Supplemental Information for "A Specialized Pathway for Erythroid Iron Delivery Through Lysosomal Trafficking of Transferrin Receptor 2" by Khalil et al.

Inventory of Supplemental Information

Supplemental Methods

Transferrin uptake assays

Analysis of transferrin internalization was performed using fluorochrome- and gold-conjugated holotransferrin. Transferrin conjugates with Alexa Fluor® 594 (T13343), fluorescein (T2871), and pHrodo® Red (P35376) were purchased from ThermoFisher Scientific. 10 nm and 30 nm gold-conjugated transferrin (AC-10-07) was purchased from Cytodiagnostics, Ontario, Canada. Biotin-conjugated transferrin (T23363) was purchased from ThermoFisher Scientific. For fluorescence microscopy, 2 x 10^5 erythroid or granulocytic cells were incubated twenty minutes at 37°C in 100 µl IMDM with 0.05% BSA and 25 µg/ml fluorochrome-labeled transferrin. The cells were then washed in PBS, resuspended in IMDM at a density of 10^6/ml, cytospun onto a glass slide (10^5 cells per slide), and fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. For electron microscopy, 2 x 10^5 erythroid cells were incubated one hour at 37°C in 100 µl IMDM with 0.05% BSA and 25 µg/ml biotin-conjugated transferrin or four hours at 37°C in 100 µl IMDM with 0.05% BSA and 25 µg/ml gold-labeled transferrin. The cells were then washed in PBS and resuspended in 2% paraformaldehyde (PFA) and 2% glutaraldehyde in PBS for 20 minutes at room temperature.

Flow cytometry and Amnis ImageStream® analysis

For flow cytometry of cultured human progenitors, cells were centrifuged, washed, and re-suspended in PBS + 1% FBS with a conjugated antibody cocktail. Antibodies were added at 2 µl per 100 µl buffer. After 30 minutes staining on ice, samples were washed with PBS + 1% FBS and run on a CyAn ADP Analyzer (Beckman Coulter, Brea, CA) or a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ), followed by compensation and analysis using FlowJo 8.8.7 software (FlowJo, Ashland, OR). Live cell gating was conducted using FSC/SSC and exclusion of Zombie-Violet (#423113, Biolegend, San Diego, CA). For flow cytometry of mouse bone marrow, femurs were flushed with PBS with 5 mM EDTA, passed through a 70 µm filter mesh, incubated five minutes at room temperature in ammonium chloride lysis buffer to remove red blood cells, and washed with PBS. Marrow was then stained with a conjugated antibody cocktail and analyzed as above, with specific gating on FSC/SSCLow erythroid cells. Fluorochrome-conjugated antibodies to human markers (CD235, CD13, CD36) and murine markers (Ter119, CD71) were purchased from BD-Pharmingen (San Jose, CA).

For Amnis ImageStream® analysis, cells were initially subjected to MitoTracker Deep Red FM® (M22426, ThermoFisher Scientific) staining at 200 nM in serum-free RPMI medium for 30 minutes. The cells were washed with PBS and stained for GPA or CD13 as described for flow cytometry, followed by fixation in 4% fresh PFA for 15 minutes at room temperature. The cells were then washed with PBS and permeabilized 30 minutes at room temperature with intracellular staining buffer: 0.06% Triton X-100, 2% BSA, and 2% FBS in PBS. Following staining for one hour at room temperature with a 1:100 dilution of FITC-conjugated anti-human-Lamp1 (#53107942 Ebioscience), the cells were washed with intracellular staining buffer, followed by incubation with 1 µg/ml DAPI for ten minutes and final washing in intracellular staining buffer. For acquisition, the samples were re-suspended at 106 cells per 50 µl and analyzed on an Amnis ImageStreamX Mark II cytometer (EMD Millipore, Billerca, MA). Data analysis was conducted using the IDEAS® software package (EMD Millipore) with gating on viable, singlet, in-focus, GPA+ or CD13+ cells.

Cell surface biotinylation

Primary human progenitors were washed with PBS and resuspended at 10^7 cells/ml in the surface-impermeable biotinylation EZ-Link™ Sulfo-NHS-LC-Biotin (#21335, ThermoFisher Scientific), dissolved at 1 mg/ml in PBS pH 8.0. The cell suspension was incubated on ice for 30 minutes, washed twice with PBS pH 8.0 with 100 mM glycine, and returned to culture medium at 37°C for 0-3 hours incubation. At the indicated time points, the cells were resuspended at a density of 10^6 cells per 150 µl in biotin lysis buffer (150 mM NaCl, 5 mM EDTA, and 10 mM Tris HCl [pH 7.4] with 1% Triton X-100), incubated for 15 minutes on ice, and centrifuged at 17,000 rpm for ten minutes at 4°C. A portion of

supernatant was directly analyzed by immunoblot as input, and the remainder combined with pre-washed NeutrAvidin resin (ThermoFisher Scientific) at a ratio of 3:1 lysate:bead slurry. Slurry/lysate mixtures were rotated for one hour at 4°C, washed twice in biotin lysis buffer, resuspended in Laemmli buffer, and eluates ("PD") underwent immunoblot as above.

Microscopy

For gross and light microscopic assessment of bone marrow, 12-week-old *Mcoln1-/-* and wild type littermates were euthanized, and femurs were photographed and fixed in formalin for 48 hours, followed by decalcification, paraffin embedding, sectioning, and H&E staining. Slides were imaged on an Olympus BX51 light microscope.

For immunofluorescent staining combined with mitochondria labeling, cells were first incubated with 200 nM MitoTracker Deep Red FM® (M22426, ThermoFisher Scientific) in serum-free RPMI medium for 30 minutes, then washed in PBS and resuspended in IMDM at 10^6 cells/ml. The cells were then cytospun onto a glass slide (10^5 per slide) and fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. The slides made from cytospins of MitoTracker or fluorescent-tranferrin loaded cells were washed in PBS, then permeabilized/blocked in immunofluorescence (IF) staining buffer (0.06% Triton X-100, 2% BSA, 2% FBS in PBS) for one hour at room temperature. Staining was performed using FITC-conjugated anti-human-Lamp1 (#53107942 Ebioscience), rabbit polyclonal anti-TfR1 (sc-9099 from Santa Cruz Biotechnology), or mouse monoclonal anti-TfR1 (sc-32272 from Santa Cruz Biotechnology) at dilutions of 1:100 in IF staining buffer overnight in a hybridization chamber at 4°C. Slides were washed three times with IF staining buffer, and Alexa Fluor®-conjugated secondary antibodies (ThermoFisher Scientific) were applied at dilution of 1:500 in IF staining buffer for one hour at room temperature, with DAPI at 1μg/ml included in the final ten minutes of the secondary stain. Slides were washed three times in IF staining buffer, once in PBS, mounted with coverslip and medium (#H-1000 Vectashield, Burlingame, CA), and viewed on a Zeiss LSM-700 confocal microscope with the 63x objective. Images were analyzed using Fiji ImageJ version 2.0.0 software. Colocalization analysis was performed using Fiji ImageJ coloc2 function to obtain Pearson's R value (above threshold) for the Tf and TfR1 signal. The percentage of Tf colocalized with TfR1 was assessed by Imaris 8.4 (Bitplane, Zurich, Switzerland) spot counting function. Spot masks were created for the Tf and TfR1 signal. The TfR1 spot mask was analyzed by distance transformation, and values mapped to the Tf spot mask, such that each Tf vesicle was given a distance value to the most proximal TfR1 vesicle. Distances closer than .4 μm were defined to be colocalized.

For transmission electron microscopy (TEM) of mouse bone marrow cells, femurs were flushed with PBS with 5 mM EDTA; extruded marrow was passed through a 70 μm filter mesh, incubated five minutes at room temperature in ammonium chloride lysis buffer to remove red blood cells, washed in PBS, and fixed in 2% PFA plus 2% glutaraldehyde in PBS for 20 minutes at room temperature. For TEM of cultured human progenitors, cells were washed in PBS and fixed in 2% PFA plus 2% glutaraldehyde in PBS for 20 minutes at room temperature. All samples were post-fixed in 1% osmium tetroxide for thirty minutes at room temperature, and embedded and polymerized at 65°C for 24 hours. 70 nM sections were placed on 200 mesh grids and stained with uranyl acetate and lead citrate. Secondary staining of biotin-conjugated transferrin was performed using 10 nm gold-conjugated streptavidin (#25269 from Electron Microscopy Sciences, Hatfield, PA) at a 1:5 dilution in incubation buffer of .2% BSA in PBS for two hours. Grids were then washed three times in incubation buffer and three times in water for 5 minutes per wash. Grids were carbon coated to minimize conductance and imaged on a JEOL 1230 (JEOL, Peabody, MA) electron microscope or TITAN Themis (FEI, Hillsboro, OR) at 80 kV. Images were taken with a Scientific Instruments of America 4K X 4K CCD camera. For quantification of gold-conjugated transferrin in organelle compartments, images were taken of unstained, processed sections to best visualize 10 nm gold particles. Images were then de-identified, scrambled and counted in a blinded manner, with MVB/lysosomes being distinguished from endosomes by having three or more intraluminal vesicles (ILVs). For quantification of direct mitochondrial-lysosomal membrane contact sites (MCSs), de-identified, scrambled images of progenitors were counted in a blinded manner, with direct contacts

defined by lack of detectable cytosol between mitochondrial and MVB/lysosome membranes. Intra-mitochondrial electron dense foci were similarly counted in a blinded manner. For quantification of mitochondria size, images were subjected to tracing of mitochondria and area measurement via Fiji ImageJ version 2.0.0.

Immunoprecipitation

For immunoprecipitations, K562 cells were washed in PBS and resuspended in IP lysis buffer (150 mM NaCl, 2 mM MgCl2, 10 mM HEPES (Gibco), 0.5% NP40, cOmplete® protease inhibitor (Roche Diagnostics), and PhosSTOP® phosphatase inhibitor (Roche Diagnostics)) at a density of 2 x 10^6 per 100 µl. Transfected HEK293T cells were washed and lysed in 500 µl IP lysis buffer per semi-confluent 10cm plate. Lysates were incubated on ice for 15 minutes then centrifuged 10 minutes at 15,000 rpm at 4°C. Protein content in the supernatant was quantified by BCA while samples were stored on ice. For immunoprecipitation of endogenous proteins in K562s, 6 µg of antibody was added to 3 mg of protein extract in 1 ml lysis buffer overnight, using the following antibodies: rabbit IgG (ab27478 from Abcam, Cambridge, UK), rabbit polyclonal anti-MFN2 (ab56889, Abcam), and rabbit monoclonal anti-TfR2 (ab185550, Abcam). Immunoprecipitations with unconjugated antibodies were captured with a 1:1 mixture of protein A (#88846 ThermoFisher Scientific) and protein G (#88847 ThermoFisher Scientific) magnetic beads, pre-washed three times in lysis buffer and added at a volume of 25 µl packed beads per 1ml sample. For immunoprecipitation of epitope-tagged recombinant proteins, 30 µl of Myc-tag Mouse monoclonal magnetic bead conjugated slurry (#5698, Cell Signaling Technology, Danvers, MA) was added to 3 mg of protein extract in 1ml lysis buffer. Bead suspensions were rotated two hours at 4°C, quickly washed twice in lysis buffer, slowly washed twice with 15 minutes of rotation in 4°C lysis buffer, again quickly washed twice in lysis buffer, resuspended in 50 µl Laemmli buffer, boiled, and analyzed by immunoblot as described.

Transfections and transduction

HEK293T cells were grown to 60% confluency in 10 cm plates (for preparation of lentivirus) and transfected in their culture medium using a calcium phosphate precipitation kit in accordance with manufacturer's instructions (#631312, Clontech, Mountain View, CA). For lentiviral packaging, pCMV-dR8.74 and pMD2.G were co-transfected with pLKO.1 shRNA vectors at a mass ratio of 3:1:4 for pCMV-dR8.74:pMD2.G:pLKO.1. pLKO.1 vectors expressing shRNAs active against TFR2 (TRCN0000063628), MFN2 (TRCN0000082683), and TfR1 (TRCN0000057658) were purchased from GE Dharmacon (Lafayette, CO). 16 hours after transfection, the medium was exchanged with Opti-MEM™ I (Gibco), and supernatants were collected 40 hours following initiation of transfection. Viral supernatants were filtered with 0.45 µm syringe filters and stored at -80°C until use. For K562 transduction, cells were incubated with viral supernatants for 24 hours, followed by puromycin selection at 2 µg/ml in growth medium for at least 48 hours. For human primary progenitors, cells were expanded in pre-stimulation medium for 48 hours and transferred to retronectin-coated 12-well plates containing viral supernatants supplemented with prestimulation cytokines. For HUDEP-2 transduction, cells were incubated with viral supernatants for 24 hours, followed by puromycin selection at 2 µg/ml in growth medium for at least 48 hours. After 2 hours at 37°C, plates were spun at 500 g for 90 minutes. Following overnight culture at 37°C, cells were transferred to fresh viral supernatants with prestimulation cytokines for a second round of incubation, spinoculation, and overnight culture. The cells were then subjected to 48 hours of puromycin selection at 2 µg/ml in prestimulation medium, and live cells were enriched by centrifugation on Ficoll-Paque PLUSTM medium (#17-1440-02, GE Healthcare, Pittsburgh, PA). Viable mononuclear cells were washed in IMDM and subjected to erythroid culture.

For immunoprecipitation of transfectants, HEK293T cells were transfected with 20 µg plasmid using a calcium phosphate precipitation kit and harvested for protein extracts 40 hours following initiation of transfection. The following expression constructs were used for cotransfection and coimmunoprecipitation in HEK293T cells: pcDNA6/myc and pcDNA6/myc human TfR2 from Dr. Paul

Schmidt (Boston Children's Hospital); Mfn2-Myc, a gift from David Chan (Addgene plasmid #23213);[1] pEYFP-C1; Rab7-GFP, a gift from Merino Zerial.[2]

Supplemental Figures

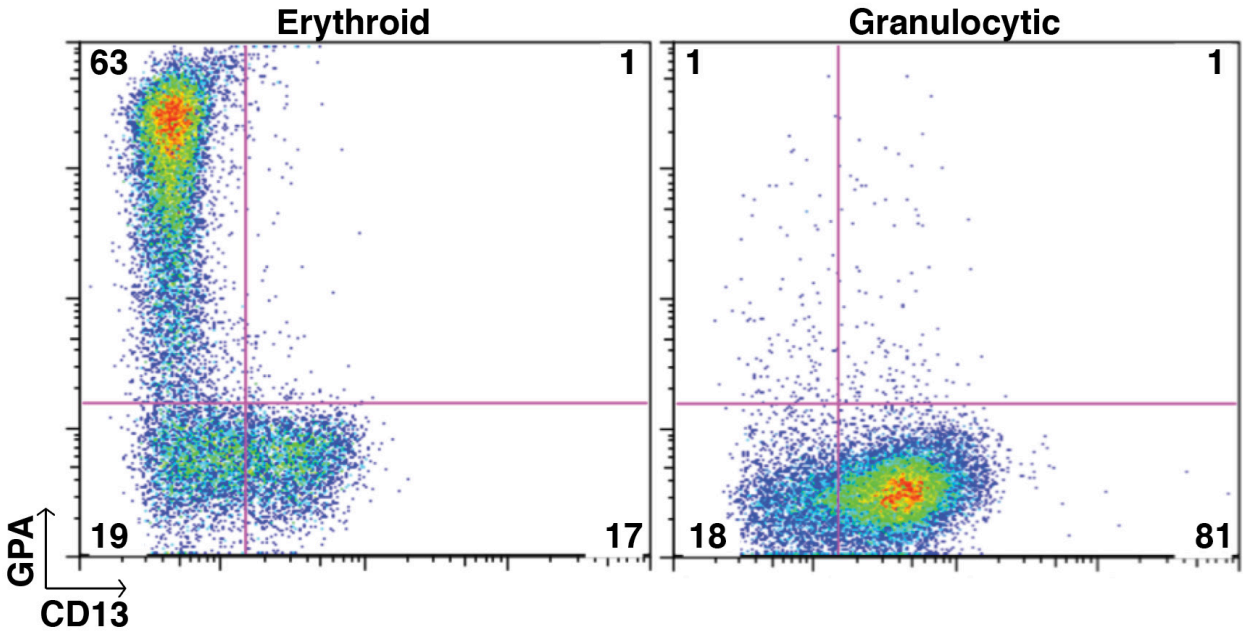

Figure S1. Differentiation of erythroid and granulocytic progenitors from purified CD34+ human bone marrow cells.
Flow cytometry of progenitors cultured four days in erythroid or granulocytic medium.

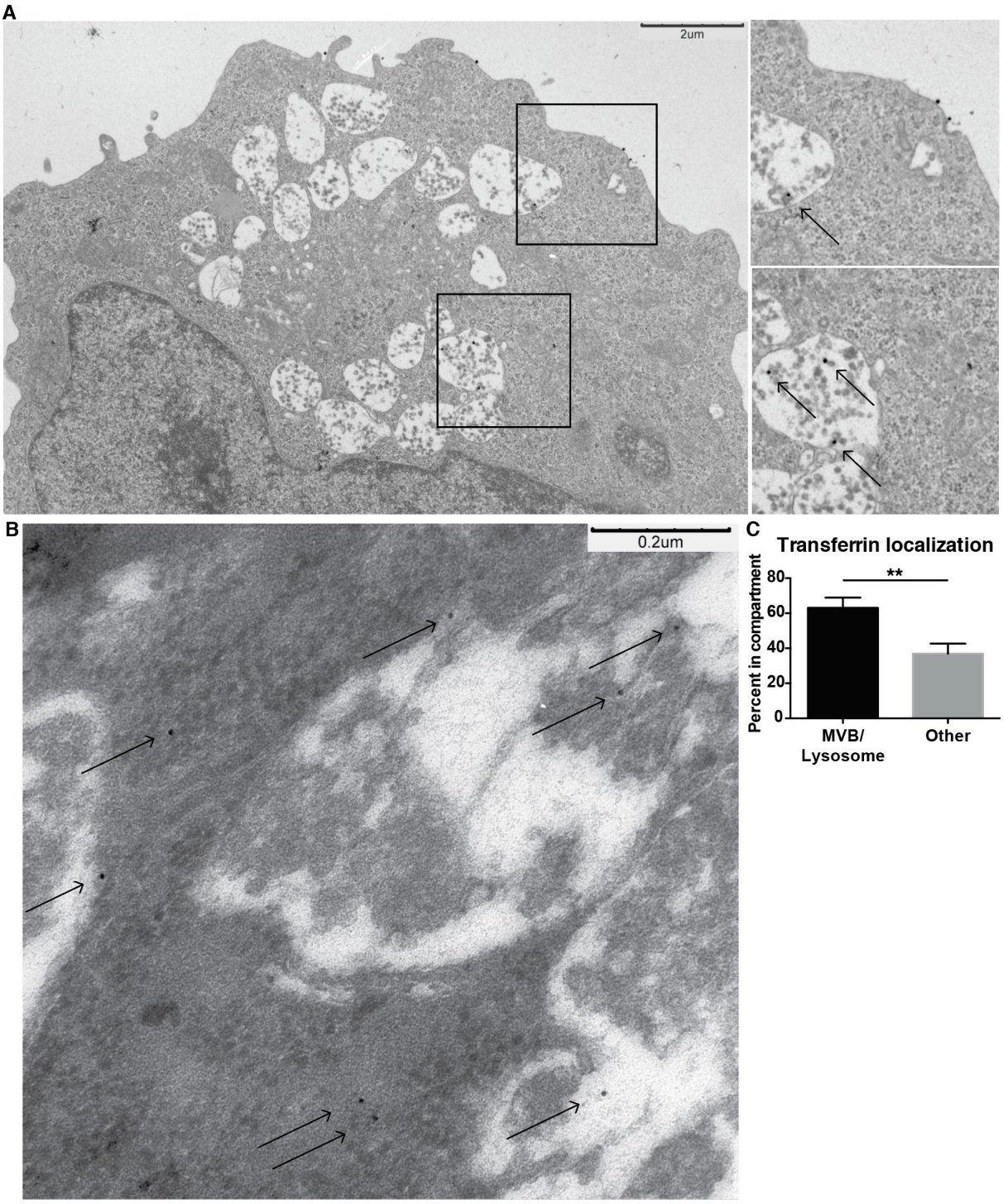

Figure S2. Distribution of transferrin in erythroid progenitors.
(A) Transmission electron microscopy of erythroid progenitors subjected to 30 nm gold-conjugated transferrin uptake, with insets highlighting specific MVB/lysosomal accumulation of transferrin particles.
(B) Transmission electron microscopy of erythroid progenitors subjected to biotin-conjugated transferrin uptake followed by gold-conjugated streptavidin staining. Arrows denote gold particles.
(C) Quantification of biotin-conjugated transferrin uptake by erythroid cells as in (B) (number of cells counted = 18 per group, $^{**}P < 0.01$).

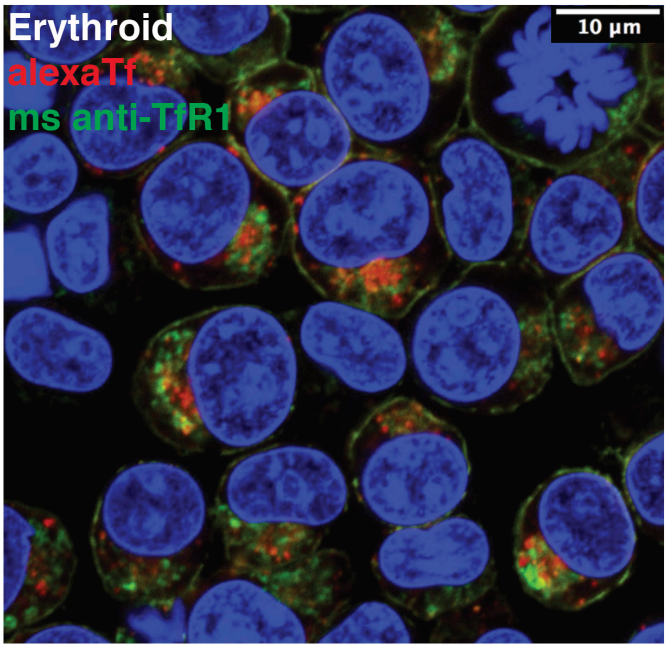

Figure S3. Confirmation of non-colocalization of transferrin with TfR1 in erythroid progenitors. Immunofluorescent staining for TfR1 using alternative mouse monoclonal anti-TfR1 (sc-32272 from Santa Cruz Biotechnology) on erythroid progenitors subjected to Alexa Fluor® 594-tagged transferrin loading (63X oil objective).

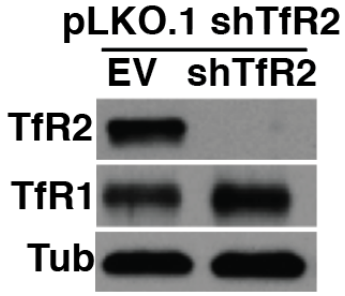

Figure S4. TfR2 knockdown in lentivirus-transduced progenitors.
Immunoblot documentation of TfR2 knockdown in erythroid progenitors transduced with lentiviral shRNA constructs (EV: empty vector).

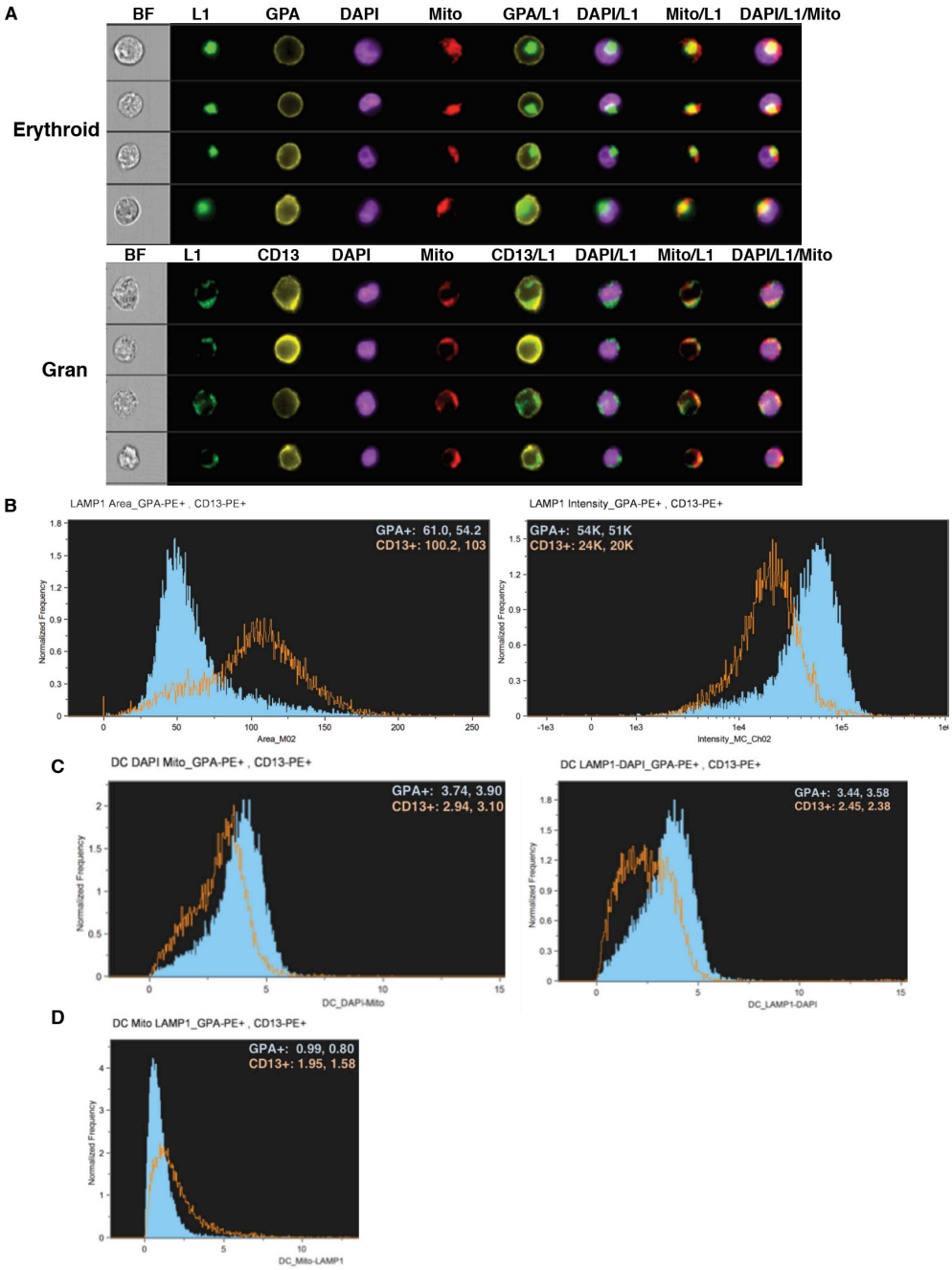

Figure S5. Demonstration by imaging cytometry of differences between erythroid and granulocytic progenitors in lysosomal-mitochondrial organization.

(A) Representative images of erythroid (GPA+) and granulocytic (CD13+) progenitors showing lysosomal (L1), mitochondrial (Mito), and nuclear (DAPI) distribution. Imaging cytometry was conducted using Amnis ImageStream®.

(B) Comparison of lysosomal LAMP1 area (left panel) and intensity (right) in gated erythroid and granulocytic progenitors. Mean, median values are shown in upper right corner.

(C) Comparisons of nuclear-mitochondrial and nuclear-lysosomal orientations using DAPI-MitoTracker XY delta-centroid (left panel) and DAPI-Lamp1 XY delta-centroid (right) in gated erythroid and granulocytic progenitors. Mean, median values are shown in upper right corner.

(D) Comparison of lysosomal-mitochondrial orientation using Lamp1-MitoTracker XY delta-centroid in gated erythroid and granulocytic progenitors. Mean, median values are shown in upper right corner.

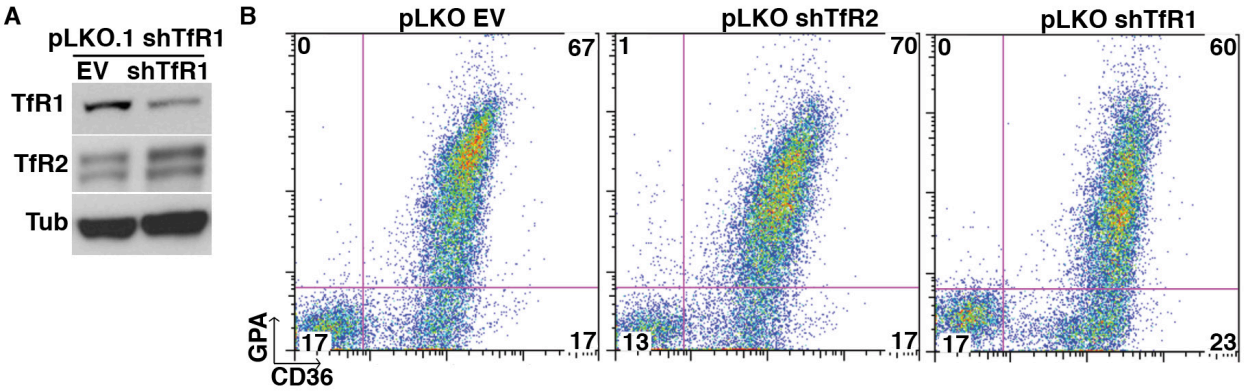

Figure S6. TfR1 knockdown in lentivirus-transduced progenitors.

(A) Immunoblot documentation of TfR1 knockdown in erythroid progenitors transduced with lentiviral shRNA constructs (EV: empty vector).

(B) Flow cytometry of progenitors subjected to transduction with lentiviral shRNA constructs and cultured four days in erythroid medium.

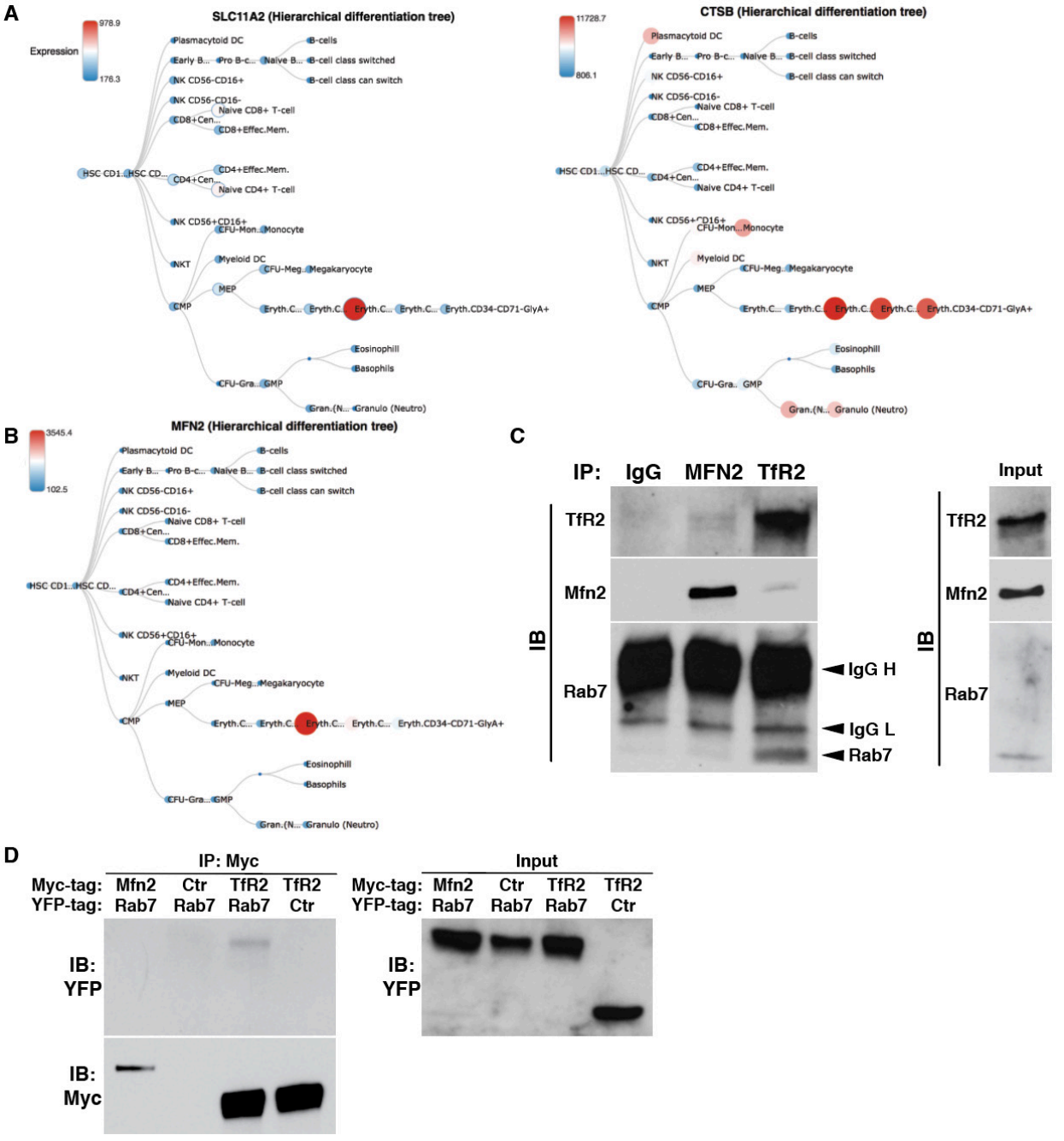

Figure S7. Interactions between lineage- and stage- specific drivers of iron uptake.
(A) Heatmap depiction of *SLC11A2* (encoding DMT1) and *CTSB* (encoding lysosomal Cathepsin B protease) transcript levels throughout normal human hematopoiesis, with highest expression in GPA+ CD71+ intermediate erythroid progenitors. The DMAP dataset was analyzed using BloodSpot (http://servers.binf.ku.dk/bloodspot).
(B) Heatmap depiction of *MFN2* transcript levels throughout normal human hematopoiesis.
(C) Immunoprecipitation (IP) with indicated antibodies of K562 cell extracts followed by immunoblot (IB).
(D) Immunoprecipitation (IP) of Myc-tagged recombinant MFN2 and TfR2 from extracts of HEK293T transfectants followed by immunoblot (IB) detection of YFP-tagged Rab7 and control YFP. Input immunoblot in left panel.

Supplemental References

1. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol*. 2003;160(2):189-200.

2. Rink J, Ghigo E, Kalaidzidis Y, Zerial M. Rab conversion as a mechanism of progression from early to late endosomes. *Cell*. 2005;122(5):735-749.