Global Transcriptional Response to Hfe Deficiency and Dietary Iron Overload in Mouse Liver and Duodenum

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Abstract
Iron is an essential trace element whose absorption is usually tightly regulated in the duodenum. HFE-related hereditary hemochromatosis (HH) is characterized by abnormally low expression of the iron-regulatory hormone, hepcidin, which results in increased iron absorption. The liver is crucial for iron homeostasis as it is the main production site of hepcidin. The aim of this study was to explore and compare the genome-wide transcriptome response to Hfe deficiency and dietary iron overload in murine liver and duodenum. IlluminaM arrays containing over 47,000 probes were used to study global transcriptional changes. Quantitative RT-PCR (Q-RT-PCR) was used to validate the microarray results. In the liver, the expression of 151 genes was altered in Hfe−/− mice while dietary iron overload changed the expression of 218 genes. There were 173 and 108 differentially expressed genes in the duodenum of Hfe−/− mice and mice with dietary iron overload, respectively. There was 93.5% concordance between the results obtained by microarray analysis and Q-RT-PCR. Overexpression of genes for acute phase reactants in the liver and a strong induction of digestive enzyme genes in the duodenum were characteristic of the Hfe-deficient genotype. In contrast, dietary iron overload caused a more pronounced change of gene expression responsive to oxidative stress. In conclusion, Hfe deficiency caused a previously unrecognized increase in gene expression of hepatic acute phase proteins and duodenal digestive enzymes.

Introduction
Iron plays crucial roles in cellular metabolism but, in excess, it can catalyze the formation of free radicals leading to oxidative stress and cell damage [1]. Iron is absorbed in the duodenum, where it crosses the apical and basolateral membranes of absorptive enterocytes to enter the blood stream [2]. There is no regulated mechanism of iron excretion, and thus the absorption of iron must be tightly regulated to maintain iron balance. HFE-related hereditary hemochromatosis (HH, OMIM-235200) is an autosomal recessive disorder in which absorption of iron is inappropriately high [3,4]. HH is characterized by high transferrin saturation and low iron content in macrophages. Iron is deposited primarily in the parenchymal cells of various organs, particularly the liver, but also the pancreas, heart, skin, and testes, resulting in tissue damage and organ failure. Clinical complications in untreated HH patients include hepatic fibrosis, cirrhosis, hepatocellular carcinoma, diabetes, cardiomyopathy, hypogonadism, and arthritis [4].

HH is characterized by inappropriately low expression of the iron-regulatory hormone hepcidin [5]. Hepcidin, a small peptide hormone expressed mainly in the liver, is a central player in the maintenance of iron balance [6]. The only known molecule capable of transporting iron out of cells is ferroportin [7–9]. This iron exporter is located in the plasma membrane of enterocytes, reticuloendothelial cells, hepatocytes, and placental cells [7]. Hepcidin binds to ferroportin and induces its internalization and degradation, therefore suppressing the transport of iron into the circulation [10]. The expression of hepcidin is induced by increased iron stores and inflammation, and suppressed by hypoxia and anemia [11,12].

Mice homozygous for a null allele of Hfe (Hfe−/−) provide a genetic animal model of HH [13]. There are several animal models of iron overload based on administration of exogenous iron [14]. According to the route of iron delivery, these can be divided into two main types: enteral (i.e. dietary) and parenteral models. For example, dietary supplementation with carbonyl iron in mice reproduces the HH pattern of hepatic iron loading, with predominantly parenchymal iron deposition [14]. Although both Hfe−/− mice and carbonyl iron-fed mice develop iron overload, there are important differences between these two models. Hfe−/− mice lack Hfe protein and therefore have decreased expression of hepcidin [15,16], while mice...
with dietary iron overload express functional Hfe protein and their hepcidin expression is elevated [12].

Current RNA microarray technology allows expression profiling of the whole transcriptome. This methodology has been used to explore the effects of Hfe gene disruption on mRNA expression in the liver and duodenum, two organs with crucial roles in iron metabolism [17]. In the present study, we used this approach to study gene expression in the liver and duodenum of Hfe<sup>−/−</sup> mice and wild-type mice, with or without dietary iron overload. This allowed the identification of genes whose expression is changed during iron overload and those genes whose expression is differentially influenced by lack of Hfe protein.

**Results**

We used global microarray analysis to study gene expression in the liver and duodenum of Hfe<sup>−/−</sup> mice and carbonyl iron loaded mice, and comparing it with that of wild-type mice fed a standard diet. This approach allowed the identification of genes whose expression is changed during iron overload and those genes whose expression is differentially influenced by lack of Hfe protein. All the mice used were males and all had the same genetic background (C57BL/6).

**Hepatic transcriptional response to Hfe deficiency and dietary iron overload**

Hepatic RNA from 3 Hfe<sup>−/−</sup> mice and 2 wild-type mice was subjected to microarray analysis. The Pearson correlation coefficient between the knock out mice and between the controls was in both cases 0.989. The results revealed 86 induced genes and 27 genes were regulated in opposite directions by these two conditions in the liver (Table 4). In some cases, several genes belonging to the same gene family showed divergent regulation (e.g., Saa1, Saa2, Saa3) with upregulation in Hfe<sup>−/−</sup> mice and downregulation by dietary iron overload.

**Altered expression of iron-related genes in the liver.** The expression of 3 iron-related genes was altered in the liver of Hfe<sup>−/−</sup> mice. The expression of Hmp1 and Tfe was decreased and that of Lox2 was induced. We confirmed these results using Q-RT-PCR and also tested the expression of Hmp2, which was downregulated (Figure 1). Dietary iron overload changed the expression of 5 iron-related genes in the liver. The expression of Hmp1, Hmp2, Lox2 and Qp was upregulated using both microarray analysis and Q-RT-PCR, while Tfe expression was down-regulated by 1.7-fold (Figure 2).

**Confirmation of hepatic microarray results by Q-RT-PCR.** Microarray analysis for the expression of several genes was confirmed by performing Q-RT-PCR on hepatic samples from 5 Hfe<sup>−/−</sup> mice, 4 wild-type control mice, 5 iron-fed mice and 4 mice fed a standard diet. For this purpose, we selected iron-related genes and others whose expression was substantially altered in the experimental groups. A total of 29 results from the hepatic microarray data, corresponding to 24 different genes, were tested by Q-RT-PCR, and 27 (93.1%) of them showed concordant results by these two methods (Figures 1 and 2). Changes in Foxj1 and Dmt1 expression were false-positives in the microarray analysis for Hfe<sup>−/−</sup> mice and dietary iron overload, respectively. The upregulation of Ltf expression by dietary iron overload observed by microarray analysis could not be confirmed by Q-RT-PCR because the expression levels in samples from all but one of the treated mice and all control mice were below the detection threshold.

**Duodenal gene expression response to Hfe deficiency and dietary iron supplementation**

Microarray analysis of duodenal RNA from 2 Hfe<sup>−/−</sup> mice and 2 wild-type mice revealed that the expression of 143 genes was upregulated when 30 genes was downregulated when a cutoff value of ±1.4-fold was used (Table 1 and Dataset S3). The fold-changes ranged from 9.83 to −3.47. Functional annotation of the gene lists highlighted the biological processes that may be modified by Hfe deficiency. This analysis revealed enrichment of heat shock proteins and proteins related to inflammatory responses or antigen processing and presentation, among others (Table 2).

Another microarray experiment was performed using hepatic RNA from 3 mice with dietary iron overload and 2 mice fed a standard diet. The similarity between samples from individual mice was measured as the Pearson correlation coefficient, which was 0.989 between iron overloaded mice and 0.991 between control mice. The expression of 123 genes was upregulated and that of 95 genes was downregulated, applying a cutoff value of ±1.4-fold (Table 1 and Dataset S2). The fold-changes ranged between 13.58 and −7.46. The list of regulated genes was functionally annotated (Table 3), showing enrichment of cytochrome P450 proteins as well as others involved in glutathione metabolism, acute-phase response, organic acid biosynthetic process and cellular iron homeostasis, among others.

There were 11 upregulated and 7 downregulated genes that were affected by both Hfe deficiency and dietary iron overload in similar fashion, while 27 genes were regulated in opposite directions by these two conditions in the liver (Table 4). In some cases, several genes belonging to the same gene family showed divergent regulation (e.g., Saa1, Saa2, Saa3) with upregulation in Hfe<sup>−/−</sup> mice and downregulation by dietary iron overload.

**Table 1. Number of genes regulated by Hfe deficiency or dietary iron overload in murine liver and duodenum.**

| Tissue | Model   | Total regulated genes | Upregulated genes | Downregulated genes | Proportion of results confirmed by Q-RT-PCR |
|--------|---------|-----------------------|-------------------|---------------------|-----------------------------------------|
| Liver  | Hfe<sup>−/−</sup> | 151 | 86 | 65 | 11/12 |
|        | Dietary Iron | 218 | 123 | 95 | 16/17 |
| Duodenum | Hfe<sup>−/−</sup> | 173 | 143 | 30 | 6/7 |
|        | Dietary Iron | 108 | 49 | 59 | 10/10 |

Genes with changes in mRNA expression greater than ±1.4-fold were considered as regulated.

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Table 2. Functional annotation of genes regulated in the liver of Hfe<sup>−/−</sup> mice.

| Functional Category                  | Gene Symbol | Description                               | GenBank Number | FC   | Q-PCR |
|--------------------------------------|-------------|-------------------------------------------|----------------|------|-------|
| Response to unfolded protein         | Hspd1       | heat shock protein 1 (chaperonin)         | NM_010477      | 1.54 |       |
|                                      | Hsp90ab1    | heat shock protein 90 kDa alpha (cytosolic), class B member 1 | NM_008302 | -1.48|       |
|                                      | Hspb1       | heat shock protein 1                      | NM_013350      | -1.66|       |
|                                      | Hspa8       | heat shock protein 8                      | NM_031165      | -1.70|       |
|                                      | Hsp90b1     | heat shock protein 90 kDa beta (Grp94), member 1 | NM_011631 | -1.71|       |
|                                      | Hsp90a1     | heat shock protein 90 kDa alpha (cytosolic), class A member 1 | NM_010480 | -1.72|       |
|                                      | Hspa5       | heat shock protein 5                      | NM_022310      | -2.14|       |
|                                      | Hsp1        | heat shock 105 kDa/110 kDa protein 1      | NM_013559      | -2.16| -2.43|
|                                      | Syvn1       | synovial apoptosis inhibitor 1, synoviolin | NM_028769      |       | -2.45|
| Inflammatory response                | Saa2        | serum amyloid A 2                          | NM_011314      | 9.83 | 39.36 |
|                                      | Saa1        | serum amyloid A 1                          | NM_009117      | 6.30 | 16.36 |
|                                      | Omn2        | orosomucoid 2                             | NM_01016       | 3.29 |       |
|                                      | Saa3        | serum amyloid A 3                          | NM_011315      | 2.89 |       |
|                                      | Omn1        | orosomucoid 1                             | NM_008768      | 1.68 |       |
|                                      | Serpina3n   | serine (or cysteine) peptidase inhibitor, clade A, member 3N | NM_009252 | 1.63 |       |
|                                      | C1s         | complement component 1, s subcomponent    | NM_144938      | 1.47 |       |
|                                      | Cxcl9       | chemokine (C-X-C motif) ligand 9           | NM_008599      | -1.57|       |
| Apolipoprotein associated with HDL   | Saa2        | serum amyloid A 2                          | NM_011314      | 9.83 | 39.36 |
|                                      | Saa1        | serum amyloid A 1                          | NM_009117      | 6.30 | 16.36 |
|                                      | Saa3        | serum amyloid A 3                          | NM_011315      | 2.89 |       |
|                                      | Apoa4       | apolipoprotein A-IV                        | NM_007468      | 2.36 |       |
| Monoxygenase activity                | Moxd1       | monoxygenase, DBH-like 1                    | NM_021509      | 4.12 |       |
|                                      | Cyp2a5      | cytochrome P450, family 2, subfamily a, polypeptide 5 | NM_007812 | 1.67|       |
|                                      | Cyp27a1     | cytochrome P450, family 27, subfamily a, polypeptide 1 | NM_024264 | 1.64|       |
|                                      | Cyp2d26     | cytochrome P450, family 2, subfamily d, polypeptide 26 | NM_029562 | 1.59|       |
|                                      | Kmo         | kynurene 3-monoxygenase (kynurenine 3-hydroxylase) | NM_133809 | 1.48|       |
|                                      | Cyp4a14     | cytochrome P450, family 4, subfamily a, polypeptide 14 | NM_007822 | -1.44|       |
|                                      | Cyp4a11     | cytochrome P450, family 3, subfamily a, polypeptide 11 | NM_007818 | -1.58|       |
|                                      | Cyp26b1     | cytochrome P450, family 26, subfamily b, polypeptide 1 | NM_175475 | -2.39 | -2.18|
| Steroid biosynthetic process         | Nsdhl       | NAD(P) dependent steroid dehydrogenase-like | NM_010941 | 1.44|       |
|                                      | Hmgs1       | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | NM_145942 | -1.42|       |
|                                      | Lss         | lanosterol synthase                        | NM_146006      | -1.45|       |
|                                      | Hmgcr       | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase | NM_008255 | -1.50|       |
|                                      | Mvd         | mevalonate (diphospho) decarboxylase       | NM_138656      | -1.67|       |
| Antigen processing and presentation  | Psmb8       | proteasome (prosome, macropain) subunit, beta type 8 (large multipeptide subunit 7) | NM_010724 | 1.50|       |
|                                      | Cd74        | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | NM_010545 | -1.59|       |
|                                      | H2-Eb1      | histocompatibility 2, class II antigen E beta | NM_010382 | -1.63|       |
|                                      | H2-Ab1      | histocompatibility 2, class II antigen A, beta 1 | NM_207105 | -1.77|       |
|                                      | H2-Aa       | histocompatibility 2, class II antigen A, alpha | NM_010378 | -1.81|       |
| Endopeptidase inhibitor activity     | Serpina12   | serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12 | NM_026535 | 2.01|       |
|                                      | Wfdc2       | WAP four-disulfide core domain 2           | NM_026323      | 1.65|       |
|                                      | Serpina3n   | serine (or cysteine) peptidase inhibitor, clade A, member 3N | NM_009252 | 1.63|       |
|                                      | Itih4       | inter alpha-trypsin inhibitor, heavy chain 4 | NM_018746 | 1.48|       |
| Carboxy-lyase activity               | Ddc         | dopa decarboxylase                         | NM_016672      | -1.48|       |
|                                      | Mvd         | mevalonate (diphospho) decarboxylase       | NM_138656      | -1.67|       |
|                                      | Csad        | cysteine sulfenic acid decarboxylase       | NM_144942      | -1.76|       |
Global transcriptional regulation was also studied in the duodenum of mice fed an iron-supplemented diet, using 3 treated mice and 2 controls. The Pearson correlation coefficient was 0.985 between treated mice and 0.983 between controls. The expression of 49 genes was induced and 59 genes were repressed, applying a cutoff value of ≥ 1.4-fold (Table 1 and Dataset S4). The fold-changes ranged between 6.07 and 5.64. Functional annotation of the gene list evidenced enrichment of genes involved in glutathione metabolism, antigen processing and presentation and inflammatory response, among others (Table 6).

We identified genes whose expression was affected by both Hfe deficiency and dietary iron supplementation in the duodenum. There were 4 genes whose expression was induced in both conditions, 3 genes whose expression was decreased, and 4 genes with opposite regulation (Table 7).

**Altered expression of iron-related genes in the duodenum.** In the duodenum of Hfe^−/−^ mice, Hamp2 expression was increased by 2.7-fold using microarray analysis. However, this could not be confirmed by Q-RT-PCR, because Hamp2 mRNA levels in the samples from wild-type mice and in one Hfe^−/−^ sample were below the detection threshold. In mice fed the iron-supplemented diet, the duodenal expression of Tfrc was downregulated and that for Hmas1 was upregulated: both of these results were validated by Q-RT-PCR (Figure 3).

**Confirmation of duodenal microarray results by Q-RT-PCR.** Q-RT-PCR analyses were done on duodenal RNA samples from 5 Hfe^−/−^ mice, 4 wild-type control mice, 5 iron-fed mice and 4 mice fed a standard diet in order to confirm the microarray results. The mRNA expression of a total of 17 different genes was tested and 16 (94.1%) showed concordant results between microarray analysis and Q-RT-PCR (Figures 3 and 4). The sole discrepant result concerned the expression of Diko1 that was downregulated according to microarray analysis, while Q-RT-PCR revealed a slight induction (1.25-fold) of expression.

**Discussion.**

The goal of this study was to explore and compare the genome-wide transcriptome response to Hfe deficiency and dietary iron overload in murine liver and duodenum. This approach allowed the identification of genes whose expression is changed during iron overload and those genes whose expression is differentially influenced by lack of Hfe protein. The global transcriptional response to Hfe deficiency has been explored previously in the liver and duodenum of two mouse strains [17]. However, it is notable that only a few analogous changes in gene expression are seen when comparing our data with those of the previous study, even for mice of the same genetic background. Two other reports have explored expression of selected genes by using dedicated arrays in Hfe^−/−^ mice and in mice with secondary iron overload produced by intraperitoneal injection of iron-dextran [19,20]. In one study, duodenum and liver samples were analyzed using an array of iron-related genes [19]. The results for duodenal gene expression in Hfe^−/−^ mice have no concordance with ours. Regulation of hepatic gene expression, on the other hand, is similar for several genes, such as Hamp1, Tfrc and Mt1. The second report focused on gene expression in the duodenum [20], and again, there is little concordance between their observations and ours. The lack of agreement between these studies is probably due to differences in the animal models (parenteral vs. enteral iron loading; mouse strains) and in the microarray methodology.

The hepatic expression of acute phase proteins (APPs) can be induced by inflammatory mediators such as interleukin-6. Interestingly, the liver of Hfe^−/−^ mice has upregulated expression of APPs such as serum amyloids, lipocalins and orosomucoids. Notably, the expression of serum amyloid genes (Saa1, Saa2, Saa3) was upregulated in the Hfe^−/−^ mice compared to being downregulated in dietary iron overload, suggesting that Hfe deficiency induces this gene expression by an iron-independent mechanism. However, hepatic interleukin-6 mRNA expression was not significantly changed by Hfe deficiency, so the potential involvement of this cytokine in the observed upregulation of APPs remains uncertain.

Lipocalin2 (human Ngal from neutrophil gelatinase-associated lipocalcin) is an APP with antimicrobial properties through a mechanism of iron deprivation by siderophore binding [21]. It can donate iron to various types of cells [22,23] and seems to be capable of intracellular iron chelation and iron excretion [24]. Furthermore, a recent study has shown that lipocalin2 is an adipokine with potential importance in insulin resistance associated with obesity [25]. We observed that Lcn2 expression is increased in the liver of both Hfe^−/−^ mice and those with dietary iron overload, suggesting that this induction is iron-related.

Dietary iron overload of the liver led to increased expression of both hepcidin genes (Hamp1, Hamp2) as previously reported [26,27], and these results were verified by Q-RT-PCR. In the liver of Hfe^−/−^ mice, Hamp1 expression was downregulated as expected [15,16,19]. We also examined the levels of Hamp2 mRNA by Q-RT-PCR and found a 1.92-fold change. The low expression of hepatic Hamp1 in Hfe^−/−^ mice is likely responsible for the increased iron absorption and low microphage iron content in these mice [15,16,19].

Inhibitor of DNA-binding/differentiation proteins, also known as Id proteins, comprise a family of proteins that heterodimerize...
Table 3. Functional annotation of genes regulated in the liver of iron-fed mice.

| Functional Category | Gene Symbol | Description | GenBank Number | FC | Q-PCR |
|---------------------|-------------|-------------|----------------|----|-------|
| Electron transport, containing heme and monooxygenase activity | Cyp2b10 | cytochrome P450, family 2, subfamily b, polypeptide 10 | NM_009999 | 13.58 | |
| | Cyp2b9 | cytochrome P450, family 2, subfamily b, polypeptide 9 | NM_010000 | 7.41 | |
| | Cyp4a14 | cytochrome P450, family 4, subfamily a, polypeptide 14 | NM_007822 | 6.97 | 16.06 |
| | Cyp26b1 | cytochrome P450, family 26, subfamily b, polypeptide 1 | NM_175475 | 2.24 | |
| | Cyp2c29 | cytochrome P450, family 2, subfamily c, polypeptide 29 | NM_007815 | 1.77 | |
| | Cyp2c54 | cytochrome P450, family 2, subfamily c, polypeptide 54 | NM_206537 | 1.76 | 2.37 |
| | Cyp2a5 | cytochrome P450, family 2, subfamily a, polypeptide 5 | NM_007812 | 1.65 | |
| | Cyp2b13 | cytochrome P450, family 2, subfamily b, polypeptide 13 | NM_007813 | 1.50 | |
| | Cyp4v3 | cytochrome P450, family 4, subfamily v, polypeptide 3 | NM_133969 | 1.82 | |
| | Cyp7b1 | cytochrome P450, family 7, subfamily b, polypeptide 1 | NM_007825 | 2.50 | |
| | Cyp4a12b | cytochrome P450, family 4, subfamily a, polypeptide 12B | NM_172306 | 1.73 | |
| | Cyp7a1 | cytochrome P450, family 7, subfamily a, polypeptide 1 | NM_007824 | 2.80 | |
| | Cyp4a12a | cytochrome P450, family 4, subfamily a, polypeptide 12a | NM_177406 | 3.62 | |
| Glutathione metabolism | Gsta1 | glutathione S-transferase, alpha 1 (Ya) | NM_008181 | 1.94 | |
| | Gstt2 | glutathione S-transferase, theta 2 | AK079739 | 1.86 | |
| | Gsta2 | glutathione S-transferase, alpha 2 (Yc2) | NM_008182 | 1.83 | |
| | Gstm6 | glutathione S-transferase, mu 6 | NM_008184 | 1.78 | |
| | Mgst3 | microsomal glutathione S-transferase 3 | NM_022556 | 1.72 | |
| | Gstm3 | glutathione S-transferase, mu 3 | NM_010359 | 1.59 | |
| | Gclc | glutamate-cysteine ligase, catalytic subunit | NM_010295 | 1.55 | |
| | Gstp1 | glutathione S-transferase, pi 1 | NM_013541 | 1.81 | |
| Acute-phase response | Il1b | interleukin 1 beta | NM_008361 | 2.04 | |
| | Saa3 | serum amyloid A 3 | NM_011315 | 1.82 | |
| | Saa4 | serum amyloid A 4 | NM_011316 | 1.91 | |
| | Saa2 | serum amyloid A 2 | NM_011314 | 2.79 | 3.36 |
| | Saa1 | serum amyloid A 1 | NM_009117 | 3.96 | 4.31 |
| Organic acid biosynthetic process | Fasn | fatty acid synthase | NM_007988 | 2.22 | |
| | Elovl6 | ELOVL family member 6, elongation of long chain fatty acids | NM_130450 | 1.87 | |
| | Acaca | acetyl-Coenzyme A carboxylase alpha | NM_133360 | 1.81 | |
| | Cd74 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | NM_010545 | 1.65 | |
| | Cyp7b1 | cytochrome P450, family 7, subfamily b, polypeptide 1 | NM_007825 | 2.50 | |
| | Elovl3 | elongation of very long chain fatty acids-like 3 | NM_007703 | 5.00 | |
| Cellular iron homeostasis | Hamp2 | hepcidin antimicrobial peptide 2 | NM_183257 | 10.03 | 24.77 |
| | Hamp1 | hepcidin antimicrobial peptide 1 | NM_032541 | 1.73 | 5.27 |
| | Tfrc | transferrin receptor | NM_011638 | 1.74 | |
| | Alas2 | aminolevulinic acid synthase 2, erythroid | NM_009653 | 2.20 | |
| Hemopoiesis and immune system development | Id2 | inhibitor of DNA binding 2 | NM_010496 | 2.92 | 5.2 |
| | Egr1 | early growth response 1 | NM_007913 | 2.55 | |
| | H2-Aa | histocompatibility 2, class II antigen A, alpha | NM_010378 | 1.81 | |
| | Gadd45g | growth arrest and DNA-damage-inducible 45 gamma | NM_011817 | 1.66 | |
| | Cd74 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | NM_010545 | 1.65 | |
| | Hbb-b1 | hemoglobin, beta adult major chain | NM_008220 | 1.45 | |
| | Pik3r1 | phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha), transcript variant 1 | NM_001024955 | 1.70 | |
| | Alas2 | aminolevulinic acid synthase 2, erythroid | NM_009653 | 2.20 | |
| | BcI6 | B-cell leukemia/lymphoma 6 | NM_009744 | 2.61 | |
| Serine-type endopeptidase inhibitor activity | Serpina7 | serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7 | NM_177920 | 2.12 | |
The upregulation of gene expression for digestive enzymes, such as elastases, carboxypeptidases, trypsins, and lipases. In contrast, feeding mice with an iron-supplemented diet did not affect the expression of any of these enzymes. Some genes of the MHC class II family.

| Functional Category | Gene Symbol | Description | GenBank Number | FC | Q-PCR |
|---------------------|-------------|-------------|----------------|----|-------|
| Serpina3m | serine (or cysteine) peptidase inhibitor, clade A, member 3 M | NM_009253 | 2.04 | |
| Spink4 | serine peptidase inhibitor, Kazal type 4 | NM_011463 | 1.52 | |
| Serpina1e | serine (or cysteine) peptidase inhibitor, clade A, member 1e | NM_009247 | 1.86 | |
| Serpina12 | serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12 | NM_026535 | 2.19 | |
| Serpine2 | serine (or cysteine) peptidase inhibitor, clade E, member 2 | AK045954 | 2.88 | |
| Antigen processing and presentation via MHC class II | H2-Aa | histocompatibility 2, class II antigen A, alpha | NM_010378 | 1.81 | |
| H2-Ab1 | histocompatibility 2, class II antigen A, beta 1 | NM_207105 | 1.68 | |
| Cd74 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | NM_010545 | 1.65 | |
| H2-Eb1 | histocompatibility 2, class II antigen E beta | NM_010382 | 1.43 | |

The solute carrier molecules constitute a large family of proteins involved in membrane transport of diverse molecules. The gene expression of many family members was affected by Hfe deficiency or dietary iron overload. In the duodenum, the expression of the sodium-coupled neutral amino acid transporter Slc46a3 was induced in Hfe<sup>-/-</sup> mice and repressed in mice fed an iron-supplemented diet. In the liver, the expression of Slc46a3 was upregulated in Hfe<sup>-/-</sup> mice. This gene belongs to the Slc46 subfamily of heme transporters. It is thus a close relative of Slc46a1 (also known as HCP1), a recently identified, although controversial, heme transporter [33,34]. The iron transporter Dmt1, encoded by Slc11a2, contains an iron-responsive element (IRE) in the 3’UTR of its mRNA. This permits the regulation of Dmt1 mRNA levels according to the cellular labile iron pool by mediation of the iron regulatory proteins, IRP1 and IRP2. Under iron-replete conditions, IRP activity is reduced rendering the Dmt1 mRNA vulnerable to degradation. The opposite is true under iron-deficient conditions, which is believed to be the situation inside the enterocytes of HH patients and Hfe<sup>-/-</sup> mice [35,36]. Accordingly, in some studies, increased expression of Dmt1 has been observed in the duodenum of HH patients [37] as well as in Hfe<sup>-/-</sup> mice [38]. However, we did not find a significant change in the expression of Dmt1 in the Hfe<sup>-/-</sup> duodenum. This may be explained by the inability of our microarray probes and PCR primers to discriminate between IRE-positive and IRE-negative transcripts.

The post-transcriptional regulation of Tfrc (transferrin receptor 1) by iron is also mediated through the IRE/IRP system [39]. Tfrc is involved in the uptake of transferrin-bound iron by cells. Analogous to our observations, suppression of Tfrc expression in the duodenum [40] and liver [27] of mice fed an iron-supplemented diet, and in the liver of Hfe<sup>-/-</sup> mice [19] has been reported previously. Our microarray analysis indicates that the expression of Tfrc was not significantly changed in the duodenum of Hfe<sup>-/-</sup> mice, a result that agrees with a previous report [19].

Excess free iron increases oxidant production [1]. Subsequently, some antioxidant defense mechanisms are upregulated in order to provide resistance to iron-related toxicity. It is notable from our data that this response is elicited in both liver and duodenum, as seen in the upregulation of glutathione S-transferase genes. Interestingly, dietary iron overload seems to induce a stronger response than Hfe deficiency, especially in the regulation of enzymes involved in glutathione-related detoxification of reactive intermediates.
Table 4. Comparison of hepatic gene regulation by Hfe deficiency or dietary iron overload.

| Gene Symbol | Description | GenBank Number | FC Hfe[^+^] | FC diet |
|-------------|-------------|----------------|-------------|---------|
| Lcn2        | lipocalin 2  | NM_008491      | 9.54        | 2.10    |
| Rgs16       | regulator of G-protein signaling 16 | NM_01267 | 4.61 | 5.06 |
| Mt1         | metallothionein 1 | NM_013602 | 4.17 | 3.95 |
| Apoa4       | apolipoprotein A-IV | NM_007468 | 2.36 | 6.56 |
| Slc2a2      | solute carrier family 2 (facilitated glucose transporter), member 2 | NM_031197 | 1.92 | 2.17 |
| Mfsd2       | major facilitator superfamily domain containing 2 | NM_029662 | 1.68 | 3.59 |
| Cyp2a5      | cytochrome P450, family 2, subfamily a, polypeptide 5 | NM_007812 | 1.67 | 1.65 |
| Gstt2       | glutathione S-transferase, theta 2 | NM_013611 | 1.58 | 1.86 |
| Ppp1r3c     | protein phosphatase 1, regulatory (inhibitor) subunit 3C | NM_016854 | 1.57 | 1.53 |
| Bhlhb2      | basic helix-loop-helix domain containing, class B2 | NM_011498 | 1.52 | 2.35 |
| Dusp1       | dual specificity phosphatase 1 | NM_013642 | 1.50 | 2.15 |
| Saa2        | serum amyloid A 2 | NM_011314 | 9.83 | 2.79 |
| Saa1        | serum amyloid A 1 | NM_009117 | 6.30 | 3.96 |
| Saa3        | serum amyloid A 3 | NM_011315 | 2.89 | 1.82 |
| Angptl4     | angiopoietin-like 4 | NM_020581 | 2.30 | 2.03 |
| Hp          | haptoglobin | NM_017370 | 2.23 | 1.69 |
| Serpina12   | serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12 | NM_026535 | 2.01 | 2.19 |
| Lpin1       | lipin 1 | NM_015763 | 1.92 | 1.59 |
| Il6ra       | interleukin 6 receptor, alpha | AK020663 | 1.70 | 2.08 |
| Dio1        | deiodinase, iodothyronine, type 1 | NM_007860 | 1.57 | 1.87 |
| Ppp1r3b     | protein phosphatase 1, regulatory (inhibitor) subunit 3B | NM_177741 | 1.55 | 1.95 |
| Dct         | dopachrome tautomerase | NM_010024 | 1.50 | 2.72 |
| Mup4        | major urinary protein 4 | NM_008648 | 1.44 | 4.28 |
| Cyp26b1     | cytochrome P450, family 26, subfamily b, polypeptide 1 | NM_175475 | –2.39 | 2.24 |
| Pldha1      | pleckstrin homology-like domain, family A, member 1 | NM_009344 | –2.20 | 1.51 |
| Godd45g     | growth arrest and DNA-damage-inducible 45 gamma | NM_011817 | –1.97 | 1.66 |
| Socs3       | suppressor of cytokine signaling 3 | NM_007707 | –1.96 | 1.89 |
| Csh         | cytokine inducible SH2-containing protein | NM_009895 | –1.93 | 2.37 |
| H2-Aa       | histocompatibility 2, class II antigen A, alpha | NM_010378 | –1.81 | 1.81 |
| Egr1        | early growth response 1 | NM_007913 | –1.77 | 2.55 |
| H2-Ab1      | histocompatibility 2, class II antigen A, beta 1 | NM_207105 | –1.77 | 1.68 |
| Gsta2       | glutathione S-transferase, alpha 2 (Yc2) | NM_008182 | –1.71 | 1.83 |
| H2-Eb1      | histocompatibility 2, class II antigen E beta | NM_010382 | –1.63 | 1.43 |
| Cd74        | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | NM_010545 | –1.59 | 1.65 |
| Cyp4a14     | cytochrome P450, family 4, subfamily a, polypeptide 14 | NM_007822 | –1.44 | 6.97 |
| Hbb-b1      | hemoglobin, beta adult major chain | AK010993 | –1.42 | 1.45 |
| Rnf186      | ring finger protein 186 | NM_025786 | –1.41 | 1.81 |
| Hamp1       | hepcidin antimicrobial peptide 1 | NM_025241 | –1.41 | 1.73 |
| Cred2       | cysteine-rich with EGF-like domains 2 | NM_029720 | –3.47 | –1.64 |
| Hsp90       | heat shock 105 kDa/110 kDa protein 1 | NM_013559 | –2.16 | –2.13 |
| Tfrc        | transferrin receptor | NM_011638 | –1.92 | 1.74 |
| Hsp70       | heat shock protein 1 | NM_013560 | –1.66 | 1.81 |
| Hhex        | hematopoietically expressed homeobox | NM_008245 | –1.55 | –2.05 |
| Mcm10       | minichromosome maintenance deficient 10 (S. cerevisiae) | NM_027290 | –1.55 | –1.55 |
| Ddc         | dopa decarboxylase | NM_016672 | –1.48 | 1.97 |

FC, fold-change; diet, iron-supplemented diet.
doi:10.1371/journal.pone.0007212.t004
In conclusion, Hfe deficiency results in increased gene expression of hepatic APPs and duodenal digestive enzymes. In contrast, dietary iron overload causes a more pronounced change of gene expression responsive to oxidative stress.

Materials and Methods

Ethics Statement

The animal protocols were approved by the Animal Care and Use Committees of Saint Louis University and the University of Oulu (permission No 102/05).

Animal care and animal models

Five male C57BL/6 mice homozygous for a disruption of the Hfe gene and 4 male wild-type control mice were fed a standard rodent diet (250 ppm of iron) and sacrificed at approximately 10 weeks of age. The generation of the Hfe\(^{+/+}\) mice has been described elsewhere [13]. In addition, 5 male C57BL/6 mice fed an iron-supplemented diet (2% carbonyl iron) and 4 male control mice fed a standard diet (200 ppm of iron) for 6 weeks were used [27]. The mice with dietary iron overload had a hepatic iron concentration that was approximately 2.5 times higher than the Hfe\(^{+/+}\) mice. The duodenum and liver samples were immediately collected from anesthetized mice and immersed in RNAlater solution (Ambion, Huntingdon, UK).

RNA isolation

Total RNA extraction and quality control have been described previously [27].

Microarray analysis

All microarray data reported in the present article are described in accordance with MIAME guidelines, have been deposited in NCBI's Gene Expression Omnibus public repository [41], and are accessible through GEO Series accession number GSE17969 [42]. Microarray experiments were performed in the Finnish DNA Microarray Centre at Turku Centre for Biotechnology using Illumina's Sentrix Mouse-6 Expression Beadchips. Duodenal and liver RNA samples from 3 Hfe\(^{+/+}\) mice and 3 mice with dietary iron overload were used. As controls, RNA samples from the duodenum and liver of 4 wild-type mice (2 controls of the Hfe\(^{+/+}\) mice and 2 controls of the mice with dietary iron overload) were used. All 10 samples were analyzed individually. The amplification of total RNA (300 ng), in vitro transcription, hybridization and scanning have been described before [27].

Figure 1. Validation of liver microarray data from Hfe\(^{+/+}\) mice by Q-RT-PCR. The expression of various mRNA species in 5 Hfe\(^{+/+}\) mice is compared to those in 4 wild-type controls. Each sample was run in triplicate. (mean±SD). *p<0.05; **p<0.025; ***p<0.01. doi:10.1371/journal.pone.0007212.g001

Figure 2. Expression of genes affected by dietary iron overload in the liver, as confirmed by Q-RT-PCR. Samples from 5 mice fed an iron-supplemented diet and 4 mice fed a control diet were used, and each sample was run in triplicate. (mean±SD). *p<0.05; **p<0.025; ***p<0.01. doi:10.1371/journal.pone.0007212.g002
Data analysis

Array data were normalized with Chipster (v1.1.1) using the quantile normalization method. Quality control of the data included non-metric multidimensional scaling, dendrograms, hierarchical clustering, and 2-way clustering (heat maps). These analyses showed that data from one of the three duodenal samples from \textit{Hfe}^2/2 mice were highly divergent from the other two. Thus, this sample was excluded from further analyses. The data were then filtered according to the SD of the probes. The percentage of data that did not pass through the filter was adjusted to 99.4%, implicating a SD value of almost 3. At this point, statistical analysis was performed using the empirical Bayes t-test for the comparison of 2 groups. Due to the small number of samples, the statistical results were considered as orientative and thus no filtering was applied to the data according to p-values. The remaining 280 probes were further filtered according to fold-change with \pm 1.4 as cut-off values for up- and down-regulated expression, respectively. The functional annotation tool DAVID (Database for Annotation, Visualization and Integrated Discovery) \cite{43,44} was used to identify enriched biological categories among the regulated genes as compared to all the genes present in Illumina’s Sentrix Mouse-6 Expression Beadchip. The annotation groupings analyzed were: Gene Ontology biological process and molecular functions, SwissProt Protein Information Resources keywords, SwissProt comments, Kyoto Encyclopedia of Genes and Genomes and Biocarta pathways. Results were filtered to remove categories with EASE (expression analysis systematic explorer) scores greater than 0.05. Redundant categories with the same gene members were removed to yield a single representative category.

Table 5. Functional annotation of genes regulated in the duodenum of \textit{Hfe}^2/2 mice.

| Functional Category                  | Gene Symbol | Description | GenBank Number | FC  | Q-PCR |
|-------------------------------------|-------------|-------------|----------------|-----|-------|
| Endopeptidase activity             | Ela3        | elastase 3, pancreatic | NM_026419 | 15.67 | 14.77 |
|                                    | Try4        | trypsin 4   | NM_011646 | 13.09 |
|                                    | RP23-395H4.4 | elastase 2A | NM_007919 | 10.20 |
|                                    | Ctr1        | chymotrypsin-like | NM_023182 | 9.99  |
|                                    | Ctrb1       | chymotrypsinogen B1 | NM_025583 | 9.68  |
|                                    | Prss2       | protease, serine, 2 | NM_009430 | 7.41  |
|                                    | 2210010C04Rik | RIKEN cDNA 2210010C04 gene | NM_023333 | 7.14  |
|                                    | Ela1        | elastase 1, pancreatic | NM_033612 | 5.84  |
|                                    | Klk1b5      | kallikrein 1-related peptidase b5 | NM_008456 | 2.90  |
|                                    | Ctrc        | chymotrypsin C (caldecrin) | NM_001033875 | 2.51  |
|                                    | Klk1b11     | kallikrein 1-related peptidase b11 | NM_010640 | 2.34  |
|                                    | Klk1        | kallikrein 1 | NM_010639 | 2.29  |
|                                    | Klk1b27     | kallikrein 1-related peptidase b27 | NM_020268 | 2.22  |
|                                    | Klk1b4      | kallikrein 1-related peptidase b4 | NM_010915 | 2.11  |
|                                    | Klk1b24     | kallikrein 1-related peptidase b24 | NM_010643 | 2.10  |
|                                    | Mela        | melanoma antigen | NM_008581 | 2.05  |
|                                    | Ctse        | cathepsin E | NM_007799 | 1.91  | 2.32  |
|                                    | Klk1b26     | kallikrein 1-related peptidase b26 | NM_010644 | 1.74  |
|                                    | Capn5       | calpain 5   | NM_007602 | 1.60  |
| Lipid catabolic function           | Cel         | carboxyl ester lipase | NM_009885 | 9.82  |
|                                    | Pnliprp1    | pancreatic lipase related protein 1 | NM_018874 | 8.17  |
|                                    | Clps        | colipase, pancreatic | NM_025469 | 5.35  |
|                                    | Pla2g1b     | phospholipase A2, group IB, pancreas | NM_011107 | 4.86  |
|                                    | Pnliprp2    | pancreatic lipase-related protein 2 | NM_011128 | 4.50  |
|                                    | Apoc3       | apolipoprotein C-III | NM_023114 | −1.79 |
| Triacylglycerol lipase activity    | Cel         | carboxyl ester lipase | NM_009885 | 9.82  |
|                                    | Pnliprp1    | pancreatic lipase related protein 1 | NM_018874 | 8.17  |
|                                    | Pnliprp2    | pancreatic lipase-related protein 2 | NM_011128 | 4.50  |
| Antimicrobial                      | Hamp2       | hepcidin antimicrobial peptide 2 | NM_183257 | 2.74  | 6.66  |
|                                    | Defcr-rs1   | defensin related sequence cryptdin peptide (paneth cells) | NM_007844 | −1.60 |
|                                    | Lyz1        | lysozyme 1  | NM_013590 | −1.68 |
|                                    | Defcr6      | defensin related cryptdin 6 | NM_007852 | −2.11 |
|                                    | Defcr20     | defensin related cryptdin 20 | NM_183268 | −2.69 |
| Metallo-carboxypeptidase activity  | Cpa1        | carboxypeptidase A1 | NM_025350 | 12.42 |
|                                    | Cpa2        | carboxypeptidase A2, pancreatic | NM_001024698 | 8.14 |
|                                    | Cpb1        | carboxypeptidase B1 (tissue) | NM_029706 | 12.51 | 14.55 |

\[\text{doi:10.1371/journal.pone.0007212.t005}\]
Table 6. Functional annotation of genes regulated in the duodenum of mice fed an iron-supplemented diet.

| Functional Category                  | Gene Symbol | Description                                                                 | GenBank Number | FC   | Q-PCR |
|-------------------------------------|-------------|-----------------------------------------------------------------------------|----------------|------|-------|
| Glutathione metabolism              | Gstm1       | glutathione S-transferase, mu 1                                            | NM_010358      | 4.42 | 4.29  |
|                                     | Gsta3       | glutathione S-transferase, alpha 3                                         | NM_010356      | 4.27 |       |
|                                     | Gsta1       | glutathione S-transferase, alpha 1 (Ya)                                   | NM_008181      | 3.51 |       |
|                                     | Gsta2       | glutathione S-transferase, alpha 2 (Yc2)                                  | NM_008182      | 2.93 |       |
|                                     | Gstm6       | glutathione S-transferase, mu 6                                            | NM_008184      | 2.80 |       |
|                                     | Gstm4       | glutathione S-transferase, mu 4                                            | NM_026764      | 2.41 |       |
|                                     | Gsta4       | glutathione S-transferase, alpha 4                                         | NM_010357      | 2.26 |       |
|                                     | Gstm3       | glutathione S-transferase, mu 3                                            | NM_010359      | 1.88 |       |
|                                     | Anpep       | alanyl (membrane) aminopeptidase                                            | NM_008486      | –1.83|       |
| Antigen processing and presentation | Cd74        | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | BC003476      | –1.95|       |
|                                     | H2-Eb1      | histocompatibility 2, class II antigen E beta                              | NM_010382      | –2.06|       |
|                                     | H2-DMa      | histocompatibility 2, class II, locus DMa                                  | NM_010386      | –2.07|       |
|                                     | Psmb8       | proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7) | NM_010724      | –2.07|       |
|                                     | H2-DMb2     | histocompatibility 2, class II, locus Mb2                                  | NM_010388      | –2.16|       |
|                                     | H2-Aa       | histocompatibility 2, class II antigen A, alpha                            | NM_010378      | –2.53|       |
|                                     | H2-Ab1      | histocompatibility 2, class II antigen A, beta 1                           | NM_027105      | –2.76|       |
| Chaperone cofactor-dependent protein folding | Cd74 | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | BC003476      | –1.95|       |
|                                     | H2-DMa      | histocompatibility 2, class II, locus DMa                                  | NM_010386      | –2.07|       |
|                                     | H2-DMb2     | histocompatibility 2, class II, locus Mb2                                  | NM_010388      | –2.16|       |
|                                     | Dnajb1      | DnaJ (Hsp40) homolog, subfamily B, member 1                               | NM_018808      | –2.62| 2.17  |
| MHCII                               | H2-Eb1      | histocompatibility 2, class II antigen E beta                              | NM_010382      | –2.06|       |
|                                     | H2-DMa      | histocompatibility 2, class II, locus DMa                                  | NM_010386      | –2.07|       |
|                                     | H2-DMb2     | histocompatibility 2, class II, locus Mb2                                  | NM_010388      | –2.16|       |
|                                     | H2-Aa       | histocompatibility 2, class II antigen A, alpha                            | NM_010378      | –2.53|       |
|                                     | H2-Ab1      | histocompatibility 2, class II antigen A, beta 1                           | NM_027105      | –2.76|       |
| T cell differentiation and activation | Cd74        | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | BC003476      | –1.95|       |
|                                     | H2-DMa      | histocompatibility 2, class II, locus DMa                                  | NM_010386      | –2.07|       |
|                                     | H2-DMb2     | histocompatibility 2, class II, locus Mb2                                  | NM_010388      | –2.16|       |
|                                     | Egr1        | early growth response 1                                                    | NM_007913      | –3.33| 2.32  |
| Inflammatory response                | Reg3g       | regenerating islet-derived 3 gamma                                         | NM_011260      | –1.56|       |
|                                     | Cxcl13      | chemokine (C-X-C motif) ligand 13                                          | NM_018866      | –1.71|       |
|                                     | C3          | complement component 3                                                    | NM_009778      | –1.78|       |
|                                     | Ccl5        | chemokine (C-C motif) ligand 5                                             | NM_013653      | –2.00|       |
| Antimicrobial                        | Pap         | pancreatitis-associated protein                                            | NM_011036      | –2.13|       |
|                                     | Defcr20     | defensin related cryptdin 20                                               | NM_183268      | 1.72 |       |
|                                     | Defcr5      | defensin related cryptdin 5                                               | NM_007851      | 1.41 |       |
|                                     | Lyzs        | lysozyme                                                                    | NM_017372      | 1.88 |       |
|                                     | Defcr-rs1   | defensin related sequence cryptdin peptide (paneth cells)                 | NM_007844      | –3.23|       |
| Lectin                              | Reg2        | regenerating islet-derived 2                                               | NM_009043      | 2.14 |       |
|                                     | Glg1        | golgi apparatus protein 1                                                  | NM_009149      | 1.43 |       |
|                                     | Reg3g       | regenerating islet-derived 3 gamma                                         | NM_011260      | 1.56 |       |
|                                     | Pap         | pancreatitis-associated protein                                            | NM_011036      | 2.13 |       |
| B cell mediated immunity            | C3          | complement component 3                                                    | NM_009778      | 1.78 |       |
|                                     | Cd74        | CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | BC003476      | –1.95|       |
Table 6. Cont.

| Functional Category                  | Gene Symbol | Description                          | GenBank Number | FC  | Q-PCR |
|--------------------------------------|-------------|---------------------------------------|----------------|-----|-------|
| Cholesterol metabolic process        | Ldlr        | low density lipoprotein receptor      | NM_010700      | 1.99|       |
|                                      | Cyp51       | cytochrome P450, family S1            | NM_020010      | 1.96|       |
| Response to heat                     | Hmgs2       | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 | NM_008256      | 1.88|       |
|                                      | Hspa1a      | heat shock protein 1A                 | NM_010479      | 1.91|       |
|                                      | Hsp90aa1    | heat shock protein 90 kDa alpha (cytosolic), class A member 1 | NM_010480 | 2.11|       |
|                                      | Hsp1        | heat shock 105 kDa/110 kDa protein 1   | NM_013559      | 5.64| 6.55  |

Quantitative Reverse-Transcriptase PCR

For this analysis, duodenal and liver RNA samples from 5 mice of each experimental group (Hfe<sup>+/−</sup> and dietary iron overload) and 4 mice from each control group (wild-type and normal diet) were used. Exceptionally, for the analysis of mRNA expression in the duodenum of Hfe<sup>+/−</sup> mice, only 4 samples were used. RNA samples (5 μg) were converted into first strand cDNA with a First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada) using random hexamer primers. The relative expression levels of target genes in the duodenum and liver were assessed by Q-RT-PCR using the LightCycler detection system (Roche, Rotkreuz, Switzerland). The reaction setup, cycling program, standard curve method and primer pairs for Angptl4, Dnajb1 and Tfrc have been described before [27]. Mouse Hmgs2, Hspa1a and Hsp90aa1 primers have also been characterized previously [26]. The primer sets for the other target genes (Dataset S5) were designed using Primer3 Basic Local Alignment and Search Tool (BLAST) [46]. To avoid amplification of contaminating genomic DNA, both primers from each set were specific to different exons, when possible. Each cDNA sample was tested in triplicate. The specificity of the primers was verified using NCBI GenBank. The specificity of the primers was verified using NCBI (Dataset S5) were designed using Primer3 Basic Local Alignment and Search Tool (BLAST) [46]. To avoid amplification of contaminating genomic DNA, both primers from each set were specific to different exons, when possible. Each cDNA sample was tested in triplicate. The mean and SD of the 3 crossing point (Cp) values were calculated for each sample and a SD cutoff level of 0.2 was set. Accordingly, when the SD of the triplicates of a sample was greater than 0.2, the most outlying replicate was excluded and the analysis was continued with the two remaining replicates. Using the standard curve method, the Cp values were then transformed by the LightCycler software into copy numbers. The expression value for each sample was the mean of the copy numbers for the sample’s replicates. This value was normalized by dividing it by the geometric mean of the 4 internal control genes, an accurate normalization method [47]. The normalization factor was always considered as a value of 100 and the final result was expressed as relative mRNA expression level.

Statistical analyses

We performed statistical analyses of the microarray data using the empirical Bayes t-test for the comparison of 2 groups, and the p-values are shown in supplementary datasets S1-S4. For the Q-RT-PCR results, we used the Mann-Whitney test to evaluate differences in group values for Hfe<sup>+/−</sup> mice vs. wild-type mice and mice with dietary iron overload vs. untreated mice. Due to the small sample sizes, the statistical significance is only considered as orientative. Values are expressed as mean±SD.

Supporting Information

Dataset S1  List of genes differentially expressed in the liver of Hfe knockout mice
Found at: doi:10.1371/journal.pone.0007212.s001 (0.04 MB XLS)

Table 7. Genes regulated in the duodenum of mice by Hfe deficiency or iron-supplemented diet.

| Gene Symbol | Description                           | GenBank | FC Hfe<sup>+/−</sup> | FC diet |
|-------------|---------------------------------------|---------|----------------------|---------|
| Increased in Hfe<sup>+/−</sup> and by diet | Reg2      | regenerating islet-derived 2         | NM_009043 | 10.34  | 2.14 |
|            | Alpi       | alkaline phosphatase, intestinal     | NM_01081082 | 2.09   | 1.71 |
|            | Akrb8     | aldo-keto reductase family 1, member B8 | NM_008012 | 1.60   | 4.17 |
|            | Mboat1    | membrane bound O-acyltransferase domain containing 1 | NM_153546 | 1.46   | 1.81 |
| Increased in Hfe<sup>+/−</sup> and decreased by diet | Reg3b      | regenerating islet-derived 3 beta   | NM_010136 | 6.87   | 2.13 |
|            | Klk1b27   | kallikrein 1-related peptidase b27   | NM_020268 | 2.22   | 1.87 |
|            | Slc38a5   | solute carrier family 38, member 5  | NM_172479 | 2.14   | 2.31 |
| Decreased in Hfe<sup>+/−</sup> and increased by diet | Defcr20    | defensin related cystatin 20         | NM_183268 | 2.69   | 1.72 |
| Decreased in Hfe<sup>+/−</sup> and by diet | Hspb1      | heat shock protein 1                 | NM_013560 | 2.07   | 2.17 |
|            | Defcr-rs1 | defensin related sequence cystatin precursor (Paneth cells) | NM_007844 | 1.60   | 3.23 |
|            | LOC620017 | PREDICTED: similar to Ig kappa chain V-V region L7 precursor | XM_357633 | 1.44   | 2.31 |

FC, fold-change; diet, iron-supplemented diet.
doi:10.1371/journal.pone.0007212.t007
Figure 3. Expression of genes regulated in the duodenum of dietary iron-loaded mice as verified by Q-RT-PCR. Samples from 5 mice fed an iron-supplemented diet and 4 mice fed a control diet were used, and each sample was run in triplicate. (mean ± SD). *p<0.05; **p<0.025; ***p<0.01. doi:10.1371/journal.pone.0007212.g003

Figure 4. Validation of the duodenal microarray results from Hfe−/− mice by Q-RT-PCR. The Hfe−/− and control groups contained samples from 4 mice, and each sample was tested in triplicate. (mean ± SD). *p<0.05; **p<0.025; ***p<0.01. doi:10.1371/journal.pone.0007212.g004

Dataset S2 List of genes differentially expressed in the liver of mice fed an iron-supplemented diet
Found at: doi:10.1371/journal.pone.0007212.s002 (0.05 MB XLS)

Dataset S3 Genes whose expression was altered in the duodenum of Hfe knockout mice
Found at: doi:10.1371/journal.pone.0007212.s003 (0.05 MB XLS)

Dataset S4 Genes whose expression was affected in the duodenum of mice fed an iron-supplemented diet
Found at: doi:10.1371/journal.pone.0007212.s004 (0.04 MB XLS)

Dataset S5 Sequences of the primers used in the Q-RT-PCR experiments performed in this study
Found at: doi:10.1371/journal.pone.0007212.s005 (0.06 MB DOC)

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Author Contributions
Conceived and designed the experiments: AR REF RSB BRB SP. Performed the experiments: AR. Analyzed the data: AR TL. Contributed reagents/materials/analysis tools: REF RSB BRB SP. Wrote the paper: AR. Critically reviewed the manuscript and approved its final version: TL REF RSB BRB SP.

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Transcription in Iron Overload

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