Erythroid cell mitochondria receive endosomal iron by a “kiss-and-run” mechanism

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In erythroid cells, more than 90% of transferrin-derived iron enters mitochondria where ferrochelatase inserts Fe2+ into protoporphyrin IX. However, the path of iron from endosomes to mitochondrial ferrochelatase remains elusive. The prevailing opinion is that, after its export from endosomes, the redox-active metal spreads into the cytosol and mysteriously finds its way into mitochondria through passive diffusion. In contrast, this study supports the hypothesis that the highly efficient transport of iron toward ferrochelatase in erythroid cells requires a direct interaction between transferrin-endosomes and mitochondria (the “kiss-and-run” hypothesis). Using a novel method (flow sub-cytometry), we analyze lysates of reticulocytes after labeling these organelles with different fluorophores. We have identified a double-labeled population definitively representing endosomes interacting with mitochondria, as demonstrated by confocal microscopy. Moreover, we conclude that this endosome-mitochondrion association is reversible, since a “chase” with unlabeled holotransferrin causes a time-dependent decrease in the size of the double-labeled population. Importantly, the dissociation of endosomes from mitochondria does not occur in the absence of holotransferrin. Additionally, mutated recombinant holotransferrin, that cannot release iron, significantly decreases the uptake of 59Fe by reticulocytes and diminishes 59Fe incorporation into heme. This suggests that endosomes, which are unable to provide iron to mitochondria, cause a “traffic jam” leading to decreased endocytosis of holotransferrin. Altogether, our results suggest that a molecular mechanism exists to coordinate the iron status of endosomal transferrin with its trafficking. Besides its contribution to the field of iron metabolism, this study provides evidence for a new intracellular trafficking pathway of organelles.

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1. Introduction

Iron is a transition metal whose properties make it useful to vital biologic functions in virtually all living organisms. Essential cellular functions include use of iron for oxygen transport, electron transfer, DNA synthesis and innumerable other purposes [1–4]. However, under physiologic conditions iron is virtually insoluble and can be extremely toxic when not properly shielded [5–7]. Hence, iron plays a role in the formation of toxic oxygen radicals that damage various cell structures. Therefore, it is critical that we gain a better understanding of the mechanisms involved in normal and abnormal intracellular iron trafficking.

Developing erythroid cells, which have a capacity to obtain and process iron with astonishing efficacy [8], are capable of concentrating iron (in the form of heme in the red cell) to approximately 7000-fold what is present in the plasma (as diferric transferrin [Fe2-Tf]). Additionally, the delivery of iron into hemoglobin occurs extremely efficiently, since mature erythrocytes contain about 45,000-fold more heme iron than non-heme iron [9]. These facts suggest that in erythroid cells the iron transport and heme biosynthetic machineries are fully integrated and are part of the same metabolic pathway that leads to a remarkably efficient production of heme. Delivery of iron to these cells occurs following the binding of Fe2-Tf to its cognate receptors (TfR) on the cell membrane. The Fe2-Tf-TfR complexes are then internalized via endocytosis, and iron is released by a process which includes both acidification and the participation of the TfR [1,9–12]. Iron, following its reduction to Fe2+ by Steap3 (six-transmembrane epithelial antigen of the prostate [13]), likely together with ascorbate [14] is then transported across the endosomal membrane by the divalent metal transporter 1, DMT1/ Nramp2 [15].
Following its egress from endosomes, iron is transported to intracellular sites of use and/or storage in ferritin, but this aspect of iron metabolism remains elusive or is at best controversial. It has been commonly believed that a low molecular weight intermediate chaperones iron from endosomes to mitochondria and other sites of utilization [16]. However, this long-sought iron binding intermediate, that would constitute the labile iron pool, has never been identified. In fact, earlier work from this laboratory demonstrated [17] that there was virtually no low molecular weight iron pool in hemoglobin-synthesizing cells and that iron present in this pool does not behave as an intermediate, thus corresponding to an end product. Although it has recently been proposed that the ferrous iron in this pool is associated with glutathione [18], this conclusion was only based on chemical interactions. Hence, the relevance of this study to the physiological mechanisms involved in the intracellular iron transport remains in doubt. Additionally, it has been proposed that human poly(rC)-binding protein 1 (PCBP1) “chaperones” iron into ferritin and non-heme iron proteins [19,20]. On the other hand, more recent reports [21,22] have proposed PCBP2, rather than PCBP1, to play an “iron-chaperone” role. This diversity is probably due to different cell types and/or experimental strategies used. However, no direct evidence has been provided that PCBPs acquire iron from transferrin-endosomes, carry it in cytosol and, more crucially, deliver it to mitochondria.

In erythroid cells, more than 90% of iron enters mitochondria where ferrochelatase, the enzyme that inserts Fe$^{2+}$ into protoporphyrin IX (PPIX), resides in the matrix side of the inner mitochondrial membrane. Importantly, in these cells, strong evidence exists for specific targeting of iron toward mitochondria. This mitochondria-directed transport is demonstrated in hemoglobin-synthesizing cells, in which iron acquired from Tf continues to flow into mitochondria even when the synthesis of PPIX is suppressed either experimentally [17,23–25] or in hereditary sideroblastic anemia caused by defects in heme synthesis [26–28]. Of note, a compelling candidate for the transport of iron through the mitochondria towards ferrochelatase, mitoferrin, has been identified [29].

Based on the above considerations, we have formulated a hypothesis that in erythroid cells a transient mitochondrion-endosome interaction is involved in iron translocation to its final destination [17,30]. We have collected the following experimental evidence to support this hypothesis: 1) iron, delivered to mitochondria via the Tf–TfR pathway, is unavailable to cytoplasmic chelators [31]; 2) Tf-containing endosomes move to and contact mitochondria [31] in erythroid cells; 3) endosomal movement is required for iron delivery to mitochondria [31,32]. We have also demonstrated that “free” cytoplasmic iron is not efficiently used for heme biosynthesis and that the endosome-mitochondrion interaction increases chelatable mitochondrial iron [31].

As already mentioned, the substrate for the endosomal transporter, DMT1, is Fe$^{2+}$, the redox form of iron which is also the substrate for ferrochelatase. These facts make our hypothesis quite attractive, since the “chaperone”-like function of endosomes may be one of the mechanisms that keeps the concentrations of reactive Fe$^{2+}$ at extremely low levels in the oxygen-rich cytosol of erythroblasts and reticulocytes, preventing ferrous iron’s participation in a dangerous Fenton reaction.

There is accumulating evidence for transfer of metabolites directly between interacting organelles [33–36]. However, the role of endosomes in distributing intracellular iron is accepted without enthusiasm [20], misinterpreted [16] or simply ignored [37]. Hence, we deemed it essential to seek further evidence for our “kiss-and-run” hypothesis.

In the current study, we used 3D live confocal imaging of reticulocytes following their incubation with MitoTracker Deep Red (MTDR) and Alexa Green Transferrin (AGTf) and demonstrated transient interactions of endosomes with mitochondria. We also demonstrate these interactions by a novel method exploiting flow sub-cytometry to analyze reticulocyte lysates labeled with MTDR and AGTf. This strategy identified a population double-labeled with both fluorescent markers representing endosomes interacting with mitochondria. FACs sorting followed by 2D confocal microscopy confirmed the association of both organelles in the double-labeled population. The results of these experiments as well as those exploiting a mutated recombinant Fe$^{2+}$-Tf unable to release iron efficiently further support the hypothesis that the efficient iron transfer to PPIX requires a transient interaction between the endosome and the mitochondrion.

2. Results

2.1. Three-dimensional confocal microscopy of live cells reveals endosome-mitochondrion interactions

Using two-dimensional (2D) confocal microscopy, we have previously shown that endosomes come in contact with mitochondria [31]. To avoid any pseudo-interactions of these two organelles, i.e., the endosome hovering over the mitochondria, we have used a Quorum WaveFx spinning disc confocal system to capture three-dimensional (3D) live cell imaging of reticulocytes with fluorescently labeled mitochondria and endosomes. An individual cell at different time intervals is presented in three different orientations (Fig. 1, A–C) and shows the proximity of endosomes and mitochondria at different angles (Link for Video; please see Supplementary Material).

2.2. Development of the flow sub-cytometry method to study endosome-mitochondrion interactions

We developed a new method, “flow sub-cytometry”, based on the standard flow cytometry method, to analyze lysates obtained from reticulocytes with fluorescently labeled mitochondria (MTDR) and endosomes (AGTf). Employing this strategy, three distinct populations: endosomes, mitochondria, and a population double labeled with both

![Fig. 1. 3D confocal microscopy demonstrates the association of transferrin-endosomes (green) with mitochondria (red). At one time interval (3 sec, marked by white arrows), endosome-mitochondria pairs are shown in three different orientations.](image-url)
fluorescent markers have been identified (Fig. 2A). To confirm the interaction between endosomes and mitochondria, we used FACS-sorting to isolate the population double-labeled with both fluorescent markers as well as MTDR-labeled population (as a negative control), followed by 2D examination using a confocal fluorescence microscope. A multichannel protocol was used to scan the samples for AGTf and MTDR labeled endosomes and mitochondria, respectively. The colocalization of the two organelles was demonstrated by overlapping the green and the red images of endosomes and mitochondria, where the merged images represent both organelles together (Fig. 2B). This experiment has unequivocally demonstrated the presence of the Tf-endosome interacting with the mitochondrion in the double-labeled population (UR). Importantly, the LR population contains exclusively MTDR-labeled mitochondria and the LL population is devoid of any fluorescence. Moreover, we have shown that the size of the double-labeled population representing endosomes associated with mitochondria (UR quadrant) increases with incubation time with AGTf and reaches a plateau in ~20 min (Fig. 3).

2.3. Holotransferrin causes the detachment of endosomes from mitochondria

To investigate whether the association of endosomes with mitochondria is reversible, we incubated reticulocytes with both fluorescent markers for 20 min, washed the cells and reincubated with or without holotransferrin (Fe2-Tf) for 60 min. The presence of non-fluorescent Fe2-Tf caused a time-dependent decrease in the size of the double-labeled population (Fig. 4A and B). Hence, AGTf-endosomes associated with mitochondria are replaced by incoming non-fluorescent Fe2-Tf-endosomes, indicating the reversibility of the interaction. AGTf-endosomes leaving mitochondria cannot be detected by FACS because of their small size. Importantly, this finding, together with the fact that endosomes remain bound to mitochondria in the absence of Fe2-Tf, suggests that the Tf-endosome drives the detachment.

2.4. Intra-endosomal transferrin saturation governs interorganelar association

We investigated whether the degree of endosomal Tf saturation with iron affects the dynamics of the endosome-mitochondrion association. When baflomycin A1, a specific V-ATPase inhibitor known to decrease acidification of endosomes [38], is added to reticulocytes, the size of the double-labeled population dramatically decreased (Fig. 5A). Baflomycin A1 has been shown to block the incorporation of 59Fe into heme from 59Fe-Tf-endosomes [32]. Additionally, heme treatment of reticulocytes also considerably decreased the size of the double-labeled population (Fig. 5B). Heme has been shown to feedback inhibit the release of iron from Tf within reticulocyte endosomes [39] and, similarly as baflomycin A1, can be expected to increase endosomal Tf saturation.

The most likely explanation for these observations is that baflomycin A1 as well as heme block Fe2+ export from endosomes already associated with mitochondria, extending their residence time on these organelles. This causes a “traffic jam” that decelerates the movement of endosomes containing fluorescent Tf towards mitochondria. Furthermore, the presence of heme during the reincubation of double-labeled reticulocytes with unlabeled Fe2-Tf slowed down the disappearance of the double population (Fig. 5C), suggesting that the efficient dissociation of endosomes requires them to be iron-free.

2.5. Calmodulin antagonist (W-7) decreases the size of double-labeled population

N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7), a calmodulin antagonist, is known to inhibit the microfilament motor, myosin, which is involved in intracellular movement of endosomes and causes significant inhibition of 59Fe incorporation from 59Fe-Tf-endosomes into heme [32]. A positive experiment presented in Fig. S2 shows that W-7, as expected, inhibits dramatically the association of endosomes with mitochondria.

2.6. Transferrin mutant unable to release iron (Holo-rTf) impairs the uptake of 59Fe from 59Fe2-Tf

To assess whether endosomes unable to release iron affect intracellular iron trafficking, reticulocytes were preincubated with Holo-rTf at 37 °C for 30 min, washed with cold PBS and incubated with 59Fe-Fe2-Tf for the indicated time intervals. After washing with cold PBS, total cell and heme 59Fe-radioactivities were measured. The presence of Holo-rTf during incubation significantly inhibited 59Fe uptake by reticulocytes from wild-type 59Fe-Fe2-Tf and also diminished 59Fe incorporation into heme (Fig. 6). This result suggests that the failure to release iron from Holo-rTf interferes with the process of endocytosis or endosomal trafficking, causing a decrease in the total 59Fe uptake by the reticulocytes as compared to controls.
2.7. Endosomes interact with mitochondria also in erythroid progenitors

In this experiment, we used murine fetal liver (FL) cells, primary mouse erythroid cells that closely recapitulate several aspects of terminal maturation in vivo, in particular very active hemoglobinization [8]. We deemed it important to ascertain whether endosomes interact with mitochondria only in differentiating erythroid cells or if this phenomenon operates also before the onset of hemoglobin synthesis. As predicted, subjecting these cells to flow sub-cytometry as performed above with reticulocytes, revealed a large pool of the double-labeled population in FL cells induced to differentiate by erythropoietin. Importantly, we also observed that the endosome-mitochondria population, although smaller than in erythropoietin-induced FL cells, is present already in undifferentiated cells (Fig. S3).

3. Discussion

During differentiation, immature erythroid cells acquire vast amounts of iron at a “breakneck” rate. Proper coordination of iron delivery and utilization in heme synthesis is essential; disruption of this process likely underlies iron loading disorders such as sideroblastic anemia and myelodysplastic syndrome with ringed sideroblasts [28]. On a per-cell basis, the rate of heme synthesis in developing erythroid cells is at least an order of magnitude higher than in the liver, the second highest heme producer in the body.

As described in the Introduction, most mammalian cells take up iron via receptor-mediated endocytosis by the Tfr. The prevailing opinion is that after its escape from endosomes, iron spreads into the cytosol [40] and mysteriously finds its way into mitochondria. Based on the fact that Tfr-bound iron is extremely efficiently used for hemoglobin synthesis, targeted into erythroid mitochondria, and that no cytoplasmic iron transport intermediate has ever been identified in erythroid cells, we have suggested a new hypothesis of intracellular iron transport [17, 30]. This model proposes [17] that after iron is released from Tfr in Hb-synthesizing cells, it would bypass the cytosol and be directly transferred from protein to protein (probably in hydrophobic environments) until it reaches ferrochelatase in the mitochondrion.

Previous work from our laboratory has suggested that iron is directly transferred from endosomes to mitochondria [31,32] likely through interorganellar interaction. However, this model has not been accepted by many [16,20]. In the present study, we used 2D and 3D confocal microscopy and developed a novel experimental strategy based on flow cytometry analysis of fluorescently labeled organelles (“flow sub-cytometry”) to provide evidence for our hypothesis.
Employing 3D confocal system, we acquired video images, allowing us to capture fluorescent Tf-endosomes interacting with labeled mitochondria in space, excluding the possibility of “pseudo-interactions” caused by a coincidental overlapping. This technique very clearly revealed contacts of endosomes with mitochondria in 3D space (Fig. 1).

Using a flow sub-cytometry to analyze lysates obtained from reticulocytes with fluorescently labeled mitochondria (MTDR) and endosomes (AGTf), we have identified three distinct populations: endosomes, mitochondria, and a population double-labeled with both fluorescent markers (Fig. 2A). The double-labeled population was sorted by FACS and shown by 2D confocal microscopy to be composed of endosomes associated with mitochondria (Fig. 2B). The size of the double-labeled population increases with the incubation time and reaches a plateau at approximately 20 min (Fig. 3).

The reversibility of the endosome-mitochondria association was demonstrated by the experiment in which we first preincubated reticulocytes with fluorescent markers for 20 min followed by their incubation (“chase”) with or without unlabeled holotransferrin (Fig. 4A and B). The presence of unlabeled Fe2-Tf caused a time-dependent decrease in the size of the double-labeled population indicating the reversibility of the endosome-mitochondria contact. Importantly, in the absence of holotransferrin, endosomes remain associated with mitochondria. Taken together, our finding indicates that Tf-endosome drives the detachment.

Both bafilomycin A1 and heme interfere with iron release from Tf and cause a significant decrease in the size of the double-labeled population (Fig. 5A and B). These agents obstruct Fe2⁺ export from endosomes already associated with mitochondria, extending the residence time of endosomes on mitochondria, thus blocking the movement of endosomes containing fluorescent Tf towards mitochondria. Additionally, hemin slows down the dissociation of labeled endosomes from mitochondria (Fig. 5C). Hence, our results suggest that a molecular mechanism exists to coordinate the iron status of endosomal Tf with its trafficking.

Moreover, the presence of a Tf mutant unable to release iron (Holo-rTf) impairs the uptake of 59Fe from 59Fe2-Tf (Fig. 6), suggesting that efficient endocytosis and/or endosomal trafficking requires the transfer of iron from endosomes to mitochondria. In other words, the endosomal mitochondrial interface plays a role in regulating the cellular iron uptake, probably by controlling Tf endocytosis.

Our finding of endosomes interacting with mitochondria in undifferentiated fetal liver cells (Fig. S3) is of considerable importance. It suggests that the endosome-mitochondrion interaction may be a common mechanism involved in iron delivery for heme synthesis. However, since FL progenitors are committed to synthesize hemoglobin, further experiments with non-erythroid cells are needed to establish whether the kiss-and-run mechanism is generally involved in the transport of Tf-borne iron to mitochondria. If the so-called kiss-and-run mechanism of iron delivery to mitochondria proves to be ubiquitous, then mitochondria...
may be the entry site of iron into the cell. In this context it needs to be pointed out that the only known function of internalized Tf is to deliver iron to its intracellular destination [31]. Hence, the described endosome mitochon-
dria association facilitates interorganellar iron transfer.

Our study is relevant to sideroblastic anemias, a heterogeneous group of disorders, characterized by mitochondrial iron overload in de-
veloping red blood cells [26–28]. The unifying characteristic of all sideroblastic anemias is the ring sideroblast, a pathological erythroid precursor containing excessive deposits of non-heme iron in mitochon-
dria with perinuclear distribution creating a ring appearance.

The majority of congenital sideroblastic anemias are X-linked; most of them are caused by mutations in the gene encoding erythroid-specific 5-
aminolevulinate synthase [26–28]. However, there is a certain proportion of patients with hereditary sideroblastic anemia who exhibit autosomal recessive inheritance; some such patients have a defect in the gene encoding the erythroid-specific mitochondrial carrier protein, SLC25A38 [41]. It has been suggested that SLC25A38 may translocate glycine into mitochondria, a proposal recently confirmed by Fernández-Murray et al. [42] and Lunetti et al. [43].

In our opinion, at least four factors are responsible for the pathogen-
esis of ring sideroblast formation in congenital sideroblastic anemias: (i) As extensively discussed in this report, the efficient delivery of iron to ferrochelatase in erythroid cells requires the direct interaction of endosomes with mitochondria. (ii) Mitochondrial iron cannot be ade-
quately utilized due to the lack of PPIX. (iii) Erythroid cells, but not non-erythroid cells, are equipped with a negative feedback mechanism in which ‘uncommitted’ heme inhibits iron acquisition from TF [30]. Al-
though the molecular mechanism of this inhibition is still unknown, the lack of heme plays an important role in mitochondrial iron accumula-
tion in erythroid cells. (iv) Iron can leave erythroid mitochondria only after being inserted into PPIX [24].

As already alluded to, there is no consensus regarding the mecha-
nism of iron transfer from plasma TF to mitochondria. One of the pro-
posed intermediates in the intracellular iron path is the iron storage protein, ferritin [44,45]. Nevertheless, a strong argument against this claim was collected and reported [46]. Somewhat surprisingly, it was suggested in a recent report [47] that the autophagic turnover of ferritin via NCOA4 is a critical process for regulating intracellular iron bioavail-
bility. However, Darshan et al. [48] demonstrated that the conditional deletion of the ferritin heavy chain in adult mice did not cause any de-
crease in hematocrit or hemoglobin levels, strongly indicating that ferri-
tin is not involved in hemoglobinization. In this context it is pertinent to men-
tion that the nuclear receptor coactivator 4 (NCOA4), also known as ARA70, was initially identified as a specific coactivator for the androgen receptor in human prostate cells [49]. Additionally, another study [50] has provided evidence that NCOA4 knockout is not lethal and NCOA4-null mice have only a mild microcytic hypochromic anemia. These find-
ings concur with our previous and current studies demonstrating that the efficient utilization of iron for hemoglobin synthesis requires a di-
rect contact of endosomes with mitochondria.

Finally, recent experimental support for the kiss-and-run hypothesis came from a study that investigated the interaction between several mitochondrial heme biosynthetic enzymes and proteins involved in iron ho-
meostasis [51]. This study revealed a partnership between ferrochelatase (a mitochondrial protein) and TfR (an endosome associated protein) that may be mediated by a yet to be identified bridge protein. It will be worth examining whether one of the PCBPs could serve as such protein [19,21,52]. Medlock et al. [45] further state, “(their) data...support a model of transient protein-protein interactions within a dynamic protein complex.” Our model, shown in Fig. 7, suggests a potential outer

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Fig. 5. Bafilomycin A1 and hemin decrease the association of endosomes with mitochondria. (A) The percentage of the double-labeled population after treatment with bafilomycin. ■ Control; ♦, bafilomycin A1 (100 nM). Results presented are representative of 6 replicate experiments. (B) The percentage of the double-labeled population after treatment with hemin. ■ Control; ▲, hemin (5 μM). Results presented are representative of 4 replicate experiments. (C) Hemin prevents the dissociation of labeled endosomes from mitochondria. ■ Control; ▲, hemin (5 μM). Error bars were determined from triplicate values.

Fig. 6. Holo-rTf inhibits 59Fe incorporation into reticulocytes and heme from 59Fe2-Tf. Error bars were determined from triplicate values. — p ≤ 0.01; --- p ≤ 0.001.
mitochondrial membrane protein(s) that may interact with the intracellular loop of DMT1 for the delivery of iron to ferrochelatase.

In summary, our study provides strong evidence that the highly efficient delivery of iron to heme in hemoglobin-synthesizing cells requires the direct interaction of transferrin-endosomes with mitochondria. To the best of our knowledge, this is the first evidence of the interorganellar interaction having the functional consequence of facilitating the transport of a micronutrient. A full understanding of the molecular mechanisms involved in the endosome-mitochondria system will require the identification of the “signal[s]” that direct iron-carrying endosomes towards mitochondria, the players involved in the docking of endosomes to mitochondria and the “signal[s]” that determine the detachment of iron-free endosomes from mitochondria. We believe that the current work provides a strong platform to pursue such studies.

4. Materials and methods

4.1. Materials

MitoTracker Deep Red 633 (MTDR) and Alexa Green Transferrin 488 (AGTf) were purchased from Molecular Probes (Invitrogen, Carlsbad, CA). Protease inhibitor was purchased from Roche (Penzberg, Germany). Pronase, heparin and bafilomycin A1 were purchased from Bioshop (Burlington, Ontario). Holotransferrin (Fe2-transferrin), avertin, phenylhydrazine, thiozole orange and N-(6-Aminohexyl)-5-chloro-1-napthalenesulfonamide hydrochloride (W7) were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent mounting media were purchased from Dako (Glostrup, Denmark). Microslides and micro coverslips were purchased from VWR (West Chester, PA). Minimum Essential Media (MEM) were purchased from Invitrogen (Gibco® Life Technologies™, Carlsbad, CA) supplemented with 25 mM HEPES. Mitochondrial isolation buffer contains 220 mM mannitol, 60 mM sucrose, 20 mM HEPES, pH 7.4, 80 mM KCl, 2 mM magnesium acetate, 0.5 mM EGTA and protease inhibitors. The human mutant recombinant Fe2-Tf unable to release iron (K206E/K534A) [53,54] was generated in Dr. Mason’s laboratory.

4.2. Animals

All the animal studies performed in this work were approved by the Lady Davis Institute animal care committee following the guidelines of the Canadian Council on Animal Care.

CD1 and C57BL/6 mice (females; 6 months old) retired breeders were obtained from Charles River Laboratories (Wilmington, MA) and were housed in microisolator cages.

4.3. Isolation of reticulocytes

Adult, female CD1 female mice were injected with neutralized phenoxyhydrazine intraperitoneally at the dose of 50 mg/kg/day for three consecutive days. Mice were then anesthetized after 2–3 days following the last injection by using 1 mL of 2.5% avertin. Blood was collected via direct cardiac puncture using heparinized syringe and resuspended in cold PBS. Cells were then washed with ice-cold PBS three times. By using this technique we obtained 30–45% reticulocytes as verified by thiozole orange staining.

4.4. Cell culture of primary mouse fetal liver cells

Primary erythroid cells were cultured as described [8]. Briefly, cells were grown from FL cells that were obtained from embryonic day 12.5 embryos of wild type C57BL/6 mice. FL cells were grown in serum-free StemPro-34 medium plus Nutrient Supplement (Invitrogen, Carlsbad, CA) and 2 mM L-glutamine, with 100 ng/mL murine recombinant stem cell factor (SCF), 10^{-5} M synthetic glucocorticoid dexamethasone (Dex), 40 ng/mL insulin-like growth factor 1 (IGF-1), 0.2% (vol/vol) gentamicin, 0.2% amphotericin, and 2 U/mL human recombinant erythropoietin (Epo). FL cells were kept either in an undifferentiated state or induced to terminal differentiation (48 h) by re-suspending them in differentiation medium (StemPro-34) containing Nutrient Supplement, 10 U/mL Epo, 2 mM L-glutamine, 4 × 10^{-4} IU/mL insulin, 3 × 10^{-5} M RU486, and 1 mg/mL iron-saturated human transferrin (Fe2-Tf, Sigma, St. Louis, MO).
4.5. Confocal microscopy

Reticulocytes were labeled with MTDR and AGTf and kept at 4 °C until their analysis by spinning disc confocal microscopy. The temperature of the chamber was kept at 37 °C. One-milliliter samples of reticulocytes suspension were resuspended in 50 μL of medium without phenol red. Cells were then mounted on a pre-warmed (30 °C) heated stage and were allowed to equilibrate for 15 min before imaging. Reticulocytes were not kept for more than 10 min in the chamber after exposing to laser. Three-dimensional images were acquired over time. Optical sections of 0.5 μm were acquired in z-plane through a ±2.5-μm (total of 5.0-μm stack). Single endosome was tracked in a cell in different planes from the start of endocytosis to its interaction with mitochondria and leaving in a different plane. Microscopy was performed with a Zeiss LSM 5 Pascal confocal inverted microscope. Live cell imaging of reticulocytes was done with Wave FX Spinning Disc Confocal (SDC) microscopy by Quorum Technologies Inc. (Guelph, Ontario). Velocity 3D Image Analysis Software from PerkinElmer was used to analyze live cell imaging. Rapid imaging of these instruments makes it possible to determine the 3D trajectory of an intra-cellular object.

4.6. Flow sub-cytometry

Reticulocytes were washed three times with ice-cold PBS, spun down at 800 g for 5 min and pellet was re-suspended in MEM. Reticulocytes were then incubated with 500 nM MTDR for 30 min at 37 °C in a shaking water-bath following which they were washed 3-times with ice-cold PBS and centrifuged at 800g for 5 min at 4 °C. Cells were re-suspended in MEM containing 500 nM AGTf and kept on ice for 30 min in dark. After incubating with AGTf samples were divided and incubated at 37 °C for the indicated time intervals. The samples were then immediately placed on ice and washed three-times with ice-cold PBS to stop endocytosis. The samples were treated with pronase (1 mg/mL) on ice to remove any fluorescent probe bound to TPR present on the cell surface. The cells were then washed three-times with ice-cold PBS, re-suspended in 1 mL of mitochondrial isolation buffer and then subjected to four cycles of rapid freeze (liquid nitrogen) and thaw. The samples were spun at 800 g for 10 min at 4 °C and supernatants were analyzed by flow cytometer (Fig. S1). Fluorescence signals were determined using Becton Dickinson FACS Caliber (BD). AGTf 488 and MTDR 633 were determined by using a 530/30 and a 660/20 band pass filter, respectively. Data was analyzed using FCS Express V3 software. Double labeled population were also sorted on a FACSAria sorter (BD) followed by 2D examination in confocal microscopy. The effects of baflomycin A1, hemin and W7 were examined by their addition to MTDR pre-labeled reticulocytes followed by the incubation with AGTf.

4.7. Iron uptake and incorporation into heme

59Fe2-Tf was made from 59FeCl3 (PerkinElmer, Santa Clara, USA; 2 μCi) as described previously [55,56]. The acid precipitation method was used to measure 59Fe in heme and non-heme fractions as described previously [23,31,32]. Reticulocytes were collected by centrifugation (4000g for 30 s), lysed in water and then boiled in 1 mL of 0.2 M HCl. Samples were the transferred to an ice-bath and 59Fe-heme-containing proteins were precipitated with ice-cold 7% trichoroacetic acid (TCA; Bioshop Canada Inc., Burlington, Canada) solution, for 2 h and collected by centrifugation (8000g for 5 min at 4 °C). Precipitated proteins (containing 59Fe in heme) and supernatants (containing non-heme 59Fe) were separated in different tubes and radioactivity in heme and non-heme fraction was used to measure 59Fe in heme and non-heme fractions as described previously. 

Author contributions

A.H. and T.M.R. performed research, analyzed data, and wrote the manuscript; T.M.K. performed research and analyzed data; A.B.M. and A.D.S. analyzed data, and wrote the manuscript; and P.P. conceived the study, analyzed data, and wrote the manuscript.

Conflict of interest

The authors declare no competing or financial interests.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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