Supplemental Information

TMEM14c is required for erythroid mitochondrial heme metabolism.

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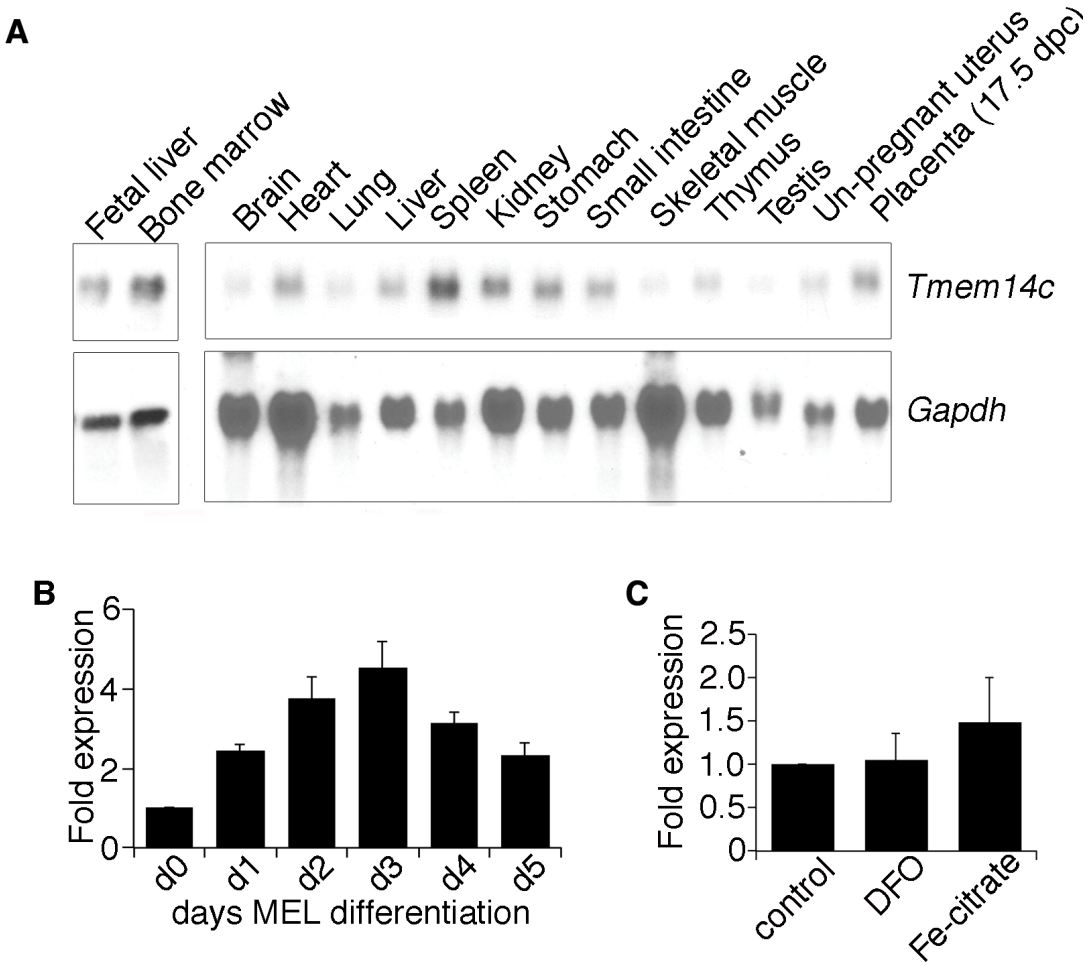
Inventory of Supplemental Information:

Age (dpc)	Total	+/+	+/gt	gt/gt	Comments
E9.5	34	9	15	10	
E10.5	26	9	9	8	
E11.5	8	2	4	2	
E12.5	73	17	31	25	
E13.5	36	10	25	1	1 +/gt dead
E14.5	34	10	17	7	3 gt/gt dead
E15.5	25	10	13	2	
E16.5	17	7	8	2	1 +/gt dead; both gt/gt dead
E18.5	18	7	11	0	3 wt dead

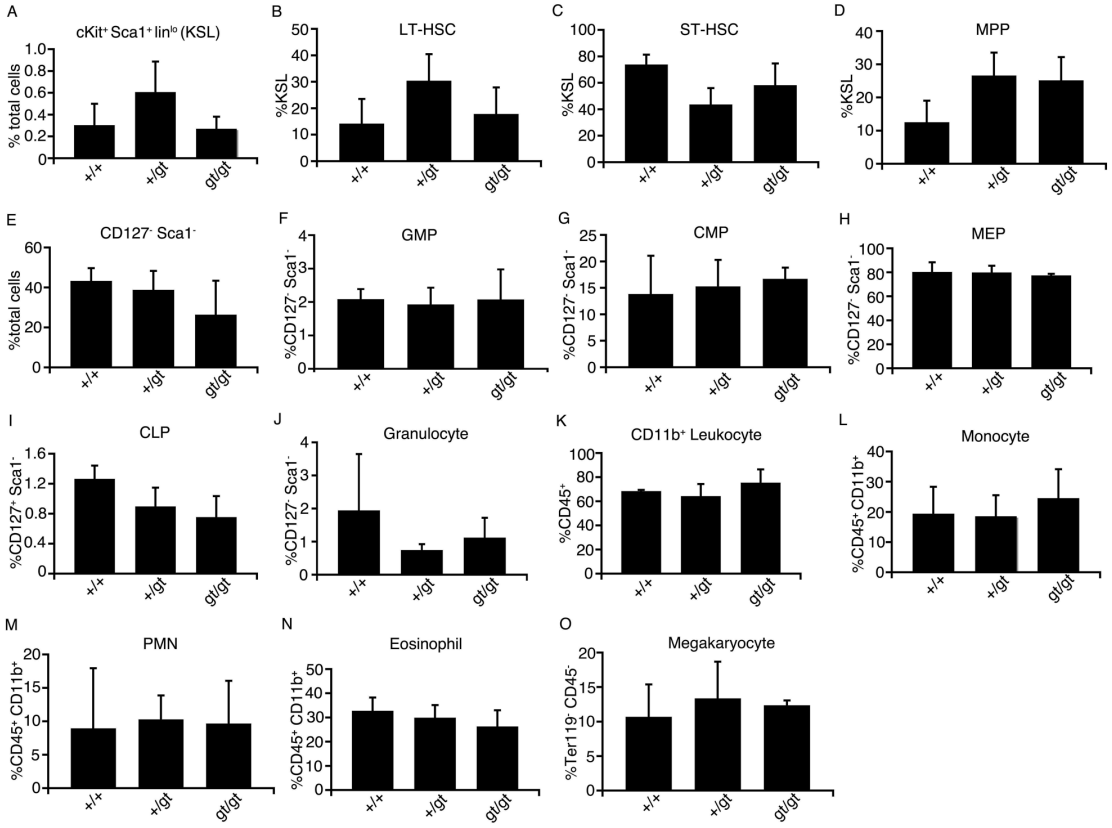
Supplemental Table S1. Timed matings of +/gt *Tmem14c* mouse show lethality from embryonic anemia. Data presented is the survival of +/+, +/gt and gt/gt genotyped embryos at various embryonic stages. Most *Tmem14c* gt/gt embryos do not survive beyond E13.5.

	WT (n = 10)	*Tmem14c* +/gt (n = 3)
RBC x10^{12}/L	10.20 ± 0.25	10.40 ± 0.54
Hgb g/dL	15.97 ± 0.39	16.30 ± 0.95
Hct %	48.25 ± 1.58	48.97 ± 2.57
MCV, fL	47.32 ± 1.13	47.07 ± 0.57
MCH, pg	15.37 ± 0.34	15.63 ± 0.31
MCHC, g/dL	33.11 ± 0.85	33.27 ± 0.32
CHCM, g/dL	31.29 ± 0.76	29.23 ± 0.42
RDW, %	13.31 ± 0.58	11.93 ± 0.23
Retic, %	3.41 ± 0.58	3.16 ± 0.44
PLT, 10^3 cells/µl	1148 ± 297	907.00 ± 213.85
MPV, fL	5.57 ± 0.46	5.17 ± 0.15

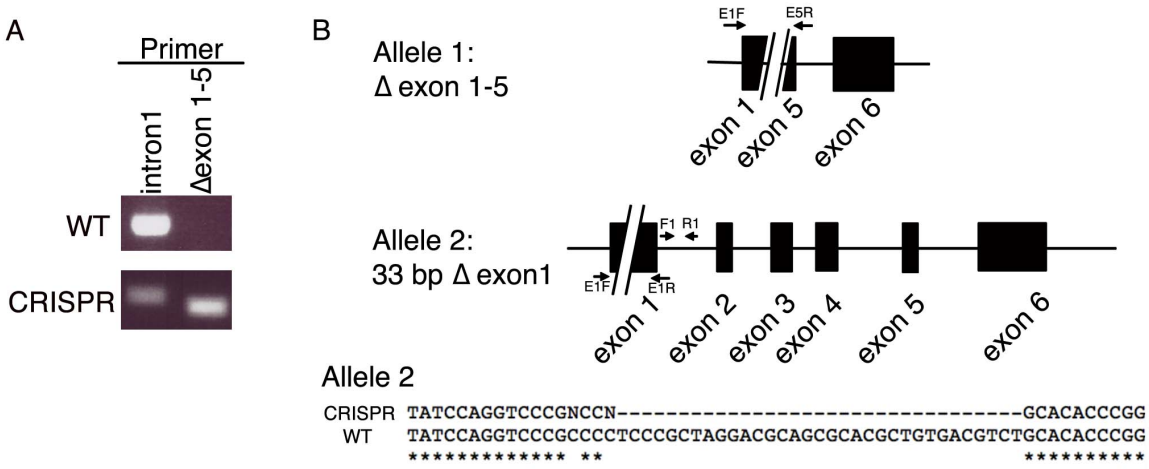
Supplemental Table S2. All values mean ± SEM. RBC, red blood cell count; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin content; CHCM, corpuscular hemoglobin concentration mean; RDW, red cell distribution width; Retic, reticulocytes; PLT, platelet count; MPV, mean platelet volume. Reference values were obtained from Sahr *et al.* (2009) (1).



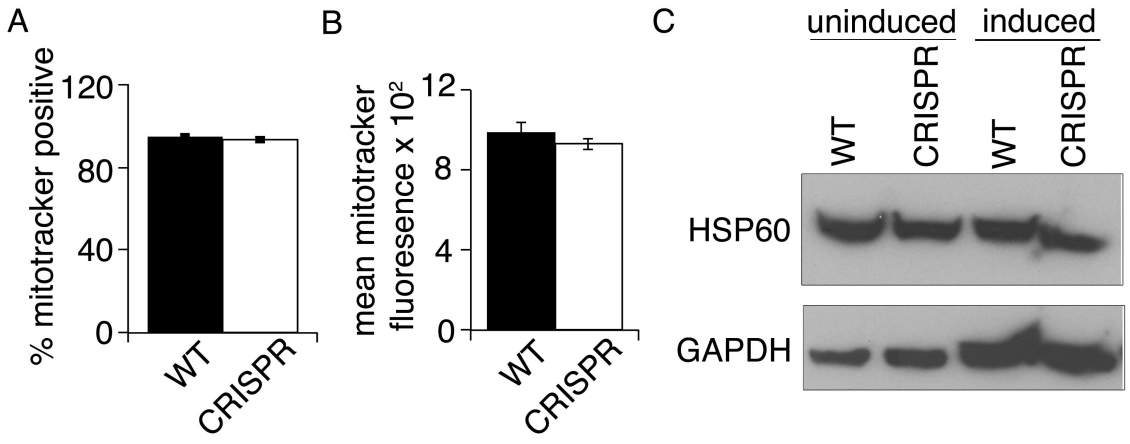
Supplemental Figure S1. ***Differential expression of Tmem14c mRNA during erythroid maturation and in various organs***. A) Tissue northern analysis shows that *Tmem14c* mRNA is enriched in hematopoietic tissues in both the fetus and the adult, namely the bone marrow, spleen, and fetal liver. Northern blots from Seegene used previously for *Gapdh* (2) were stripped and reprobed for *Tmem14c*. B) *Tmem14c* mRNA is induced in differentiating MEL cells. qRT-PCR analysis for *Tmem14c* mRNA normalized to *Hprt* mRNA from MEL cells induced to undergo erythroid differentiation with DMSO. C) qRT-PCR analysis shows that *Tmem14c* mRNA expression in MEL cells is not altered by either iron depletion (desferrioxamine [DFO] treatment) or iron supplementation (Fe-citrate).



Supplemental Figure S2. ***Tmem14c deficiency does not impact non-erythroid hematopoietic lineages.*** FACS analysis was carried out to quantify the proportion of hematopoietic cell lineages in E12.5 murine fetal livers from +/+, +/gt, and gt/gt animals. The following lineages were quantitated: A) KSL, B) Long-term repopulating hematopoietic stem cells (LT-HSC), C) Short-term repopulating hematopoietic stem cells (ST-HSC), D) Multipotent progenitors (MPP), E) $CD127^{-}$ $SCA1^{-}$, F) Granulocyte-macrophage progenitor (GMP), G) Common myeloid progenitor (CMP), H) Megakaryocyte-erythroid progenitor (MEP), I) Common lymphoid progenitor (CLP), J) Granulocyte, K) $CD11B^{+}$ leukocyte, L) Monocyte, M) Polymorphonuclear neutrophils (PMN), N) Eosinophil and O) Megakaryocyte. The frequency of these lineages was unaffected by the absence of *Tmem14c* mRNA.



Supplemental Figure S3. ***CRISPR-mediated targeting of the mouse Tmem14c locus.*** A) Genomic DNA was extracted from the wild type (WT) and CRISPR cell lines and analyzed by PCR. The primer pair F1/R1, binding to intron 1 (panel B), was used to detect the wild type allele. The primer pair E1F and E5R was used to detect the allele, which contained an excision of Δexons 1-5. B) Structure of CRISPR modified *Tmem14c* alleles in CRISPR clone (top, middle panels). In this compound heterozygote clone, Allele 1 has exons 1-5 completely excised (Δexon 1-5 in panel A). In the second allele (Allele 2), 33 bp of exon 1 is deleted as shown by sequencing (bottom panel). Position of PCR primers used to genotype alleles (WT: F1, R1; Allele 1, Δexon1-5: E1F, E5R; Allele 2, exon 1 33-bp deletion: E1F, E1R) are indicated.



Supplemental Figure S4. ***TMEM14C is not required for maintenance of normal mitochondrial physiology***. A) Wild-type (WT) and CRISPR cells have similar levels of cell viability, as shown by the numbers of cells which positively stained for Mitotracker dye. B) Mitotracker-positive wild type (WT) and CRISPR cells have similar average levels of fluorescence, indicating no difference in the numbers of functional mitochondria in each cell. C) Western analysis of whole cell extracts showing similar amounts of mitochondrial protein in wild-type and CRISPR cells whether they are induced to differentiate or not. Protein loading is normalized to GAPDH, as a control for total cellular protein.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mitochondrial fractionation and trypsin protection assay

Intact mitochondria were isolated from MEL cells using a mitochondria isolation kit from Pierce.

Sub-mitochondrial fractionations were done as previously described (3). HeLa cells were transfected with FLAG-TMEM14C. Cells were homogenized in buffer (20 mM HEPES pH 7.4, 220 mM mannitol, 70 mM sucrose, 0.2% BSA and 0.5 mM PMSF) and centrifuged at 770 x g. Mitochondria were obtained by centrifuging post-nuclear supernatant at 10,000 x g. Isolated mitochondria were re-suspended with homogenizing buffer. Hypotonic swelling of mitochondria was carried out in 20mM HEPES pH 7.4. Trypsin digestion was carried out by treating mitochondria with 0.25 μg/ml of trypsin for 30 min on ice. Trypsin was quenched with soybean trypsin inhibitor.

Northern analysis

Mouse tissue northern blot was obtained from Seegene (Seoul, Korea). The nylon filters were serially probed with random-prime labeled (Roche) mouse *Tmem14c* and *Gapdh* cDNA probes with [α-^{32}P]dCTP (6000 Ci/mmol; Perkin-Elmer) using standard procedures for hybridization and washing in 0.1x SSC, 0.1% SDS at 65^{o}C.

Mouse *in situ* hybridization

The protocol of mouse *in situ* hybridization was modified from a previous report (4). Briefly, ^{33}P-UTP-labeled cRNA probes were synthesized using MAXIscript Kit from Ambion (Austin, TX). Five micron sections from paraformaldehyde-fixed,

paraffin-embedded outbred mouse embryos were pretreated with proteinase K and acetic anhydride, followed by hybridizing with the RNA probe overnight at 47°C. Following stringent washes and RNase treatment, slides were dipped in Kodak NTB emulsion (Carestream) and exposed for 5-25 days. Brightfield and darkfield images were captured and pseudocolored as described (5).

Quantitative RT-PCR analysis

The stable shRNA and CRISPR clones were chemically differentiated with DMSO for 3 days. Total RNA was extracted by Qiagen kit and subjected to qRT-PCR analysis using TaqMan probes (Applied Biosystems). The threshold cycle (CT) values of *Tmem14c* were normalized to the internal control *Hprt1* and were further normalized to the control clone. qRT-PCR was performed on an iQ5 Real-Time PCR Detection System (Bio-Rad).

To examine the effects of cellular iron status on the expression of *Tmem14c* and *Hprt*, undifferentiated wild type MEL cells were incubated with 100 μM DFO or 200 μM iron citrate for 16 hours. Total RNA was extracted. The qRT-PCR assays were performed to analyze the expression of *Tmem14c* and *Hprt* using TaqMan probes.

Western analysis

For the mitochondrial sub-fractionation assay in Figure 2C, we used the following antibodies to detect the respective proteins: anti-FLAG (Gilbertsville, PA), anti-TOM20 (Santa Cruz, Santa Cruz, CA), Anti-TIM23 (BD Biosciences, San Jose, CA) and Anti-HSPA9 (Santa Cruz).

For western blots in Figure S4C, we used the following antibodies: anti-HSP60 (K19, Santa Cruz) and GAPDH (Pierce).

Immunoblotting of TMEM14C was performed using a custom anti-mouse TMEM14C polyclonal antibody generated against two peptides, (C)-MQKDSGPLMPLHYFGFG and (C)-YQLSQDPRNVWVFLATSGT), and immune-affinity purified against these antigenic peptides (Genemed Synthesis, Inc., San Antonio, TX). PPOX polyclonal antibodies were generated in-house by H.A.D. (University of Georgia). FECH polyclonal antibody (C-20) was obtained from Santa Cruz (Athens, GA).

MEL chemical differentiation and *o*-dianisidine cytospin staining for heme

To induce their differentiation into erythrocyte-like cells, MEL cells were treated with 1.5% dimethyl sulfoxide (DMSO). *o*-dianisidine staining was performed at day 3 after DMSO-induced differentiation. The stained cells were mounted onto slides by cytospin centrifugation as previously described (6) and the images were acquired in a Nikon Eclipse E600 microscope connected to a Leica DC500 camera.

Silencing of *Tmem14c* in mouse primary fetal liver cells (MPFL)

The methods for *Tmem14c* silencing and hemoglobin quantification in MPFL were described previously (7). The retroviral plasmids expressing shRNAs for *Tmem14c* were transfected in a packaging HEK293T cell line. The collected retroviral supernatants were added to erythroid precursor cells purified from mouse fetal liver. The retrovirally

transduced cells were sorted for GFP expression using a FACSAria machine (BD Biosciences), and the hemoglobin content was quantified with Drabkin's reagent.

Confocal immunofluorescence microscopy

Confocal microscopy was performed at the Harvard Digestive Disease Center Imaging Facility (Boston Children's Hospital). Images were taken using a spinning disk confocal head (CSU-X1, Perkin Elmer Co., Boston, MA) coupled to a fully-motorized inverted Zeiss Axiovert 200M microscope equipped with a 63X lens (Pan Apochromat, 1.4 NA). Solid-state lasers (λ473 nm, λ568 nm and λ660 nm; Crystal Laser, Reno, NV) coupled to the spinning head through a fiber optic were used as light source. An acoustic-optical tunable filter (AOTF) was used to switch between different wavelengths. The imaging system operates under control of SlideBook 5 (Intelligent Imaging Innovations Inc., Denver, CO) and includes a computer controlled spherical aberration correction device (SAC, Intelligent Imaging Innovations, Inc., Denver, CO) installed between the objective lens and the CCD camera (Orca ER, Hamamatsu). Acquisition of sequential optical sections spaced 0.4 μm apart was achieved with the aid of a motorized piezo-driven stage.

Mouse Gene Trap ES Cells

Heterozygous *Tmem14c* gene trap (gt/+) mouse ES cells (E295C12) were obtained from the German Gene Trap Consortium (Helmholtz Zentrum München, Neuherberg, Germany). ES cells were maintained on gelatin-coated dishes in ES media.

Homozygous gt/gt clones were selected using G418 selection as previously described (2, 8).

Mouse blastocyst injections and knockout mouse generation

All mouse experiments were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) regulations at The Jackson Laboratory (Protocol #11006; Bar Harbor, ME). Blastocyst injection and embryo transfer were performed using standard techniques (8, 10). Male chimeras were mated to C57BL/6J females to generate heterozygotes. All mice for this study were maintained on a segregating C57BL/6J-129/Sv (B6;129) hybrid genetic background. C.Cg-Fech^{m1Pas}/J (Fech^{m1Pas}; Stock# 002662) mice were purchased from JAX® Mice and Services. Embryos ranging in age from E7.5 to E16.5 were collected for analyses with noon of the vaginal plug date designated as E0.5. The genotypes of *Tmem14c* gene trap and *Fech* mice were determined by real-time genomic PCR analysis of tail tip or yolk sac biopsies. All mice were maintained in climate-controlled rooms (12-hour light cycle) and provided acidified water and chow (NIH 5K52) *ad libitum*.

Embryonic microscopy and flow cytometry analyses

Whole-mount β-galactosidase (LacZ) staining was performed as described (11).

Fetal liver cells were prepared for cytospins as follows. Single cell suspensions from fetal livers were prepared by incubation in 0.1% collagenase/20% fetal bovine serum/phosphate-buffered saline (StemCell Technologies, Inc., Vancouver, BC, Canada) at 37°C for 30 min and then pipetted up and down with a 1 mL pipet tip. Cytospin

preparation of cell suspensions onto poly-L-lysine coated slides and May-Grünwald staining were performed using standard techniques.

To visualize autofluoresence in fetal liver tissue, fetal livers were dissected out in phosphate-buffered-saline containing 10% fetal bovine serum and viewed using glass bottom dishes (MatTek Corp., Ashland, MA, USA) on an inverted confocal microscope (SP2, Leica Microsystems, Wetzlar, Germany) using λ405 nm UV excitation and λ620 nm emission filters.

For flow cytometry analysis, fetal liver cells from E12.5 embryos were immunostained for CD71 and TER119 to assess erythroid differention via flow cytometic analysis (LSR II, BD Biosciences, San Jose, CA, USA) as described (12).

***In vitro* colony assays**

Wild-type *E14* and *Tmem14c* gene-trap ES clones were generally split 1-day after thawing with 10^6 mES cells plated onto gelatinized 100-mm plates in mES media as previously described (2). The following day, mES cells were maintained in "switch media". 2-days after the split, the cells were collected by trypsinization and replated on untreated 100-mm dishes at a density of $3x10^3$ cells/ml (total $6x10^4$ cells) in embryoid body (EB) media with mVEGF (10 ng/ml) on Days 1 and 5 as described (8). ALA was added to EB cultures for the last 12-24 hours of Day 6 primitive differentiation. Hemoglobinization of the primitive differentiated cells by *o*-dianisidine staining as previously described (9).

To achieve a definitive wave of hematopoietic differentiation, the EB medium was supplemented with mVEGF (10 ng/ml), rEPO (10 IU/ml), mSCF (5 ng/ml). Day 5

EB's were supplemented with rEPO (5 U/ml), mIL3 (5 ng/ml), mIL6 (10 ng/ml) and mSCF (100 ng/ml), and harvested on day 6. EB's were dissociated with 0.25% trypsin, disaggregated by trituration via a 18g syringe. The disaggregated EB's were replated on 3D methylcellulose culture for CFU-E colony analysis (2). The picked CFU-E's were morphologically analyzed by cytospin and Wright-Giemsa staining.

CRISPR design and cloning

CRISPR guide sequences were designed to direct two cleavages at the *Tmem14c* locus to generate a chromosomal deletion (13-14). CRISPR guide sequences were designed to have a unique 12 bp seed sequence 5'-NNNNNNNNNNNN-NGG-3' in the mouse genome (http://www.genome-engineering.org) to minimize off-target cleavages. The exon 1 targeting sequence was: 5'-GTGCGCTGCGTCCTAGCGGG 3' and the exon 5 targeting sequence was 5' TGGGACCTTGGCCGGAATTA 3'. CRISPR guides were cloned into pX330 plasmid (Addgene) with BbsI ligation as previously described (15).

Cell targeting

CRISPR/Cas9 constructs were delivered to mouse erythroleukemia (MEL) cells by electroporation. One million MEL cells resuspended in BTX solution plus 2 μg of each of two CRISPR/Cas9 constructs (designed to generate flanking cleavages resulting in a deletion) as well as 0.2 μg pmaxGFP (Lonza) was transferred to a 2 mm cuvette and electroporated at 250V and 5 ms using a BTX ECM 830 electroporator (Harvard Apparatus). Cells were placed immediately in 5 ml media at 30 degrees. One to three days later cells were sorted by FACS gating on the 1-3% brightest GFP+ live events.

Cells were plated by limiting dilution at 0.3 cells per well in 96-well plates and cultured at 37^{o}C. Clones were screened by gDNA isolation (QuickExtract, Epicentre) and PCR for the deletion allele. Clones were validated by PCR of both the deleted and non-deleted alleles (14).

Primers

The wild-type *Tmem14c* allele was verified by primers: F1 5'-TCT TGA CTG CTC TGA CCT CTC CTC-3'; R1 5'-CCT ACA AAC TCA ACA TGG CAC GAA-3'. The gene-trap allele was verified by F2 5'-CGG TGG TGG GTC GGT GGT C-3' and R1. The Tmem14c exon 1-5 CRISPR deletion allele was amplified by primers: E1F 5'-GAG TAC GGA CCC CAA ACT CA-3' and E5R 5'-GCT TCT GCA GCC TTT GCT AC-3'. *Tmem14c* exon 1 was amplified with primers: E1F and E1R: 5'-TGG CGC GGA AGC AGC GC-3'.

Mitotracker Red staining

Cells were stained with 200 nM Mitotracker Red 580 according to the manufacturer's protocol. Flow cytometry was performed on the BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA) using the BD FACSDiva Software (BD Biosciences).

Fe radio-labeling and radio Fe-heme measurements

^{55}Fe- or ^{59}Fe-saturated transferrin was incubated with MEL cells in DMEM (high glucose) media with dialyzed, heat-activated fetal calf serum (Invitrogen) for 8h. When indicated, 5uM DP (Frontier Scientific) was included in the labeling media. 0.1mM ALA (Sigma) was included in all labeling experiments except those in Figure 7. Radiolabeled mitochondrial iron was quantitated from lysates of labeled cells. Fe-heme was extracted with cyclohexanone after acidification with 0.1 N HCl from lysed cells or isolated mitochondria. Radioactivity was quantitated using either a liquid scintillation count or γ-ray quantitated for ^{55}Fe and ^{59}Fe isotopes, respectively (2, 16).

ICP analysis of mitochondrial iron content

Mitochondrial iron was measured as previously described (17). Isolated mitochondria were treated with nitric acid and sample iron content was determined by using a Perkin-Elmer Inductively Coupled Plasma (ICP) Optical Emission Spectrometer. The results were normalized to mitochondrial protein content.

HPLC analysis of heme and porphyrins

HPLC analysis was carried out with MEL cells that were differentiated for 72h with 1.5% DMSO. Where indicated, cells were treated with ALA (2mg/ml) 24h prior to HPLC (18). A cell pellet spun down from a 30-50 mL culture was mixed with water to about 200 μL in a microfuge tube and sonicated for 12 cycles of 5-sec intervals at 50% duty (about 2.5 sec on, 2.5 sec off) using a microtip. A 50-μL aliquot was mixed vigorously with 200 μL of an extraction mixture of ethyl acetate (4 volumes) and glacial acetic acid (1 volume). The phases were separated by microcentrifugation for 1 min at

maximum speed. The upper organic layer was immediately analyzed simultaneously for protoporphyrin IX and heme in the HPLC. For porphyrins, 80 µL of the sonicated cell homogenate was mixed with 80 µL 3M HCl, incubated at 37˚C for 1 hour, and then microcentrifuged at maximum speed for 10 minutes. The supernatant was analyzed for porphyrins in the HPLC.

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