Supplemental Figure Legends

Figure S1. Confirmation of the specificities of primer/probe designs for different human and murine KCC isoforms. Representative amplification curves for plasmids containing 10^6 copies of different human KCC cDNA (pKCC1, pKCC1b, pKCC3a, and pKCC3b) or 10^7 copies of murine KCC cDNA (pmKCC1, pmKCC3 and pmKCC4) are shown in duplicate (arrows). The amplification curves from cDNA of human HEK293 cells or murine erythroleukemia cells are shown (arrow heads) as endogenous positive expression controls. Background amplification with plasmids containing 10^6 copies of all other human KCC or 10^7 copies of all other murine KCC isoforms, and no-template controls are also shown (open arrows), or being off the range (when more than 30 of cycle number).

Figure S2. Validation of optimized multiplex quantification for simultaneous amplification of human KCC1 and GAPDH. Varying amounts of total RNA from HEK 293 cells were plotted against cycle number at threshold value (Ct) using primer/probe concentrations identified by optimization experiments. All curves were derived from two standard sample sets, each was amplified twice in triplicate.

Figure S3. KCC expression during *in vitro* erythroid maturation. Human $CD34^+$ cells were isolated from fresh bone marrow of healthy donors with hapten-conjugated anti-CD34 monoclonal antibody using LS Separation Columns (Miltenyi Biotec), followed by cryopreservation. Cryopreserved $CD34^+$ cells (>90% purity) were thawed and cultured under erythropoietic conditions as described by Giarratana et al (Ref.1) for 20 days before complete withdraw of cytokines. . **A.** Representative flow cytograms of ex vivo erythropoiesis cultures stained with CD71-PECy5 and GPA-PE at indicated days demonstrate the temporal loss of immature cells ($CD71^{high}GPA^{low}$) from 24% on Day 8 to 11% on Day 20, and the appearance of more mature red cells ($CD71^{low}GPA^{high}$) from 1% to 23%. Thus the progressive, if not synchronized, erythroid differentiation is confirmed in the *ex vivo* erythroid-inductive culture. **B.** RT-QPCR analyses in cultured erythroid cells at the indicated days demonstrate the decrease of KCC1 and KCC3b, as well as the increase of KCC3a and KCC4. Data are shown as mean ± SD derived from three independent culture experiments with two separate RT reactions performed for each sample, followed by QPCR reactions in triplicates.

Figure S4. Immunoblots of HEK 293 cells overexpressing myc-tagged KCC proteins. Whole cell lysates were prepared from the cells characterized in Figure 6. Equivalent protein loads were applied to each lane. Staining was with anti-myc antibody as described in Materials and Methods. Removal of carbohydrate by N-glycanase treatment eliminated multiple bands seen in native protein and revealed similar protein expression among the three cell lines.

Figure S5. Immunofluorescence of HEK 293 cells expressing KCC proteins. HEK cells stably expressing KCC1, KCC3a, or KCC4 constructs labeled at the amino terminal with myc-tag were plated on polylysine treated coverslips, fixed in 3.7% formaldehyde in PBS, permeabilized in 0.5% Triton X-100 in PBS, and quenched with 0.1 M glycine in

PBS. They were blocked in SuperBlock (Pierce) and incubated with a rabbit anti-cMyc antibody (Abcam), which was detected with an Alexa-488 labeled donkey anti-rabbit secondary antibody (Invitrogen) (green). After staining with 4',6-diamidino-2-phenylindole (DAPI) for nuclei (blue), the cells were mounted in SlowFade Gold antifade reagent (Invitrogen). They were examined and imaged using a Leica DMI6000 fluorescence microscope equipped with Open Lab software.

Figure S6. Co-expression of KCC3 with KCC1 or KCC1Δ117. KCC3a DNA was inserted into a tetracycline-inducible vector integrated into the FlipIn T-REX HEK 293 cell line (See Materials and Methods). This KCC3 cell line was then transfected with a SF91-eGFP-PRE vector encoding KCC1 Δ117 or full length KCC1, sorted for GFP positivity by flow cytometry and cultured. Control cells were transfected with empty SF91-eGFP-PRE vector. Where indicated KCC3 expression was induced by 48 hr incubation with 1 ng/ml doxycycline prior to membrane preparation for immune blotting. Full length KCC1 and KCC3 were detected with N-terminal antibodies described in Materials and Methods. KCCΔ117 was detected with a C-terminal goat polyclonal antibody that recognizes all KCC proteins (Santa Cruz Biotechnology. The low M_R of KCCΔ117 (~100 kDa) permitted its distinction from KCC3a (M_R ~150 kDa). "Endogenous" refers to cells transduced with control GFP vector and not exposed to tetracycline. Note that KCC1 expression was similar whether or not KCC3 was expressed (KCC1only vs KCC3 + KCC1), and that KCC3 expression was unchanged by KCC1 expression (KCC3 only vs KCC3 + KCC1). The same was true when KCC1 Δ117 was expressed with KCC3.

Reference:

1. Giarratana, M. C., Kobari, L., Lapillonne, H., Chalmers, D., Kiger, L., Cynober, T., Marden, M. C., Wajcman, H., and Douay, L. (2005) *Nat Biotechnol* **23**, 69-74