Brief Critical Review

July 2007: 335–340

Steap Proteins: Implications for Iron and Copper Metabolism

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Erythroid cells of the bone marrow, the most avid consumers of iron in the body, acquire ferric (Fe^{3+}) iron exclusively via the transferrin cycling pathway. A long-standing fundamental molecular question of how ferric iron is handled in this pathway has been recently resolved by the identification of Steap3 (six-transmembrane epithelial antigen of the prostate 3) as an endosomal ferrireductase needed for efficient utilization of transferrin-delivered iron. Further characterization of Steap3 and other Steap proteins reveals a possible greater role of Steap proteins in iron and copper metabolism.

Key words: anemia, cupric reductase, DMT1, ferrireductase

doi: 10.1301/nr.2007.jul.335–340

INTRODUCTION

Iron and copper have several similarities, including a facile ability to alternate between two common oxidation states. The metabolism of iron and copper are intimately linked. The two metals can compete for a common intestinal transporter (e.g., divalent metal transporter 1 [DMT1]); they can function in concert in the same protein (e.g., cytochrome oxidase); they can function as reactive centers of enzymes (e.g., copper in ceruloplasmin); and they can participate in each other's oxidation/reduction. The recent identification of six-transmembrane epithelial antigen of the prostate 3 (Steap3) as an essential component of the transferrin-transferrin receptor cycle provides additional possible links between iron and copper homeostasis.

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MOLECULAR ASPECTS OF THE TRANSFERRIN CYCLE

Iron circulates through the plasma bound to the glycoprotein transferrin, which solubilizes the ferric (Fe^{3+}) ion and mitigates its reactivity. Cells acquire iron when iron-loaded holotransferrin binds to the transferrin receptor at the cell surface, and the entire complex is internalized into endosomes. Endosomal acidification promotes iron to dissociate from transferrin, and the metal is then reduced to ferrous iron (Fe^{2+}) and transported into the cytosol by DMT1. The iron-free apotransferrin remains bound to the transferrin receptor and returns to the cell surface, where transferrin dissociates from its receptor and reenters the bloodstream, able to acquire ferric iron for another cycle of iron transport. This entire process of transferrin receptor-mediated endocytosis completes within a few minutes, with transferrin undergoing 100 to 200 such cycles during its lifetime in the circulation.[1]

Cells take up iron in proportion to their levels of cell-surface transferrin receptors, which vary with physiologic and developmental needs for iron. Developing erythroid cells of the bone marrow express very high levels of transferrin receptor and account for 80% of the body's transferrin-bound iron uptake.[2] The iron is utilized primarily for the synthesis of heme for red cell hemoglobin. Each day, approximately 20 mg of iron is taken up by 200 billion erythroid cells as they differentiate into mature erythrocytes. The key role of transferrin in the delivery of iron to developing erythroid cells was first demonstrated in 1959 by Jandle et al.,[3] who showed that in vitro immature red cells readily incorporate serum transferrin-bound iron, but not free cationic iron, into hemoglobin. The in vivo requirement for transferrin to supply adequate amounts of iron for red blood cell synthesis is illustrated by the fact that both mice and humans with hereditary hypotransferrinemia have severe hypochromic microcytic anemia.[4,5] An absolute requirement for the transferrin receptor in red cell production was formally demonstrated by producing transferrin receptor knockout mice. Transferrin receptor-null mice have a more severe phenotype than mice lacking trans-

ferrin; they die in utero and are characterized by anemia and defective erythropoiesis.[6]

Efficient utilization of iron taken up into the endosome during the transferrin cycle depends upon the activity of DMT1, a transmembrane protein that was first identified as a candidate intestinal iron importer in iron-deficient rodents.[7,8] The observation that mice and rats with mutations in DMT1 had not only defective absorption of dietary iron, but also defects in assimilating iron from the transferrin cycle in erythroid cells, suggested that DMT1 functions in the utilization of transferrin-bound iron as well. Based on these observations, Fleming et al.[9] proposed that DMT1 additionally serves to transport iron out of the transferrin cycle endosome. The essentiality of DMT1 in both intestinal iron uptake and erythroid iron utilization was shown recently by Gunshin et al.,[10] who inactivated the DMT1 gene in mice. DMT1 knockout neonatal mice display severe anemia and usually die within a week. To test if DMT1 plays a direct role in iron acquisition by erythroid cells, hematopoietic stem cells (HSCs) isolated from wild-type or DMT1-null mouse liver were transplanted into lethally irradiated mice. As expected, recipients of DMT1-null HSCs, but not wild-type HSCs, displayed abnormal erythrocyte morphology and anemia, which did not resolve over time.

Although the studies using DMT1-null mice clearly demonstrated that developing erythroid cells require DMT1 for adequate utilization of transferrin-bound iron, a key question remained. As indicated by its name, the divalent metal transporter DMT1 transports iron only in the divalent ferrous iron (Fe^{2+}) state, yet transferrin delivers iron as ferric iron (Fe^{3+}) to cells. In removing iron from transferrin, the cell faces two obstacles: first, the formidable affinity of iron for transferrin (approximately 10^{21} M^{-1}),[11] and second, the insolubility of aqueous Fe^{3+} at physiologic pH. One way to overcome the latter involves reducing the iron to the more soluble Fe^{2+}. Indeed, studies done 25 years ago demonstrated that lipid-soluble ferrous iron chelators block iron utilization by immature erythroid cells,[12,13] suggesting that reduction of Fe^{3+} to Fe^{2+} probably occurs during the intracellular process of iron translocation into the cytosol. Subsequent studies using isolated membranes provided evidence that this process occurs enzymatically by a protein demonstrating NAD(P)H:ferricyanide oxidoreductase activity.[14] The identity of the protein involved, however, proved elusive.

STEAP3 AND ERYTHROID IRON METABOLISM

The application of genetic and molecular biological techniques to the study of mice with iron-transport defects has proved invaluable in identifying and characterizing the functions of proteins involved in iron metabolism.[15] One example is DMT1, which was identified in 1998 by Fleming et al.,[9] who used positional cloning to identify the iron transporter defective in microcytic anemia (*mk*) mice. More recently, Fleming's group used a similar strategy to identify the gene defect underlying the genetic anemia of *nm1054* mice.[16] Mutant *nm1054* mice have microcytic, hypochromic anemia, but unlike *mk* mice, have elevated liver iron stores and are therefore not systemically iron deficient.[17] Studies with isolated reticulocytes from *nm1054* mice demonstrated a marked decrease in the assimilation of iron from transferrin, suggesting that the anemia likely results from inefficient utilization of transferrin-bound iron by erythroid cells. Positional cloning revealed that the iron utilization defect was associated with a large genomic deletion encompassing several genes on mouse chromosome 1. Further studies of transgenic mice expressing the deleted genes individually showed that the anemia in *nm1054* mice could be corrected by a single gene previously identified as Steap3. Formal proof that the anemia of *nm1054* mice resulted from the lack of Steap3 was provided by the generation of Steap3-null mice, which recapitulated the *nm1054* phenotype.[16] As expected, the Steap3-null reticulocytes had markedly decreased transferrin-bound iron utilization, similar to those from *nm1054* mice. Three more lines of evidence further implicated Steap3 in erythroid iron metabolism: 1) Steap3 is expressed abundantly in fetal liver, the site of mid-gestational hematopoiesis, as well as in bone marrow; 2) intracellular Steap3 partially co-localizes with the three known players in the transferrin cycle—transferrin receptor, transferrin, and DMT1; and 3) cells transfected with Steap3 cDNA display enhanced iron uptake and ferrireductase activity, which can be completely abrogated by mutating a predicted oxidoreductase domain.

Collectively, these studies demonstrate that Steap3 most probably represents the long-sought endosomal ferrireductase and functions in the transferrin cycle (Figure 1).

DOES STEAP4 ALSO PLAY AN IMPORTANT ROLE IN IRON METABOLISM?

Although *nm1054* and *Steap3*$^{-/-}$ mice are anemic, hemoglobin levels are reduced by only 40% relative to wild-type mice (8.0 vs. 12.5 g Hb/L). Moreover, in vitro, *nm1054* and *Steap3*$^{-/-}$ reticulocytes retain some ferrireductase activity and the ability to take up iron and incorporate it into heme. These observations indicate that erythroid cells have other ways of reducing iron in the transferrin-cycle endosome.

It is possible that in addition to Steap3, other mem-

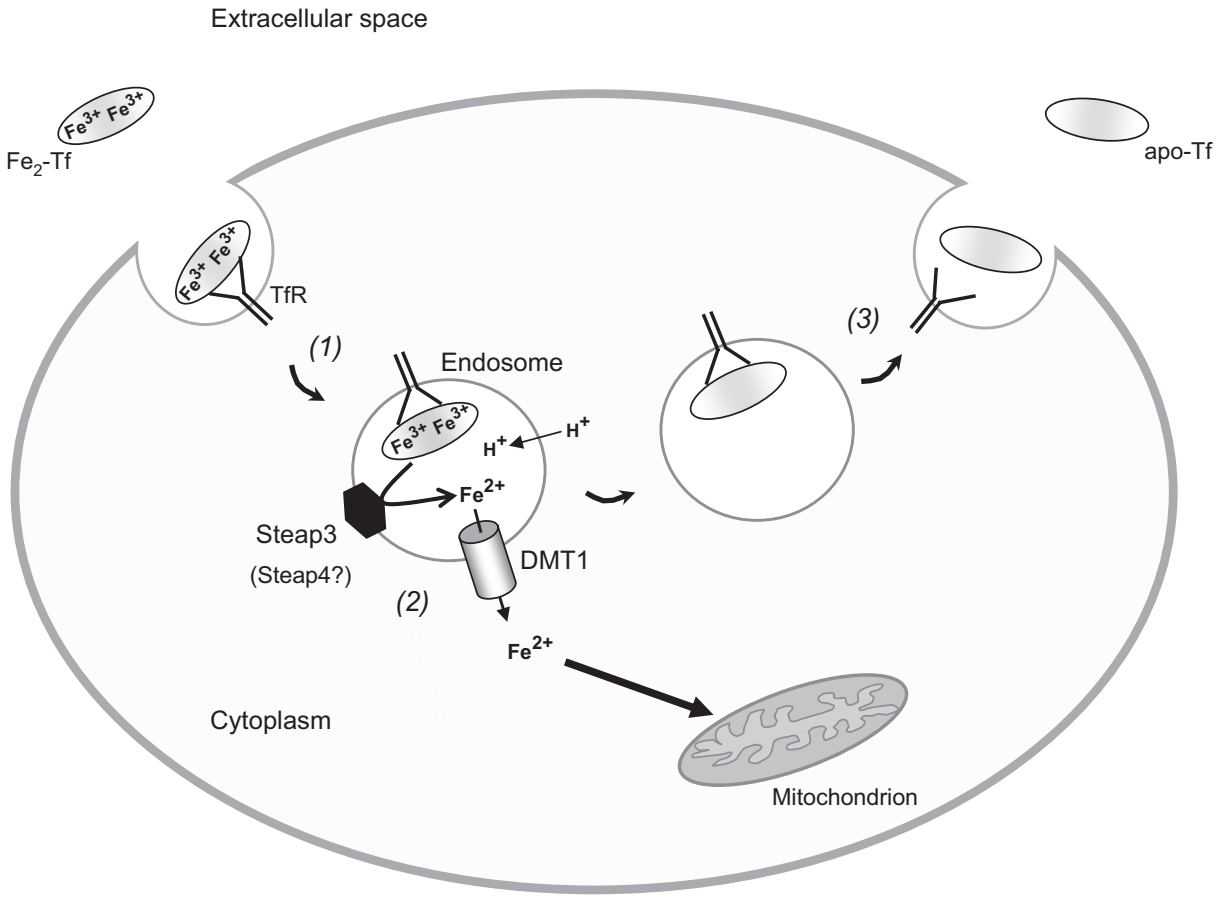

Figure 1. Uptake of transferrin-bound iron by a developing erythroid cell. (1) Transferrin receptor (TfR) at the cell surface binds holotransferrin (Fe_2-Tf) and the complex is internalized into endosomes. (2) Acidification of the endosome and reduction of Fe^{3+} by Steap3, and possibly by Steap4, promotes the release of iron from transferrin. Fe^{2+} is translocated into the cytosol by DMT1, and the iron is taken up by mitochondria and used mainly for heme synthesis. The Tf-TfR complex returns to the cell surface, and apoTf dissociates from its receptor to become free to bind more iron and cycle again.

bers of the Steap protein family may contribute ferrireductase activity in reticulocytes. Steap3 is at least 60% similar to three other proteins designated Steap1, Steap2, and Steap4.[18] Steap3, along with Steap1 and Steap2, were initially identified by their high expression in human prostate tumors.[19-21] The last member of the group, Steap4, was initially identified in a screen for genes induced by tumor necrosis factor alpha (TNFα) in differentiating preadipocytes.[22] Although Steap proteins exhibit markedly different expression profiles, all have been localized to either the plasma membrane and/or endosomes (Table 1). Multiple sequence alignment reveals that Steap2, Steap3, and Steap4 each contain a putative N-terminal oxidoreductase domain with an NAD(P)H binding motif, consistent with previous studies showing that cytosolic NAD(P)H can serve as an electron donor for transmembrane electron transport.[23] Indeed, all three of these Steap proteins, but not Steap1, which lacks an NAD(P)H binding motif, demonstrate ferrireductase activity when overexpressed in human embryonic kidney (HEK) cells. The abundant expression of Steap4 in bone marrow, and its localization to endosomes and plasma membrane (Table 1), makes it a likely candidate for conferring residual ferrireductase activity in Steap3-null erythroid cells.

STEAP PROTEINS AND COPPER METABOLISM

Sequence analysis of Steap proteins reveals distant but significant homology to the yeast FRE (Fe^{3+} reductase) family of proteins, cell-surface reductases required for efficient uptake of ferric iron by *Saccharomyces cerevisiae*.[24] Interestingly, yeast utilize FRE proteins not only for the uptake of iron, but also for the reduction and uptake of environmental copper, which exists chiefly in the oxidized cupric (Cu^{2+}) state. The ability of Steap proteins to serve as cupric reductases was investigated by transient expression studies in HEK cells.[18] It was found that overexpression of all Steap proteins, except Steap1, resulted in increased cupric reductase activity in vitro. Importantly, enhanced activity resulted in increased up-

Table 1. Expression and Functional Activity of Steap Proteins*						
			Reductase Activity		Stimulation of Uptake	
Protein	Tissue Expression	Subcellular Localization	Ferric	Cupric	Fe	Cu
Steap1	prostate >> fetal liver = liver > kidney = muscle	E, PM	–	–	–	–
Steap2	prostate >> pancreas = brain > fetal liver = duodenum	E, PM	+	+	+	+
Steap3	liver >> fetal liver = pancreas > bone marrow = placenta	E, PM	++	++	++	+
Steap4	bone marrow >> placenta = fetal liver	PM, E	+++	+++	+++	+

*Table generated based on published data.[16,18] Relative expression levels were determined by quantitative PCR of human cDNA from 15 different tissues. Subcellular localization was performed by immunofluorescence analysis of epitope-tagged proteins (E, endosome; PM, plasma membrane). In vitro reductase activity and uptake measurements were performed by spectrophotometric analysis of formation of reduced-metal/chelator complexes and by gamma counting, respectively, in transiently transfected HEK293 cells.

take of copper into the cells (Table 1), indicating that Steap proteins may play a role in cellular copper assimilation.

Ohgami et al.[18] note that preliminary data suggest no decrease in copper concentration of Steap3-deficient erythrocytes, but more sensitive indicators of cellular copper status, such as erythrocyte copper chaperone for SOD1 (CCS),[25] may reveal more subtle defects in cellular copper status. It is conceivable therefore that changes in copper status may still contribute, at least in part, to the defective erythroid iron metabolism in *nm1054* and Steap3-null mice. It is well known that copper deficiency produces hypochromic, microcytic anemia in pigs, rats, and mice. Most studies conclude that copper-deficiency anemia arises from insufficient delivery of iron to the bone marrow, resulting from either decreased intestinal iron absorption, decreased iron mobilization from storage, or a combination thereof. These mechanisms, however, do not adequately explain the observations that copper-deficient reticulocytes display impaired iron uptake and reduced heme synthesis.[26] These observations in the reticulocyte suggest that cellular copper availability can directly influence cellular iron uptake and metabolism and that future research in this area is warranted, including the role of Steap proteins in the anemia of copper deficiency.

Steap proteins may also influence intestinal copper absorption. Cells acquire copper via copper transport protein (Ctr1), a ubiquitously expressed transmembrane protein that resides at the plasma membrane and in intracellular vesicular compartments.[27,28] Studies in Caco-2 cells, a colonic adenocarcinoma cell line, indicate that intestinal cells can also take up copper partly via DMT1.[29] Nonetheless, the recent generation of intestinal-epithelial cell-specific Ctr1 knockout mice demonstrates that adequate intestinal copper absorption requires Ctr1.[30] However, with either transporter, only the reduced cuprous (Cu^{+}) form is transported. How the reduction of dietary Cu^{2+} occurs in the intestine is unknown, but may involve duodenal cytochrome b (Dcytb), which exhibits ferrireductase activity.[31] Alternatively, intestinal copper reduction, and therefore intestinal copper absorption, may be mediated by a Steap protein. Steap2 appears to be the most likely candidate because it is the only abundantly expressed Steap transcript in the duodenum (Table 1) and it localizes to epithelial cells of the gastroduodenal junction.[18] Steap 2 may also influence iron and copper uptake into the central nervous system. Abundant Steap2 transcripts are detected in the choroid plexus, the site of cerebrospinal fluid synthesis in the brain. Epithelial cells of the choroid plexus control the exchange of molecules and ions between the brain tissue and the cerebrospinal fluid. The choroid plexus expresses a high density of Ctr1,[32,33] as well as other copper-related proteins,[34] suggestive of dynamic copper metabolism in this structure. In addition, the choroid plexus appears to serve as the main entry point for both transferrin-bound and non-transferrin bound iron into the central nervous system.

SOME FUTURE RESEARCH OPPORTUNITIES

As highlighted throughout this review, the metabolism of iron and copper are closely interrelated. Future

studies need to characterize more fully copper metabolism in Steap3 knockout mice, and the generation of mice lacking Steap2 and Steap4 will help to elucidate the in vivo function of these proteins. Because members of the Steap family may have redundant functions, interbreeding of Steap2-, Steap3-, and Steap4-null mice to create double and triple knockouts will be particularly informative. Also warranted are nutritional studies of the effect of copper and/or iron deficiency on the expression and subcellular localization of these proteins. The discovery of Steap proteins, along with further characterization of their biochemical activities and expression patterns, will help us to better understand the interrelationship of these two essential nutrients.

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