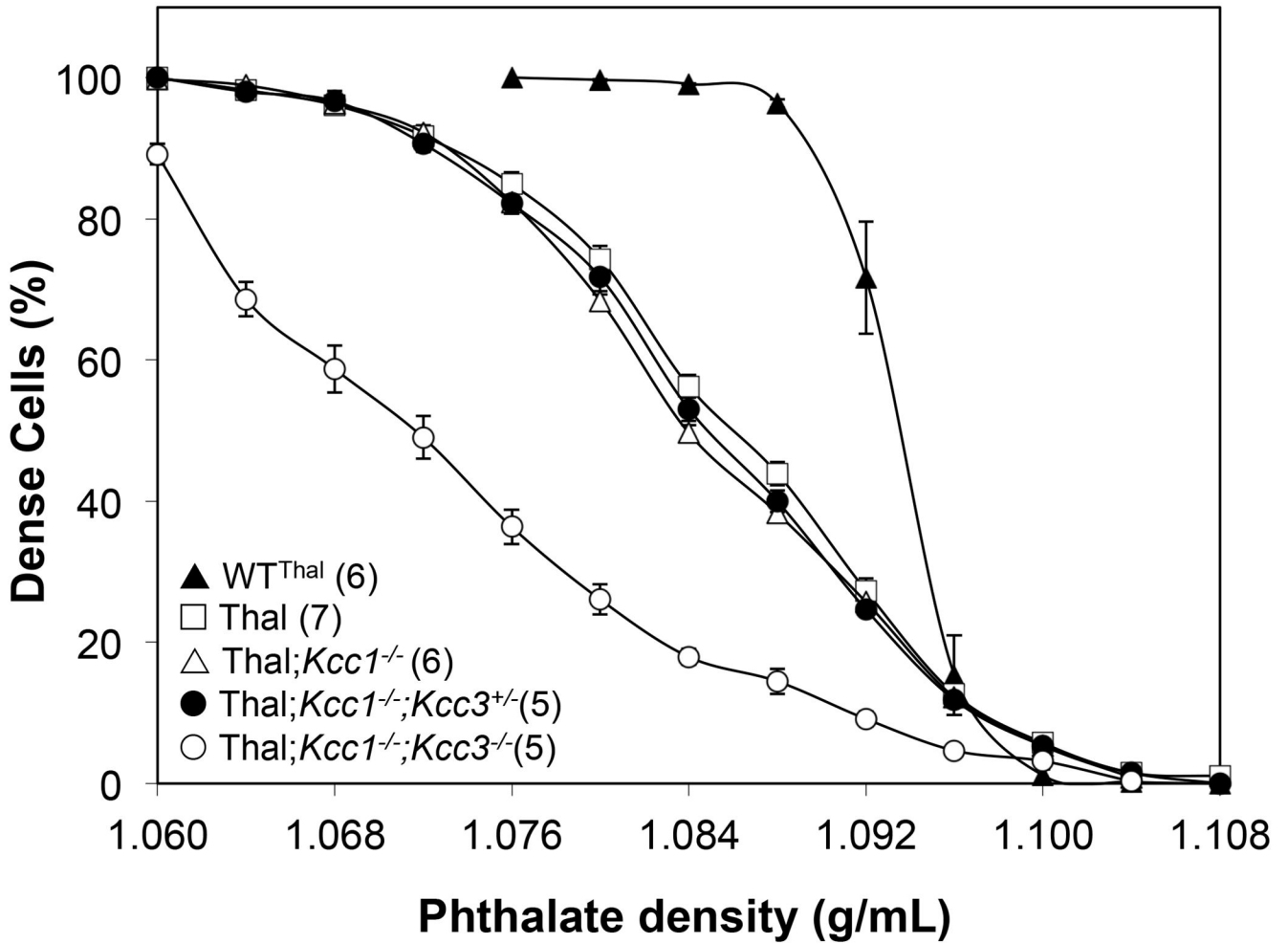
Figure 4. Red cell phthalate density profiles as function of genotype.
A. Red cells from WT^{Thal} (black triangles) and β-Thal mice (open square) compared with red cells from Thal;$Kcc1^{-/-}$ (open triangles), Thal;$Kcc1^{-/-}$;$Kcc3^{+/-}$ (black circles), and Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ mice (open circles). Values are means ± SEM for (n) independently tested pooled samples of indicated genotype.
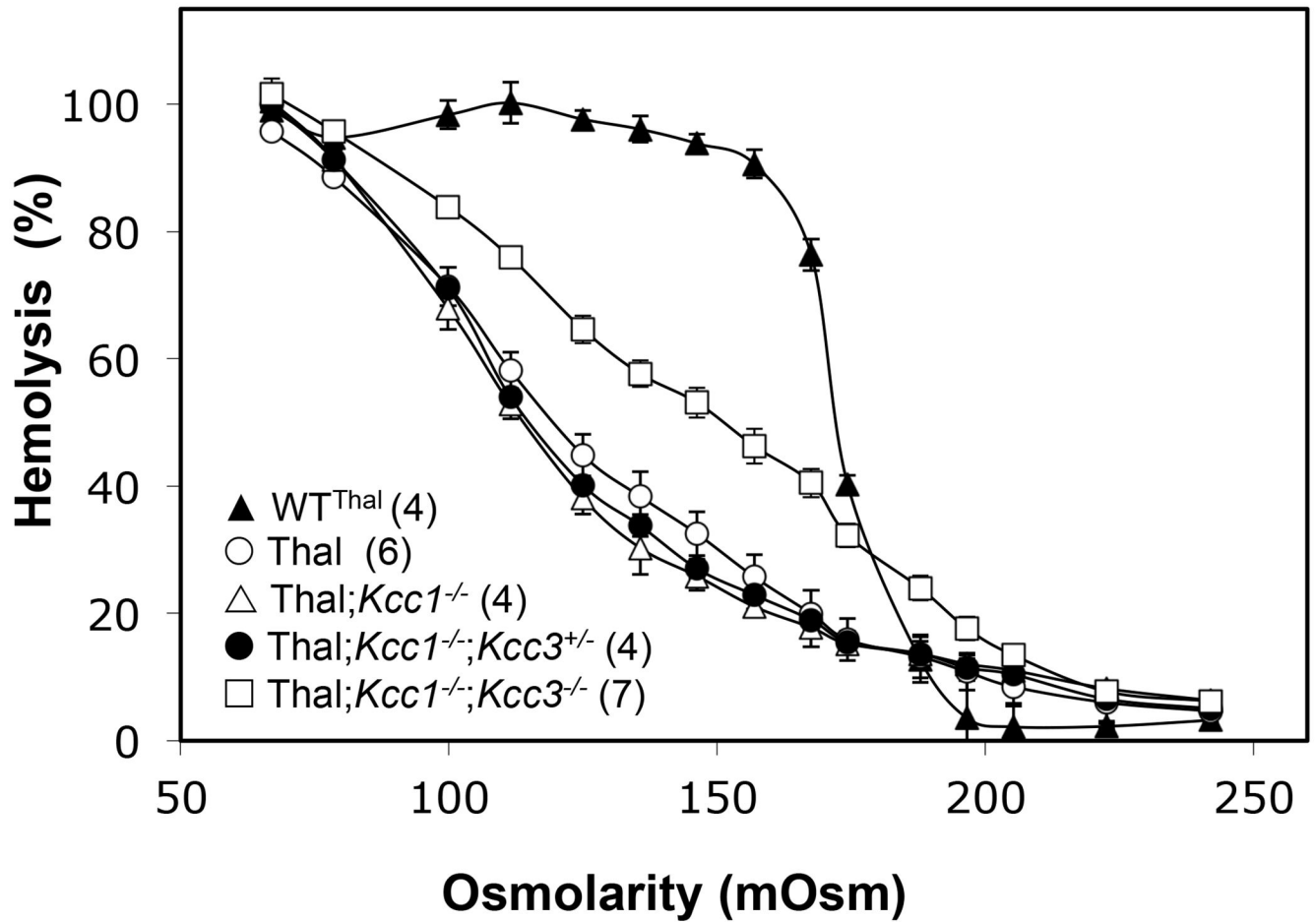
Figure 5. Red cell susceptibility to osmotic lysis as function of genotype.
Osmotic lysis curves of red cells from WT^{Thal} mice (black diamonds) compared with those of red cells from β-Thal mice (open triangles), Thal;$Kcc1^{-/-}$ mice (black circles), Thal;$Kcc1^{-/-};Kcc3^{+/-}$ mice (open circles), and Thal;$Kcc1^{-/-};Kcc3^{-/-}$ mice (open squares). Values are means ± SEM for (n) independently tested pooled samples from each genotype.
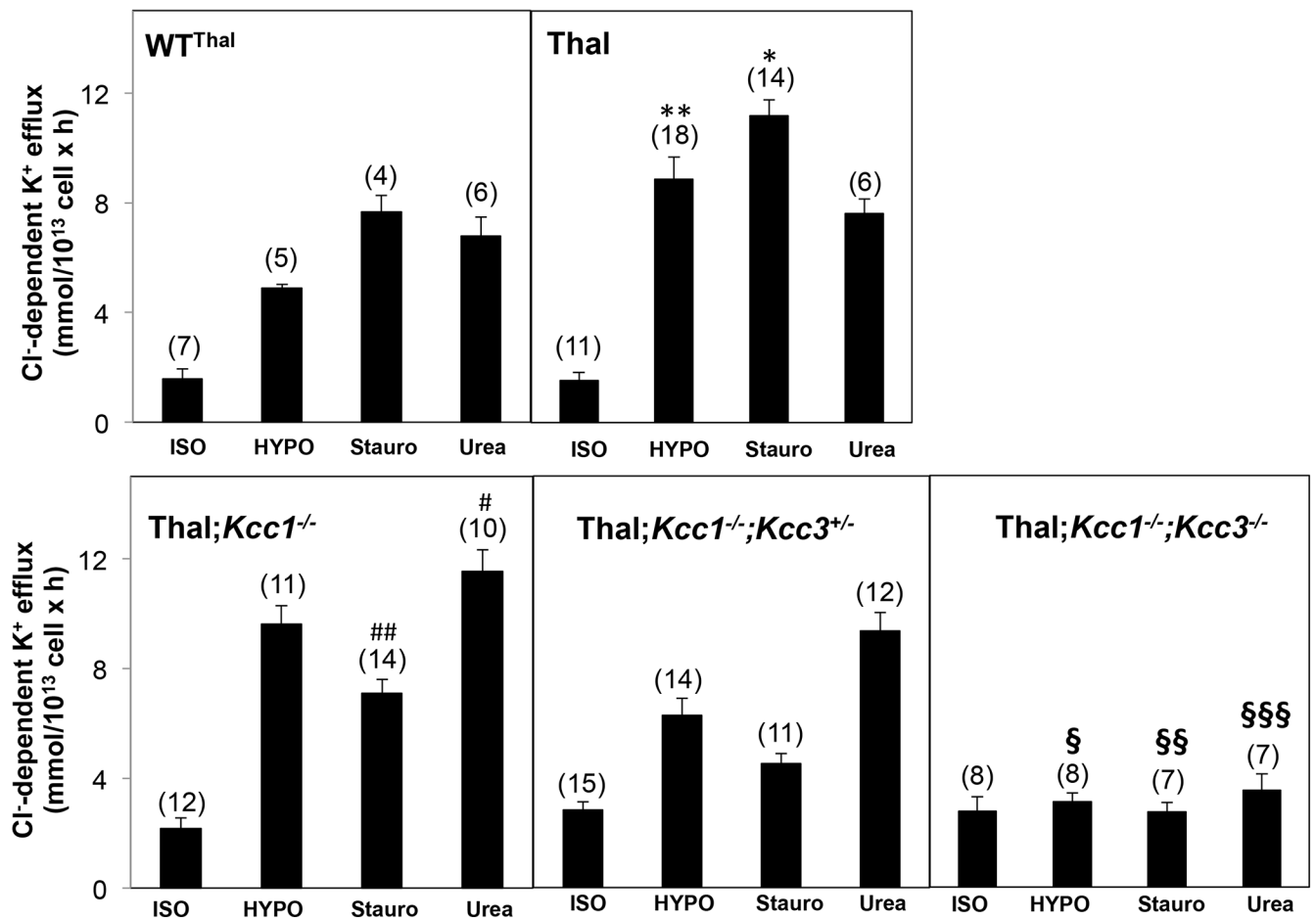
Figure 6. K-Cl cotransport activity in WT, Thal, Thal;*Kcc1*$^{-/-}$, Thal;*Kcc1*$^{-/-}$;*Kcc3*$^{+/-}$, and Thal;*Kcc1*$^{-/-}$;*Kcc3*$^{-/-}$ erythrocytes.

Chloride-dependent net K^+ efflux from mouse erythrocytes of the indicated genotypes was measured in isotonic medium (ISO), hypotonic medium (Hypo), isotonic medium containing 1 μM staurosporine (Stauro), and isotonic medium containing added 500 mM urea (Urea). Values are means ± SEM for (n) independent experiments at the indicated condition, representing the difference between the higher K^+ efflux measured in chloride and the lower K^+ efflux measured in sulfamate in each paired experiment (see Supplementary Figs. 1 and 2). *, p<0.0011 vs. WT; **, p<0.0001 vs. WT; #, p<0.0017 vs. Thal; ##, p=0.0004 vs. Thal; non-parametric Mann-Whitney test. §, p <0.019; §§, p<0.0025; §§§, p<0.0001, all vs. Thal;*Kcc1*$^{-/-}$;*Kcc3*$^{+/-}$; non-parametric Mann-Whitney test. (K-Cl cotransport activity in isotonic medium was higher in Thal;*Kcc1*$^{-/-}$;*Kcc3*$^{+/-}$ cells than in either Thal (p=0.0014) or WT^{Thal} cells (p=0.0011; p values absent from Fig. 6 for clarity). In contrast, isotonic activity of Thal;*Kcc1*$^{-/-}$;*Kcc3*$^{-/-}$ cells did not differ statistically from isotonic activity of Thal or WT^{Thal} cells.)

Table 1.

Erythrocyte indices from WT and β-Thal mice of indicated genotypes.

Values are means ± SEM for indicated (n). Hb: hemoglobin; MCV: Mean Corpuscular Volume; CHCM: Corpuscular Hemoglobin Concentration Mean; RDW: Red cell distribution Width; Hyper: Hyperchromic red cells; Retic: Reticulocytes. ***, p<0.0001 vs WT[Thal]. [#], p<0.01, [##], p<0.001; [###]; p<0.0001 vs. Thal. One-way ANOVA, Kruskal-Wallis test with Dunn's multiple comparison test.

Genotype	Hematocrit (%)	Hb (g/dL)	MCV (fL)	CHCM (g/dL)	RDW (%)	Hyper (%)	Retic (%)
WT[Thal]	42.0 ± 0.4 (55)	11.7±0.1 (51)	49.2 ± 0.1 (68)	27.9 ± 0.1 (69)	12.8 ± 0.1 (69)	0.17 ± 0.03 (69)	3.0 ± 0.1 (68)
Thal	28.6 ± 0.2*** (70)	7.9±0.1*** (68)	35.9 ± 0.2*** (98)	27.5 ± 0.1 (98)	33.7 ± 0.2*** (97)	3.7 ± 0.2*** (99)	18.6 ± 0.4*** (85)
Thal;$Kcc1^{-/-}$	31.4 ± 0.5[#] (40)	8.6±0.1[###] (39)	39.0 ± 0.3[###] (43)	27.5 ± 0.2 (44)	33.9 ± 0.4 (44)	3.5 ± 0.3 (43)	26.0 ± 1.3[##] (21)
Thal;$Kcc1^{-/-}$;$Kcc3^{+/-}$	30.3 ± 0.4 (37)	8.0±0.1 (36)	40.2 ± 0.4[###] (42)	26.5 ± 0.1[###] (44)	34.6 ± 0.3 (42)	3.6 ± 0.3 (41)	25.9 ± 1.4[#] (25)
Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$	40.3 ± 0.7[###] (22)	8.7±0.2[###] (22)	53.4 ± 0.5[###] (26)	21.6 ± 0.2[###] (26)	29.3 ± 0.3[###] (26)	2.6 ± 0.3 (26)	27.8 ± 2.0[#] (6)

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Table 2.

Reticulocyte indices from WT and β-Thal mice of indicated genotypes. Values are means ± SEM for indicated (n). MCV_r: Mean Corpuscular Volume of reticulocytes; $CHCM_r$: Corpuscular Hemoglobin Concentration, Mean of reticulocytes ; RDW_r: Reticulocyte Distribution Width; $Hyper_r$: Hyperchromic reticulocytes. *p<0.003;**, p<0.001 vs. WT^{Thal}. ***, p<0.0003 vs. Thal. One-way ANOVA, Kruskal-Wallis test with Dunn's multiple comparison test.

Genotype	MCV_r (fL)	$CHCM_r$ (mg/dL)	RDW_r (%)	$Hyper_r$ (%)
WT^{Thal} (61)	58.0 ± 0.4	26.6 ± 0.2	15.4 ± 0.3	0.6 ± 0.1
Thal (88)	52.2 ± 0.4***	24.0 ± 0.2***	20.5 ± 0.2***	0.9 ± 0.1**
Thal;$Kcc1^{-/-}$ (21)	53.6 ± 0.7	24.1 ± 0.3	20.4 ± 0.4	1.3 ± 0.3
Thal;$Kcc1^{-/-}$;$Kcc3^{+/-}$ (25)	52.9 ± 0.6	24.3 ± 0.3	20.8 ± 0.3	1.3 ± 0.2
Thal;$Kcc1^{-/-}$;$Kcc3^{-/-}$ (5)	58.4 ± 1.2*	22.3 ± 0.4	19.6 ± 0.6	0.5 ± 0.4

Table 3.

Erythrocyte ion contents from WT and β-Thal mice of indicated genotypes.

Values are mean ± SEM of (n) samples. Ion content was measured by atomic absorption spectrophotometry. *p=0.003 vs. WT^{Thal}. **p<0.001 vs. Thal. ***p<0.0001 vs. Thal. One-way ANOVA, Kruskal-Wallis test with Dunn's multiple comparison test.

Genotype	K_i (mmol/kg Hb)	Na_i (mmol/kg Hb)	Mg_i (mmol/kg Hb)
WT^{Thal}	444.1±8.0 (19)	20.8 ± 0.4(18)	9.7±0.6 (6)
Thal	452±6.7 (37)	30.3 ±1.0* (38)	13.9±1.3 (6)
Thal;*Kcc1*$^{-/-}$	444.2 ± 6.2 (28)	38.4 ± 1.1** (27)	17.9±1.7 (5)
Thal;*Kcc1*$^{-/-}$;*Kcc3*$^{+/-}$	467.5 ± 5.3 (23)	38.9 ± 0.7** (21)	15.8±0.5 (15)
Thal;*Kcc1*$^{-/-}$;*Kcc3*$^{-/-}$	640.1±14.0*** (13)	42.4 ± 2.4** (13)	21.2±0.9** (9)