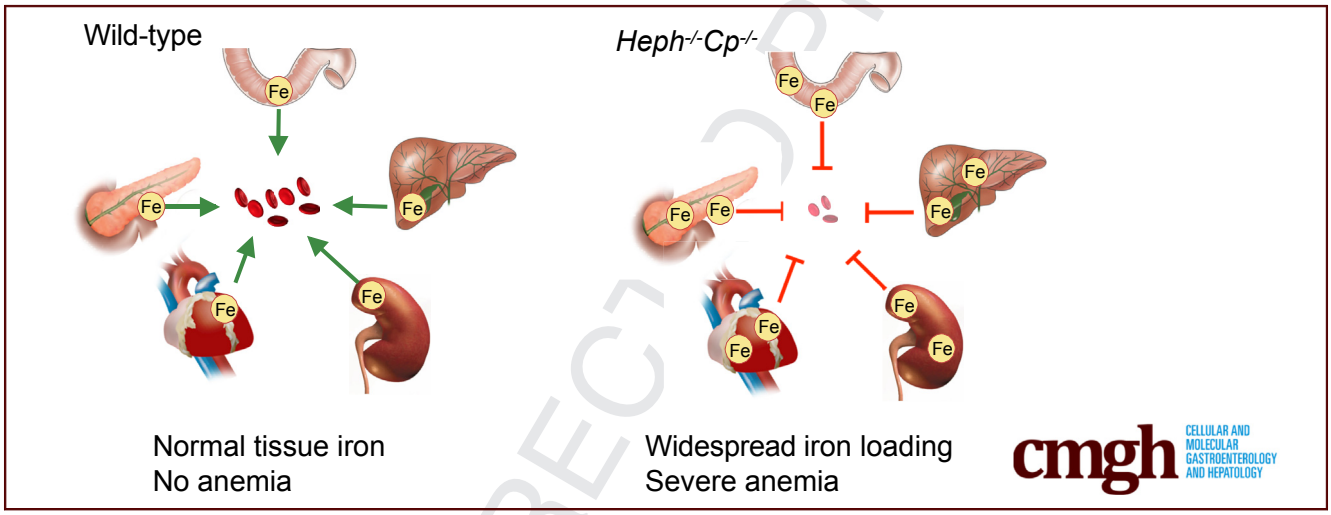
cmgh
CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Severe Iron Metabolism Defects In Mice With Double Knockout of the Multicopper Ferroxidases Hephaestin and Ceruloplasmin

Brie K. Fuqua,[1,2,a] Yan Lu,[2,a] David M. Frazer,[2] Deepak Darshan,[2] Sarah J. Wilkins,[2] Linda Dunn,[2] Alex V. Loguinov,[1] Scott C. Kogan,[3] Pavle Matak,[4] Huijun Chen,[5] Joshua L. Dunaief,[6] Chris D. Vulpe,[1] and Gregory J. Anderson[2,7]

[1]Department of Nutritional Science and Toxicology, University of California, Berkeley, Berkeley, California; [2]Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia; [3]Department of Laboratory Medicine and Helen Diller Comprehensive Cancer Center and Mouse Pathology Core, University of California, San Francisco, California; [4]Department of Pharmacology and Cancer Biology, Duke University, Duke University Medical Center, Durham, North Carolina; [5]Medical School, Nanjing University, Nanjing, Jiangsu Province, China; [6]FM Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania, Philadelphia, Pennsylvania; and [7]School of Chemistry and Molecular Bioscience, University of Queensland, Brisbane, Queensland, Australia



SUMMARY

Mice lacking 2 multicopper ferroxidases, hephaestin and ceruloplasmin, exhibit both tissue iron overload and severe systemic iron deficiency anemia. These studies reveal the importance of these proteins and an extra-intestinal role of hephaestin in ensuring proper systemic iron distribution.

BACKGROUND & AIMS: Multicopper ferroxidases (MCFs) facilitate intestinal iron absorption and systemic iron recycling, likely by a mechanism involving the oxidization of Fe^{2+} from the iron exporter ferroportin-1 for delivery to the circulating Fe^{3+} carrier transferrin. Hephaestin (HEPH), the only MCF known to be expressed in enterocytes, aids in the basolateral transfer of dietary iron to the blood. Mice lacking HEPH in the whole body (*Heph*$^{-/-}$) or intestine alone (*Heph*$^{int/int}$) exhibit defects in dietary iron absorption but still survive and grow. Circulating ceruloplasmin (CP) is the only other known MCF likely to interact with enterocytes. Our aim was to assess the effects of combined deletion of HEPH and CP on intestinal iron absorption and homeostasis in mice.

METHODS: Mice lacking both HEPH and CP (*Heph*$^{-/-}$*Cp*$^{-/-}$) and mice with whole-body knockout of CP and intestine-specific deletion of HEPH (*Heph*$^{int/int}$*Cp*$^{-/-}$) were generated and phenotyped.

RESULTS: *Heph*$^{-/-}$*Cp*$^{-/-}$ mice were severely anemic and had low serum iron, but they exhibited marked iron loading in duodenal enterocytes, the liver, heart, pancreas, and other tissues. *Heph*$^{int/int}$*Cp*$^{-/-}$ mice were moderately anemic (similar to *Cp*$^{-/-}$ mice) but were iron loaded only in the duodenum and liver, as in *Heph*$^{int/int}$ and *Cp*$^{-/-}$ mice, respectively. Both double knockout models absorbed iron in radiolabeled intestinal iron absorption studies, but the iron was inappropriately distributed, with abnormally high percentage retained in the liver.

CONCLUSIONS: These studies indicate that HEPH and CP, and likely MCFs in general, are not essential for intestinal iron absorption but are required for proper systemic iron distribution. They also point to important extra-intestinal roles for HEPH in maintaining whole-body iron homeostasis. *(Cell Mol Gastroenterol Hepatol 2018;■:■–■; https://doi.org/10.1016/j.jcmgh.2018.06.006)*

Keywords: Iron Deficiency Anemia; Iron Overload; Intestinal Iron Absorption; Non-Transferrin Bound Iron.

Multicopper ferroxidases (MCFs) enhance the efficiency of iron transport across biological membranes and are important for intestinal iron absorption and systemic iron recycling in vertebrates.[1] Evidence suggests that MCFs oxidize ferrous iron from the iron export protein ferroportin 1 (FPN1) (also known as SLC40A1), enabling the iron to bind to the ferric-specific iron carrier in the blood, transferrin (TF). The MCF ceruloplasmin (CP), which exists in both a soluble circulating form as well as in a GPI-linked form, assists in the release of iron from a variety of cell types including hepatocytes, macrophages, Sertoli cells, and astrocytes.[2–4] The membrane-bound MCF hephaestin (HEPH) exhibits highest expression in intestinal enterocytes and aids in the basolateral transfer of dietary iron to the circulation.[5] The primary role of zyklopen (also known as HEPHL1), the only other known mammalian MCF besides HEPH and CP, is currently unknown, although its tissue distribution suggests it could play a role in the brain, retina, kidney, and testes.[6]

To determine the importance of MCFs in intestinal iron absorption, we previously generated mice with whole-body ($Heph^{-/-}$) and intestine-specific ($Heph^{int/int}$) knockout of HEPH, the only MCF known to be expressed in intestinal enterocytes.[7] Ablation of HEPH perturbed, but did not abolish, intestinal iron absorption. Young knockout mice retained iron in their duodenal enterocytes and were anemic, but their anemia resolved with age as body iron requirements declined. The phenotype was much less severe than that reported for mice with whole-body knockout of FPN1 (unviable) or tamoxifen-induced postnatal ablation of FPN1 in the intestine, which results in a near complete block in intestinal iron absorption and the consequent development of very severe iron deficiency in young mice.[8] The survival and relatively mild phenotype of $Heph^{-/-}$ mice suggest that another MCF or some other ferroxidase may be able to partially compensate for loss of HEPH or, alternatively, that a ferroxidase is not absolutely required for intestinal iron absorption. In support of the former hypothesis, several studies in other cell types have indicated that FPN1 requires a ferroxidase to remain on the cell surface and properly export iron.[9–11] In addition, the ferric specificity of the iron carrier TF suggests a critical role for ferroxidases in the rapid and specific delivery of iron to this protein.[12]

The most likely ferroxidase that could at least partially compensate for HEPH ablation is the circulating form of the MCF CP. Although *Cp* is not known to be expressed at the mRNA level in intestinal enterocytes, CP has been detected by immunofluorescence in the lamina propria of duodenal villi and inside enterocytes.[13] Most notably, CP has been shown to augment intestinal iron absorption in mice in cases of extreme iron need.[13] CP also shares a high degree of sequence similarity with HEPH and co-immunoprecipitates with FPN1.[5,9] Conversely, zyklopen is not likely to play a role in intestinal iron absorption because no zyklopen expression has been detected in the small intestine or in the serum of mice.[6]

To test the potential contribution of CP to intestinal iron absorption in mice lacking HEPH, we generated mice with genetic ablation of both ferroxidases ($Heph^{-/-}Cp^{-/-}$). A mouse model lacking CP and expressing only the sex-linked anemia (*sla)* mutant form of HEPH ($Heph^{sla/sla}Cp^{-/-}$) was previously reported to be viable and to exhibit a tissue iron-loading phenotype.[14] However, no intestinal iron absorption studies were reported, and because the *sla* mutant may be a hypomorph and not equivalent to a null *Heph* allele, it was not possible to use that model to unequivocally determine whether HEPH and CP together are required for intestinal iron absorption or, if not, the importance of the role of CP. To differentiate between phenotypes because of ablation in the intestine versus other tissues, we also generated a mouse model with whole-body knockout of CP but knockout of HEPH only in the intestine ($Heph^{int/int}Cp^{-/-}$).

Results

$Heph^{-/-}Cp^{-/-}$ Mice Are Visibly Small and Severely Anemic Throughout Life

$Heph^{-/-}Cp^{-/-}$ double knockout (DKO) mice and control littermates were generated by crossing wild-type (WT), $Heph^{-/-}$, $Heph^{-/-}Cp^{-/-}$, $Cp^{+/-}$, and $Cp^{-/-}$ male mice with $Heph^{+/-}Cp^{-/-}$ and $Heph^{+/-}Cp^{+/-}$ females. Because *Heph* is located on the X chromosome, the presence of the *Heph* knockout allele in male pups is only dependent on the dam. $Heph^{-/-}Cp^{-/-}$ pups were viable and could be easily differentiated from their littermates by eye both before weaning and throughout adulthood because of their small size and marked pallor (Figure 1*A* and *B*). The urine of the $Heph^{-/-}Cp^{-/-}$ mice, like that of WT mice made severely iron-deficient by an iron-deficient diet, was clear instead of yellow. In addition, whereas WT, $Heph^{-/-}$, and $Cp^{-/-}$ littermates developed yellow to brown iron deposits on their incisors, the teeth of $Heph^{-/-}Cp^{-/-}$ mice remained white throughout life (Figure 1*C*). Unlike the $Heph^{-/-}Cp^{-/-}$ mice, however, $Heph^{int/int}Cp^{-/-}$ mice, generated by crossing $Heph^{int/int}Cp^{-/-}$ males with $Heph^{fl/fl}Cp^{-/-}$ females, could not be reliably distinguished by eye from their $Heph^{fl/fl}Cp^{-/-}$ littermates (although some were noted to have lighter teeth and paler paws than littermates), suggesting a milder phenotype (data not shown).

[a]Authors share co-first authorship.

Abbreviations used in this paper: **CP, ceruloplasmin; $Cp^{-/-}$, mice lacking CP in the whole body; DAB, 3,3′-diaminobenzidine; FDR, false discovery rate; FPN1, ferroportin 1; GI, gastrointestinal; HCl, hydrochloric acid; HEPH, hephaestin; $Heph^{-/-}$, mice lacking HEPH in the whole body; $Heph^{-/-}Cp^{-/-}$ or DKO, double-knockout mice lacking both HEPH and CP; $Heph^{fl/fl}$, mice with floxed Heph alleles; $Heph^{fl/fl}Cp^{-/-}$, mice with floxed Heph alleles and lacking CP in the whole body; $Heph^{int/int}$, mice lacking HEPH in the intestine alone; $Heph^{int/int}Cp^{-/-}$, mice lacking HEPH in the intestine alone and lacking CP in the whole body; $Heph^{sla/sla}Cp^{-/-}$, mice lacking CP in the whole body and expressing only the sla mutant form of HEPH; MCF, multicopper ferroxidase; NTBI, non-transferrin bound iron; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SD, standard deviation; sla, sex-linked anemia; TBST, Tris-buffered saline with 0.1% Tween-20; TF, transferrin; TIBC, total iron binding capacity; WT, wild-type.**

2352-345X
https://doi.org/10.1016/j.jcmgh.2018.06.006