The Molecular Circuitry Regulating the Switch between Iron Deficiency and Overload in Mice* [8]

Received for publication, September 7, 2005, and in revised form, December 26, 2005 Published, JBC Papers in Press, January 17, 2006, DOI 10.1074/jbc.M509857200

Henry Mok a, Agnieszka E. Mlodnicka b, Matthias W. Hentze c, Martina Muckenthaler d, and Armin Schumacher e

From the a Department of Molecular and Human Genetics and b Medical Scientist Training Program, Baylor College of Medicine, Houston, Texas 77030, c European Molecular Biology Laboratory, 69117 Heidelberg, Germany, and the d Department of Pediatric Oncology, Hematology, and Immunology, University of Heidelberg, 69120 Heidelberg, Germany

Recent positional cloning of the radiation-induced polycythemia (Pcm) mutation revealed a 58-bp microdeletion in the promoter region of ferroportin 1 (Fpn1), the sole cellular iron exporter identified to date. Here we report a molecular definition of the regulatory mechanisms governing the dynamic changes in iron balance in Pcm heterozygous mice between 3 and 12 weeks of age. Hepatic and/or duodenal response patterns of iron metabolism genes, such as Tfrc, cybrd1, and Slc11a2, explained the transition from early postnatal iron deficiency to iron overload by 12 weeks of age. A significant delay in developmental up-regulation of hepcidin (Hamp), the pivotal hormonal regulator of iron homeostasis, correlated with high levels of Fpn1 expression in hepatic Kupffer cells and duodenal epithelial cells at 7 weeks of age. Conversely, upon up-regulation of Hamp expression at 12 weeks of age, Fpn1 expression decreased, indicative of a Hamp-mediated homeostatic loop. Hamp regulation due to iron did not appear dependent on transcription-level changes of the murine homolog of Hemojuvelin (Rgmc). Aged cohorts of Pcm mice exhibited low levels of Fpn1 expression in the context of an iron-deficient erythropoiesis and profound iron sequestration in reticuloendothelial macrophages, duodenum, and other tissues. Thus, similar to the anemia of chronic disease, these findings demonstrate decreased iron bioavailability due to sustained down-regulation of Fpn1 levels by Hamp. We conclude that regulatory alleles, such as Pcm, with highly dynamic changes in iron balance are ideally suited to interrogate the genetic circuitry regulating iron metabolism.

In mammals, iron bioavailability for erythropoiesis and other vital organ systems is regulated at three principal sites: placental or duodenal uptake, release from hepatic stores, and recycling of scavenged iron from senescent red blood cells via reticuloendothelial (RES) macropathies (1). Iron recycling from macropathies generates the vast majority of iron for daily consumption, amounting to ~22 mg of iron per day under steady state conditions in humans (2). Ferroportin 1 (Fpn1; also known as MTP1, IREG1, SLC11A3, SLC39A1) plays a pivotal role at all three sites and functions as the sole cellular iron exporter identified to date (3–5).

Both cellular and systemic hormonal mechanisms impinge upon Fpn1 regulation. Predominant modes of cellular regulation include transcriptional as well as translational control. Several studies document changes in Fpn1 mRNA levels and transcription rates in mice and cultured cells under conditions of iron overload or deficiency (6–14). Translational regulation involves a highly conserved sequence in the 5’-untranslated region of Fpn1 mRNA known as the iron-responsive element (1). Trans-acting iron regulatory proteins bind to the iron-responsive element stem-loop structure, inhibiting Fpn1 mRNA translation under low intracellular iron conditions in tissue culture cells (6, 7, 15). Hepcidin (Hamp), the principal hormonal regulator of iron homeostasis (1), controls Fpn1 expression levels at the cell surface by a post-translational mechanism. Upon Hamp binding to cell surface-bound Fpn1, the iron exporter becomes internalized and degraded in lysosomes (16). Importantly, this implicates the Hamp-Fpn1 axis in a homeostatic loop wherein, under conditions of high intracellular iron in hepatic stores, Hamp-mediated degradation of Fpn1 decreases duodenal iron uptake and macrophage iron release, preventing organismal iron overload.

Coding region mutations in human FPN1 cause the autosomal dominant ferroportin disease (also referred to as hemochromatosis, type IV) (17). Furthermore, two mutant mouse models for Fpn1 function were recently described (3, 5). Constitutive or conditional deletion of several transmembrane domains confirmed Fpn1 function in maternal-to-fetal iron transport, export from duodenal enterocytes, and iron release from RES macrophages (5). The other mouse model involves a regulatory allele of Fpn1 generated by radiation mutagenesis. Positional cloning identified a 58-bp microdeletion in the Fpn1 promoter in polycythemia (Pcm) mutant mice (3). Depending on the developmental stage, tissue, and genotype, the microdeletion caused dynamic dysregulation of Fpn1 expression, resulting in both hypo- and hypermorphic phenotypes (3, 4). During late gestation, decreased Fpn1 protein expression in placental syncytiotrophoblast cells resulted in a severe neonatal iron deficiency and a hypochromic, microcytic anemia in Pcm homozygotes, providing the first definitive evidence for Fpn1 function in maternal-to-fetal iron transport in mammals (4). Postnatally, aberrant transcription initiation eliminated the iron-responsive element in the 5’-untranslated region of Fpn1 mRNA, increasing hepatic and duodenal Fpn1 protein levels (3). The latter governed augmentation of intestinal iron uptake, reversing the perinatal iron deficiency to a tissue iron overload by 12 weeks of age. Strikingly, the majority of Pcm heterozygous animals displayed a transient erythropoietin-independent polycythemia with peak hematocrits of up to 80% during young adulthood, eponymous of the mutant strain (3).

Here we report the molecular mechanisms underlying the iron homeostasis defects in Pcm mice. The “IronChip” microarray platform...
and real-time quantitative RT-PCR accurately defined the hepatic and duodenal gene response patterns that governed the transition from early postnatal iron deficiency to tissue iron overload in young adult Pcm mice. In support of a Hamp-Fpn1 homeostatic loop in vivo, Pcm mice manifested an inverse correlation between Hamp mRNA levels and Fpn1 expression in RES macrophages and duodenal epithelial cells. Aged cohorts of Pcm mutant mice exhibited an iron-deficient erythropoiesis in the context of profound iron sequestration in RES macrophages and other cell types, constituting the end point of Pcm disease pathogenesis.

EXPERIMENTAL PROCEDURES

Mice and Genotyping—Pcm mice on a partially congenic A/J background (N5 and later) were used for analysis and genotyped as described previously (3). Cohorts of Pcm and wild-type animals were aged to 8–19 months and fed a standard chow. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Microarray Analysis—Total RNA was isolated from liver and duodenal samples using TRizol reagent (Invitrogen) and prepared according to standard methods. Pools of total liver RNA from 6 Pcm heterozygotes and 6 wild-type mice at 3, 7, and 12 weeks of age were analyzed using the Mouse Version 6.0 of the IronChip as described previously (11). Likewise, pools of total duodenal RNA from 6 Pcm heterozygotes and 6 wild-type mice were analyzed at 7 and 12 weeks of age. Expression values were calculated from dye swap experiments (18). Genes were represented by either single or multiple clones on the microarray platform. In the latter case, the average ratios and standard deviations were determined. The entire data set representing the expression values of all genes represented on the IronChip cDNA microarray platform will be submitted to Array express (European Bioinformatics Institute).

Real-time RT-PCR Analysis—Reactions and signal detection were performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) using the following primers: Fpn1 (Mm00489837_m1), Hamp (Mm00519025_m1), Trfr (Mm00441941_m1), Scl11a2 (Mm00435363_m1), and Rgmc (Mm00510148_s1) (all from Applied Biosystems). 18 S rRNA assay was conducted using primers 5’-AGTCCTCGTT-3’ (forward), 5’-CCCTCCATG-GATCCTCGTT-3’ (reverse), and TaqMan MGB probe 5’-ATGC-CACCTTAAATCCTT-3’ labeled with VIC (Applied Biosystems). mRNA levels were expressed as a difference of cycles to threshold (∆CT) between the gene-specific probe and 18 S standard; mean wild-type values were centered at zero.

Histology and Immunohistochemistry—Liver and duodenal samples from various stages were prepared as described previously (3). A polyclonal Fpn1 antibody was generated in rabbit against oligopeptide sequence GPDEKEVTDENQPNTS at the carboxyl terminus of Fpn1 and immunoaffinity purified. Immunohistochemistry for Fpn1 and F4/80 (clone CI:A3–1; Serotec) was performed as described (4). For Trfr antigen retrieval, sections were boiled in 0.01 M citric acid, pH 6.0, and blocking was achieved with horse serum. Primary antibody incubation was performed overnight at 4 °C using mouse α-TrfR at 1:1000 dilution (Invitrogen). Prussian blue staining for iron was accomplished using the Accustain iron staining kit (Sigma) according to the manufacturer’s protocol. For comparison of protein expression levels, wild-type and Pcm mutant samples were mounted side by side on the same slide. Images were acquired with a Sony 085 CCD color RGB sensor digital camera mounted on a Zeiss Axioplan 2 microscope.

Iron Determination and Blood Cell Analyses—Non-heme tissue and serum iron were determined as described previously (3). Hematocrit (Hct) measurements, expressed as a percentage, were obtained by standard microcapillary determination. Red cell parameters, including mean corpuscular volume and mean corpuscular hemoglobin concentration, as well as white blood cell (WBC) counts were determined on the ADVIA 120 hematology system (Bayer).

Statistical Analyses—All data are reported as the mean ± S.D. All comparisons were made versus wild-type cohorts and analyzed for significant differences using the Student’s unpaired t-test.

RESULTS

To elucidate the consequences of dynamic Fpn1 dysregulation on the genetic circuitry governing iron metabolism, oxidative stress, hypoxia, and inflammation we used a highly sensitive and accurate cDNA microarray platform, the IronChip (11). The analysis focused on gene expression in liver and duodenum, respectively, as sites of iron storage and absorption at 3, 7, and 12 weeks of age. In the context of the striking polycythemia phenotype by young adulthood, heretofore not described in animal models of dysregulated iron metabolism, molecular analyses were directed toward Pcm heterozygotes. In comparison with previous microarray studies on mouse models of iron balance abnormalities (11, 19), Pcm mice presented discrete but highly reproducible differences in hepatic and duodenal gene expression during postnatal development (Fig. 1). To validate the microarray findings based on pooled RNA, the individual samples were subjected to real-time RT-PCR analysis using the most relevant probes. The average changes of differential gene expression along with the respective standard deviations are shown in supplemental Table S1.

The Hamp-Fpn1 Homeostatic Loop—Neither microarray analysis nor quantitative RT-PCR detected statistically significant differences in duodenal and hepatic Fpn1 transcript levels between Pcm heterozygous and wild-type mice at 3, 7, and 12 weeks of age (Figs. 1 and 2, A and B). The lack of Fpn1 transcript up-regulation in 7-week Pcm heterozygous liver demonstrated a post-transcriptional mechanism of Fpn1 protein up-regulation in polycythemic Pcm heterozygotes (3).
Iron Metabolism in polycythaemia Mice

Importantly, hepatic *Hamp* mRNA levels were significantly reduced in *Pcm* heterozygotes at 7 weeks of age (Figs. 1 and 2C). However, this reflected a delay in developmental *Hamp* up-regulation because at 12 weeks of age *Hamp* mRNA levels in *Pcm* heterozygotes were significantly higher (microarrays) or similar (RT-PCR) to wild-type levels (Fig. 1). Additionally, developmental up-regulation of *Hamp* is conducive to persistent elevation in *Fpn1* protein expression in polycythaemic 7-week *Pcm* heterozygotes (3). These results underscore the responsiveness of *Hamp* regulation to the dynamic changes in hepatic iron levels in the context of the *Fpn1* regulatory allele. Therefore, to the best of our knowledge, the *Pcm* mouse model provides the strongest evidence to date for the *Fpn1* homeostatic loop in vivo.

Interestingly, despite statistically significant down-regulation of hepatic *Hamp* levels in polycythaemic 7-week-old *Pcm* heterozygotes, no difference in mRNA expression of *Rgmc*, the mouse homolog of *Hemojuvelin* and potential upstream regulator of *Hamp* (20), was observed in liver (*Pcm*/+ΔCT *Rgmc* 0.39 ± 0.46 (n = 6) versus +/+ΔCT *Rgmc* 0 ± 0.83 (n = 6); p = 0.33). These results support recent evidence for differential regulation of *Hamp* and *Rgmc* in response to iron balance or erythropoietin application (21).

Response Patterns of Iron Metabolism Genes—In good agreement with the severe neonatal iron deficiency in *Pcm* mice (3, 4), microarray analysis detected increased hepatic *Trfr* (transferrin receptor) mRNA expression in 7- and 7-week-old *Pcm* heterozygotes (Fig. 1). Quantitative RT-PCR showed no statistically significant difference between *Pcm* heterozygotes and wild-type controls at 7 weeks (Fig. 2E). However, both the IronChip and quantitative RT-PCR demonstrated a strong decrease in *Trfr* mRNA expression by 12 weeks of age (Figs. 1 and 2F). This result is consistent with destabilization of *Trfr* mRNA by a mechanism dependent on iron regulatory proteins (1) in response to hepatic iron accumulation in *Pcm* liver at 12 weeks of age (3), obviating *Trfr*-dependent cellular iron uptake. Likewise, increased *Hmxox1* (*home oxygenase 1*) mRNA levels in 7- and 12-week-old *Pcm* heterozygotes can be explained by the increased need for heme catabolism in polycythaemic animals, heralding hepatic iron accumulation (Fig. 1). Consistent with marked systemic iron requirement and low hepatic *Hamp* mRNA expression, duodenal *Trfr*, *cybrd1* (duodenal cytochrome b), and *Slc11a2* (solute carrier 11a2; also known as *Nramp2*, *Dmt1*) mRNA levels of *Pcm* heterozygotes at 7 weeks of age were increased (Figs. 1 and 2G). Increased expression of these iron transporters reverted to wild-type levels at 12 weeks of age (Figs. 1 and 2H). Therefore, coordinated shifts in transcriptional expression patterns of these genes are sufficient to explain the transitory iron loading in *Pcm* heterozygotes.

Response Patterns of Selected Iron Metabolism Proteins in Liver and Duodenum—At 3 weeks of age, *Trfr* was expressed at moderate levels in wild-type liver, whereas *Pcm* heterozygotes displayed significant up-regulation of this protein (Fig. 3A; compare with mRNA results in Fig. 1). Inspection under high magnification suggested significant augmentation of *Trfr* levels in Kupffer cells and sinusoidal endothelial cells, contrasting with a more subtle increase in expression in hepatocytes (data not shown). At 12 weeks, *Pcm* heterozygotes demonstrated significantly lower *Trfr* levels compared with wild-type liver (Fig. 3B), consistent with the mRNA results (Fig. 1). Elevated *Trfr* protein expression in duodenum from 7-week-old *Pcm* heterozygotes restored *Trfr* to levels indistinguishable from wild-type littermates at 12 weeks of age (Fig. 3B; compare with mRNA results in Fig. 1). Thus, immunohistochemistry corroborated the *Trfr* mRNA response patterns in liver and duodenum. At 7 weeks of age, *Pcm* heterozygous duodenum exhibited significant elevation of *Fpn1* expression compared with wild-type littermates (Fig. 3E). Increased expression was particularly evident at the basolateral membrane of the epithelial cells. Conversely, at 12 weeks of age, *Pcm* heterozygotes were remarkable for markedly lower *Fpn1* expression levels compared with wild-type (Fig. 3F). Because *Fpn1* mRNA levels were indistinguishable between *Pcm* heterozygotes and wild-type at both 7 and 12 weeks of age (Fig. 1), the changes in protein levels strongly supported the notion of *Hamp*-mediated post-translational regulation of *Fpn1* expression.
Down-regulation of Fpn1 Expression and Reticuloendothelial Iron Overload—We previously demonstrated a significant augmentation of Fpn1 protein expression in Pcm mutant liver during early postnatal development (3). By immunohistochemistry, increased Fpn1 expression localized predominantly to punctate, stellate-shaped cells at 3 weeks of age (Fig. 4A). Conversely, expression of Trfr in Pcm heterozygotes was significantly lower at 12 weeks compared with wild-type liver (B). Augmented Trfr expression in 7-week Pcm heterozygous duodenum (C) reverted to wild-type levels by 12 weeks of age (D). Immunohistochemistry for Fpn1 revealed elevated expression in 7-week duodenum from Pcm heterozygotes compared with wild-type (E). Note the distinct Fpn1 expression along the basolateral membrane of the epithelial cells. In contrast, at 12 weeks of age, Pcm heterozygotes demonstrated lower levels of Fpn1 expression compared with wild-type duodenum (F). Original magnification ×400 for all panels.

**FIGURE 3.** Trfr and Fpn1 expression in Pcm liver and duodenum. Immunohistochemistry detected higher levels of Trfr expression in Pcm heterozygous liver at 3 weeks of age compared with wild-type (A). Conversely, expression of Trfr in Pcm heterozygotes was significantly lower at 12 weeks compared with wild-type liver (B). Augmented Trfr expression in 7-week Pcm heterozygous duodenum (C) reverted to wild-type levels by 12 weeks of age (D). Immunohistochemistry for Fpn1 revealed elevated expression in 7-week duodenum from Pcm heterozygotes compared with wild-type (E). Note the distinct Fpn1 expression along the basolateral membrane of the epithelial cells. In contrast, at 12 weeks of age, Pcm heterozygotes demonstrated lower levels of Fpn1 expression compared with wild-type duodenum (F). Original magnification ×400 for all panels.

**FIGURE 4.** Fpn1 expression and iron overload in Pcm liver. Compared with wild-type, immunohistochemistry detected increased levels of Fpn1 expression in Kupffer cells in 3-week-old Pcm homozygotes (A), whereas no differences were observed by 12 weeks of age (B). Prussian blue staining revealed iron accumulation in Kupffer cells in 12-week liver of Pcm homozygotes (C). By immunohistochemistry, Fpn1 expression remained near base-line levels in aged Pcm homozygous liver (D), whereas Prussian blue staining showed significant iron accumulation, predominantly in Kupffer cells (E). Immunohistochemistry for F4/80 revealed a similar distribution and number of macrophages in livers from aged wild-type and Pcm homozygous animals (F). Original magnification ×200 for all panels.

Iron Metabolism in polycythaemia Mice

Down-regulation of Fpn1 Expression and Reticuloendothelial Iron Overload—We previously demonstrated a significant augmentation of Fpn1 protein expression in Pcm mutant liver during early postnatal development (3). By immunohistochemistry, increased Fpn1 expression localized predominantly to punctate, stellate-shaped cells at 3 weeks of age (Fig. 4A). Based on a similar staining pattern for F4/80 (data not shown), the antibody to which recognizes a macrophage-restricted glycoprotein of the epidermal growth factor 7 family (22), marked up-regulation of Fpn1 in Pcm mutants localized to Kupffer cells. Likewise, persistent elevation of Fpn1 protein expression at 7 weeks of age localized predominantly to Kupffer cells in polycythaemic Pcm heterozygotes (data not shown). In contrast, the small subset of Pcm heterozygotes with normal hematocrit as well as Pcm homozygotes displayed wild-type levels of hepatic Fpn1 expression (data not shown). By 12 weeks of age, the Fpn1 expression pattern was indistinguishable between wild-type and Pcm mutant liver (Fig. 4B). This down-regulation of Fpn1 expression correlated with RES iron accumulation in Pcm homozygous liver by 12 weeks of age (Fig. 4C). Furthermore, hepatic
Iron Metabolism in polycythemia Mice

**FIGURE 5. Iron levels in aged Pcm mice.** Non-heme iron levels were elevated in Pcm mutant liver (A), kidney (B), duodenum (C), and heart (D). Although no differences in non-heme iron levels were observed in brain (E), Pcm homozygotes showed decreased serum iron levels (F). White columns depict wild-type; gray columns Pcm heterozygous; black columns Pcm homozygotes. *p < 0.01; **p < 0.001; ***p < 0.0001.

Fpn1 expression remained near base-line levels in aged Pcm homozygotes (Fig. 4D), whereas significant iron accumulation persisted (Fig. 4E). Large accumulations of iron, consistent with hemosiderosis, were observed in cells staining against F4/80 (Fig. 4F). Notably, the overall number of macrophages appeared similar between aged wild-type and mutant livers (Fig. 4F), and collagen staining revealed no evidence of widespread fibrosis (data not shown).

The Disease End Point in Aged Pcm Animals—Several parameters of iron homeostasis were determined in Pcm mice between 8 and 19 months of age. Analysis of age subgroups did not reveal any phenotypic difference, indicating that by 8 months of age all Pcm mice had reached the disease end point (data not shown). Hepatic iron levels remained many-fold elevated in aged Pcm animals (Fig. 5A) in a predominantly punctate pattern of distribution (Fig. 4E). Other tissues, including kidney (Fig. 5B), duodenum (Fig. 5C), and heart (Fig. 5D), also demonstrated significant elevation in iron content. Strikingly, no difference in brain iron levels was observed in aged Pcm mutants (Fig. 5E). This was also observed in the context of normal brain iron balance in humans with hereditary hemochromatosis or secondary iron overload (23).

Importantly, serum iron levels were significantly reduced in aged Pcm homozygotes (Fig. 5F). Microcapillary determination of Hct revealed a graded severity of anemia in aged Pcm mutants (+/+, Hct 39.9 ± 1.8% (n = 19); Pcm/+ Hct 35.0 ± 3.4% (n = 16), p < 0.0001; Pcm/Pcm Hct 32.5 ± 2.2% (n = 22), p < 0.0001). Pcm homozygotes also displayed significant microcytosis (Pcm/Pcm mean corpuscular volume 41.5 ± 1.3 fl (n = 17) versus +/+ mean corpuscular volume 52.1 ± 4.0 fl (n = 15); p < 0.0001); no differences in mean corpuscular hemoglobin concentration were observed (data not shown). Consistent with RES iron accumulation and reduced serum iron levels in aged animals, real-time RT-PCR analysis of hepatic Hamp transcript levels revealed a trend toward increased expression, albeit not at statistically significant levels (+/+

ΔCT Hamp 0 ± 3.27 (n = 8); Pcm/+ ΔCT Hamp 1.74 ± 0.61 (n = 7), p = 0.19; Pcm/Pcm ΔCT Hamp 1.84 ± 0.74 (n = 8); p = 0.14).

Red cell parameters and white blood cell counts in aged cohorts of Pcm animals are shown in supplemental Table S2. A modest but significant elevation in WBC was observed in both heterozygotes (Pcm/+ WBC 1.2 ± 0.42 10^3 cells/μl (n = 10) versus +/+ WBC 0.87 ± 0.32 10^3 cells/μl (n = 15); p = 0.027) and homozygotes (Pcm/Pcm WBC 1.7 ± 0.99 10^3 cells/μl (n = 17); p = 0.0044). This was primarily reflective of an increase in absolute lymphocyte count and, to a lesser degree, neutrophil count. No statistically significant differences in monocyte, eosinophil, or basophil counts were observed. In the context of a semidominant defect in spleen development in Pcm animals (4), it is conceivable that the increased presence of circulating white blood cells in aged Pcm mice results from the defects in this significant repository site for immune cells.

**DISCUSSION**

In recent years, positional cloning of iron disease loci in both humans and mutant mouse models, complemented by transgenic and gene knock-out studies in mice, have identified many genes involved in iron metabolism (1). The challenge now resides in dissecting the function of the encoded proteins and their integration into the regulatory circuits governing cellular and organismal iron homeostasis. These investigations will benefit greatly from the availability of mouse strains, such as Pcm, with regulatory mutations in critical components of the pathways.

The Pcm mouse mutant exhibits the gamut of iron balance disorders, ranging from iron deficiency at birth to tissue iron overload by young adulthood (3, 4). The present study defined, in molecular terms, the regulatory interferences underlying the dynamic changes in iron homeostasis in Pcm mice. For example, duodenal and/or hepatic expression of Fpn1 and Trfr protein, as well as Hamp, cybrd1 and Slc11a2 mRNA, explained the transition from early postnatal iron deficiency to iron overload in 12-week old Pcm mice. In comparison with previous studies (10, 11), these results demonstrate transcriptional responsiveness of the duodenal iron transport system to organismal and/or cellular iron balance. These changes in gene expression are likely to result directly or indirectly from alterations in Hamp signaling. Recently, it was shown that, under conditions of augmented erythropoiesis and decreased Hamp expression, mRNA and protein levels of Slc11a2 and Cybrd1 increased (24). Furthermore, genetically Hamp-deficient mice demonstrated up-regulation of Slc11a2, Cybrd1, and Fpn1 expression (25). Our results indicate that polycythemic, 7-week-old Pcm heterozygotes demonstrate decreased Hamp expression in the context of significant alterations in mRNA levels of iron-related genes, such as iron transporters. In addition, cellular regulation responsive to Fpn1-mediated elevated iron efflux from intestinal enterocytes leading to increased mRNA expression of iron transporters in Pcm heterozygotes must be considered (26).

Genetic evidence suggests that Rgmc, a second causative gene for juvenile onset hereditary hemochromatosis (type II), functions upstream of Hamp (20). Conceivably, profound down-regulation of Hamp in polycythemic Pcm heterozygotes at 7 weeks of age constitutes an appropriate in vivo context to discern expression differences in putative upstream regulators, including Rgmc. However, mRNA levels of Rgmc were indistinguishable between wild-type and 7-week old Pcm heterozygotes. Similarly, work by Krijt et al. (21) revealed no differences in Rgmc transcript levels following treatment of mice with iron or erythropoietin, known regulators of Hamp expression. Thus, Hamp regulation in response to iron status is not likely to depend on changes in Rgmc transcription.
Iron Metabolism in polycythaemia Mice
dynamic nature of iron homeostasis, analyses of in vivo models should
require consideration of various stages in pre- and postnatal development
as the phenotypic richness and regulatory intricacies cannot be appreciated by the description of one or two stages alone.

Acknowledgments—We thank Dr. Josef Prchal and members of our laboratories for helpful discussions and critical reading of the manuscript.

REFERENCES
1. Hentze, M. W., Muckenthaler, M. U. & Andrews, N. C. (2004) Cell 117, 285–297
2. Knutson, M. & Wessling-Resnick, M. (2003) Crit. Rev. Biochem. Mol. Biol. 38, 61–88
3. Mok, H., Jelinek, J., Pai, S., Cattanach, B. M., Prchal, J. T., Youssoufian, H. & Schumacher, A. (2004) Development 131, 1859–1868
4. Mok, H., Mendoza, M., Prchal, J. T., Balogh, P. & Schumacher, A. (2004) Development 131, 4871–4881
5. Donovan, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S. & Andrews, N. C. (2005) Cell Metabol. 1, 191–200
6. Abboud, S. & Haile, D. J. (2000) J. Biol. Chem. 275, 19906–19912
7. McKie, A. T., Marciani, P., Rolfs, A., Brennan, K. N., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T. J., Farzanefar, F., Hediger, M. A., Hentze, M. W. & Simpson, R. J. (2000) Mol. Cell 5, 299–309
8. Zoller, H., Theurl, L., Koch, R., Kaser, A. & Weiss, G. (2002) Blood Cells Mol. Dis. 29, 488–497
9. Martini, L. A., Tchack, L. & Wood, R. J. (2002) J. Nutr. 132, 693–696
10. Dupic, F., Fruchon, S., Bensaid, M., LoreaI, O., Bristot, P., Borot, N., Roth, M. P. & Coppin, H. (2002) Gut 51, 648–653
11. Muckenthaler, M., Roy, C. N., Custodio, A. O., Minana, B., deGraaf, J., Montross, L. K., Andrews, N. C. & Hentze, M. W. (2003) Nat. Genet. 34, 102–107
12. Adams, P. C., Barber, Y. P., Khan, Z. A. & Chakraborti, S. (2003) Blood Cells Mol. Dis. 31, 256–261
13. Bridle, K. R., Frazer, D. M., Wilkins, S. J., Dixon, J. L., Pardie, D. M., Crawford, D. H., Subramanian, V. N., Powell, L. W., Anderson, G. J. & Ramm, G. A. (2003) Lancet 361, 669–673
14. Knutson, M. D., Vafa, M. R., Haile, D. J. & Wessling-Resnick, M. (2003) Blood 102, 4186–4197
15. Li, X. B., Hill, P. & Haile, D. J. (2002) Blood Cells Mol. Dis. 29, 315–326
16. Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, B. M., Donovan, A., Ward, D. M., Ganz, T. & Kaplan, J. (2004) Science 306, 2090–2093
17. Pietrangeli, A. (2004) Blood Cells Mol. Dis. 32, 131–138
18. Richter, A., Schwager, C., Hentze, S., Ansorge, W., Hentze, M. W. & Muckenthaler, M. (2002) BioTechniques 33, 620–628, 630
19. Muckenthaler, M., Richter, A., Gunes, C., Riedel, D., Polycarpou-Schwarz, M., Hentze, S., Falkenhahn, M., Stremmel, W., Ansorge, W. & Hentze, M. W. (2003) Blood 101, 3690–3698
20. Celer, P. (2005) J. Mol. Med. 83, 521–525
21. Krijt, J., Vokurka, M., Chang, K. T. & Necas, E. (2004) Blood 104, 4308–4310
22. McKnight, A. J., Macfarlane, A. J., Dri, P., Turley, L., Willis, A. C. & Gordon, S. (1996) J. Biol. Chem. 271, 486–489
23. Ponka, P. (2004) Ann. N. Y. Acad. Sci. 1012, 267–281
24. Latunde-Dada, G. O., Vulpe, C. D., Anderson, G. J., Simpson, R. J. & McKie, A. T. (2000) Biochem. Biophys. Acta 1609, 169–176
25. Vattner, L., Lesbordes-Briou, J. C., Lou, D. Q., Bennoun, M., Nicolas, G., Kahn, A., Canonne-Hergaux, F. & Vaulont, S. (2005) Blood 105, 4861–4864
26. Frazier, D. M. & Anderson, G. J. (2003) Blood Cells Mol. Dis. 30, 288–297
27. Jurado, R. L. (1997) Clin. Infect. Dis. 25, 888–895
28. Drakesmith, H., Schimanski, L. M., Ormerod, E., Merryweather-Clarke, A. T., Viprakasit, V., Edwards, J. P., Sweetland, E., Bastin, J. M., Cowley, D., Chinthammitr, Y., Robson, K. J. & Townsend, A. R. (2005) Blood 106, 1092–1097
29. Schimanski, L. M., Drakesmith, H., Merryweather-Clarke, A. T., Viprakasit, V., Edwards, J. P., Sweetland, E., Bastin, J. M., Cowley, D., Chinthammitr, Y., Robson, K. J. & Townsend, A. R. (2005) Blood 105, 4096–4102
30. Liu, X. B., Yang, F. & Haile, D. J. (2005) Blood Cells Mol. Dis. 33, 33–46
31. De Domenico, I., Ward, D. M., Nemeth, E., Vaughn, M. B., Mucsi, G., Ganz, T. & Kaplan, J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 8955–8960