

# Perturbation of hepcidin expression by BMP type I receptor deletion induces iron overload in mice

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**Bone morphogenetic protein (BMP) signaling induces hepatic expression of the peptide hormone hepcidin. Hepcidin reduces serum iron levels by promoting degradation of the iron exporter ferroportin. A relative deficiency of hepcidin underlies the pathophysiology of many of the genetically distinct iron overload disorders, collectively termed hereditary hemochromatosis. Conversely, chronic inflammatory conditions and neoplastic diseases can induce high hepcidin lev-**

**els, leading to impaired mobilization of iron stores and the anemia of chronic disease. Two BMP type I receptors, Alk2 (*Acvr1*) and Alk3 (*Bmpr1a*), are expressed in murine hepatocytes. We report that liver-specific deletion of either Alk2 or Alk3 causes iron overload in mice. The iron overload phenotype was more marked in Alk3- than in Alk2-deficient mice, and Alk3 deficiency was associated with a nearly complete ablation of basal BMP signaling and hepci-**

**din expression. Both Alk2 and Alk3 were required for induction of hepcidin gene expression by BMP2 in cultured hepatocytes or by iron challenge in vivo. These observations demonstrate that one type I BMP receptor, Alk3, is critically responsible for basal hepcidin expression, whereas 2 type I BMP receptors, Alk2 and Alk3, are required for regulation of hepcidin gene expression in response to iron and BMP signaling. (*Blood*. 2011; 118(15):4224-4230)**

## Introduction

The hepatic hormone hepcidin regulates serum iron levels in mice and humans by inducing degradation of the iron exporter, ferroportin.<sup>1,2</sup> Low ferroportin levels reduce intestinal iron absorption and the release of iron from macrophage stores. Human hereditary hemochromatosis is characterized by low hepcidin levels, leading to iron accumulation in liver, heart, and endocrine organs.<sup>1,3</sup> Similarly, hepcidin deficiency causes hepatic iron overload in mice.<sup>2,4</sup> In contrast, high hepcidin levels contribute to the anemia of chronic disease (ACD) by reducing iron bioavailability for erythropoiesis.<sup>5,6</sup>

Recent studies have demonstrated a critical role for bone morphogenetic protein (BMP) signaling in the regulation of hepcidin expression by iron.<sup>7-11</sup> Binding of BMP ligands to type I and type II BMP receptors induces the type II receptor to phosphorylate and activate the type I receptor. The activated type I receptor, in turn, phosphorylates intracellular signaling molecules, including SMADs 1, 5, and 8. Phosphorylated SMADs 1, 5, and 8 bind SMAD4 and together translocate to the nucleus, where they activate the expression of genes, including hepcidin and the Id family of transcription factors.<sup>12</sup> Deficiency of Smad4,<sup>10</sup> the BMP coreceptor hemojuvelin,<sup>13,14</sup> or BMP6<sup>15,16</sup> in hepatocytes reduces expression of hepcidin<sup>17,18</sup> and induces iron overload. In addition, BMP signaling appears to have an important role in the induction of hepcidin expression by inflammatory mediators that are involved in ACD.<sup>11,19,20</sup>

There are 4 type I BMP receptors: Alk1, Alk2, Alk3, and Alk6. The identity of the type I BMP receptor(s) responsible for iron-dependent signaling and the regulation of hepcidin expression in hepatocytes are unknown. Alk1 is predominantly expressed in the endothelium. Alk6 is expressed at low levels in murine liver,<sup>21</sup> and global Alk6 deficiency does not induce iron overload in mice (D. R. Campagna, P. J. Schmidt, and M.D.F., unpublished observations, January 2011). In contrast, Alk2 and Alk3 are abundantly expressed in hepatocytes.<sup>21</sup> To identify the type I BMP receptor required for the BMP-mediated regulation of hepatic hepcidin expression and iron homeostasis, we characterized the phenotypes of mice with liver-specific deletion of either the Alk2 or Alk3 receptor. In addition, we studied the impact of Alk2 or Alk3 deficiency on BMP signaling in isolated hepatocytes and on the response to iron challenge in mice.

## Methods

### Animals

All mouse experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. *Alk2*<sup>+/-</sup> mice on a mixed C57BL/6; SV129 background or *Alk3*<sup>+/-</sup> mice on a C57BL/6 background<sup>22,23</sup> were bred to B6.Cg-Tg<sup>(Alb-Cre)</sup>21Mgn/J mice (The Jackson Laboratory) to generate compound heterozygous animals that were intercrossed to obtain second-generation (F<sub>2</sub>) animals homozygous for the *Alk2*<sup>fl</sup> or *Alk3*<sup>fl</sup>

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alleles with or without the *Alb-Cre* transgene. Mice were fed a standard, iron-replete diet (Prolab 5P75 Isopro 3000, 380 ppm iron). Twelve-week-old female mice were used for phenotypic characterization, and 8- to 12-week-old female mice were used in iron challenge experiments.

For studies of the impact of iron challenge on hepcidin gene expression, mice were injected with 0.2 g/kg dextran or iron dextran (Sigma-Aldrich) via a tail vein, as described previously,<sup>11</sup> and blood and tissue samples were harvested 2 hours after injection.

### Hematologic and iron parameters

Blood for serum iron parameters and complete blood count was obtained by retro-orbital puncture. Serum iron concentrations and transferrin saturations were determined using Iron/UIBC Kit (Thermo Scientific). Complete blood counts were measured using a HemaVet Veterinary Analyzer (Heska). After mice were killed, liver, kidney, and spleen tissues were harvested, and non-heme tissue iron levels were determined, as previously described.<sup>13</sup>

### Hepatic mRNA levels

Total RNA was extracted from mouse hepatocytes and liver tissues using Trizol (Invitrogen). Reverse RNA transcription was accomplished using MMLV-RT (Promega). Quantitative RT-PCR was performed using SYBR Green (Thermo Fisher Scientific) or Kapa Probe (for TaqMan primers) on a Mastercycler Realplex2 (Eppendorf) using primers listed in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The level of target transcripts was normalized to the level of 18S rRNA using the relative  $C_T$  method.

### Prussian blue staining and diaminobenzidine enhancement

Prussian blue staining was performed on paraffin-embedded sections, as previously described.<sup>13</sup> To enhance iron staining, paraffin-embedded hepatic sections from *Alk2<sup>fl/fl</sup>* and *Alk2<sup>fl/fl</sup>; Alb-Cre* mice were stained with Prussian blue and then incubated with diaminobenzidine for 2 minutes at room temperature, yielding a brown reaction product. Sections were analyzed by light microscopy with a Nikon Eclipse 80i microscope (Nikon Instruments).

### Primary hepatocyte cultures

Hepatocytes were isolated and cultured for 2 days using a bottom and top layer of collagen, called a “collagen sandwich,” as described previously.<sup>24</sup> After serum starvation for 6 hours in 0.1% FBS, hepatocytes were stimulated with and without BMP2 (100 ng/mL), and RNA was harvested 2 hours later.

### Statistics

All values were expressed as mean  $\pm$  SEM. Data were analyzed using the Student *t* test and 1-way ANOVA with Bonferroni posthoc tests, when applicable. Statistical significance was considered for *P* values < .05.

## Results

### Liver-specific deletion of Alk2 and Alk3 leads to iron overload in mice

Mice that are globally deficient in either Alk2 or Alk3 are not viable.<sup>25-28</sup> Therefore, to characterize the potential roles of each BMP type I receptor in the regulation of hepatocellular hepcidin expression and systemic iron metabolism, we studied mice with liver-specific deletion of Alk2 or Alk3. To delete Alk2 or Alk3 selectively in hepatocytes, we generated mice homozygous for conditionally targeted (“floxed”) Alk2 (*Alk2<sup>fl/fl</sup>*) or Alk3 (*Alk3<sup>fl/fl</sup>*) alleles that also carried a Cre recombinase transgene under the control of the albumin gene promoter (*Alb-Cre*).<sup>29</sup>

Alk2 and Alk3 mRNA levels were markedly reduced in the livers of *Alk2<sup>fl/fl</sup>; Alb-Cre* mice and *Alk3<sup>fl/fl</sup>; Alb-Cre* mice, respec-

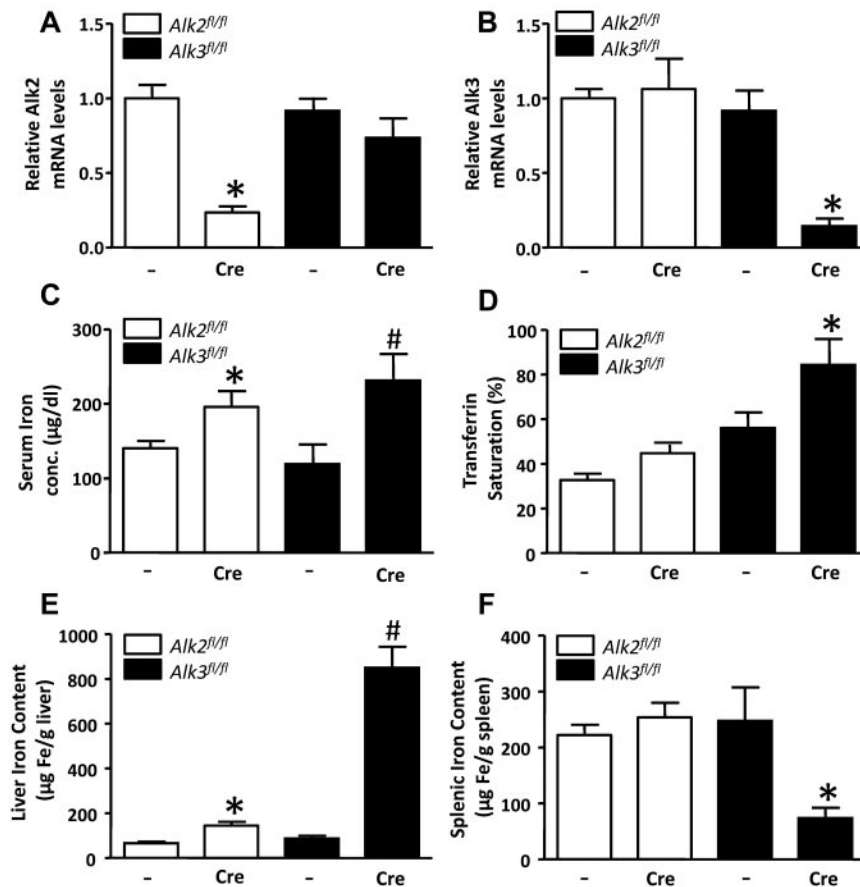
tively (Figure 1A-B). Serum iron levels were higher in *Alk2<sup>fl/fl</sup>; Alb-Cre* mice than in *Alk2<sup>fl/fl</sup>* control mice without the Cre transgene, but transferrin saturation did not differ (Figure 1C-D). In contrast, both serum iron level and transferrin saturation were higher in *Alk3<sup>fl/fl</sup>; Alb-Cre* mice than in *Alk3<sup>fl/fl</sup>* controls (Figure 1C-D). Liver iron content was modestly greater in *Alk2<sup>fl/fl</sup>; Alb-Cre* mice than in *Alk2<sup>fl/fl</sup>* mice. In contrast, liver iron content was dramatically greater in *Alk3<sup>fl/fl</sup>; Alb-Cre* mice than in *Alk3<sup>fl/fl</sup>* controls (Figure 1E). Splenic iron content did not differ in *Alk2<sup>fl/fl</sup>; Alb-Cre* and *Alk2<sup>fl/fl</sup>* mice but was markedly less in *Alk3<sup>fl/fl</sup>; Alb-Cre* than in *Alk3<sup>fl/fl</sup>* mice (Figure 1F). Of note, the ratio of liver iron content to splenic iron content was increased in both *Alk2<sup>fl/fl</sup>; Alb-Cre* mice and *Alk3<sup>fl/fl</sup>; Alb-Cre* mice (supplemental Figure 1). Kidney tissue iron content was also greater in *Alk3<sup>fl/fl</sup>; Alb-Cre* mice than in *Alk3<sup>fl/fl</sup>* mice (supplemental Figure 2A). There were no hematologic abnormalities suggestive of the presence of a secondary, anemia-induced iron overload phenotype in mice with liver-specific Alk2 or Alk3 deficiency (supplemental Figure 3). Moreover, hepatic expression of hereditary hemochromatosis disease-causing genes, including transferrin receptor-2 (*Tfr2*),<sup>30</sup> *Hfe*,<sup>31,32</sup> hemojuvelin (*Hjuv*),<sup>9,13,14,33</sup> and ferroportin 1 (*Fpn1*),<sup>34</sup> did not differ between genotypes (supplemental Figure 4). These results demonstrate a mild iron overload phenotype in *Alk2<sup>fl/fl</sup>; Alb-Cre* mice and a severe iron overload phenotype in *Alk3<sup>fl/fl</sup>; Alb-Cre* mice and indicate that both Alk2 and Alk3 have roles in the regulation of systemic iron homeostasis.

### Histologic staining reveals periportal hepatocellular iron accumulation in *Alk2<sup>fl/fl</sup>; Alb-Cre* mice, but centrilobular hepatocellular iron accumulation in *Alk3<sup>fl/fl</sup>; Alb-Cre* mice

In recessive hemochromatosis syndromes, iron is deposited preferentially in periportal hepatocytes. In the livers of *Alk2<sup>fl/fl</sup>; Alb-Cre* mice, we observed low levels of iron accumulation with the typical periportal pattern (Prussian blue staining with diaminobenzidine enhancement; Figure 2A-B). In contrast, there was abundant iron accumulation in the livers of *Alk3<sup>fl/fl</sup>; Alb-Cre* mice (Prussian blue staining alone), and the iron deposition was most prominent in the centrilobular region (Figure 2C-D). In neither *Alk2<sup>fl/fl</sup>; Alb-Cre* nor *Alk3<sup>fl/fl</sup>; Alb-Cre* animals was there substantial iron present in Kupffer cells (Figure 2B,D), the resident macrophages of the liver that usually do not become overloaded with iron until the late stages of hemochromatosis disease progression.<sup>1</sup> Iron storage in splenic macrophages was reduced in *Alk3<sup>fl/fl</sup>; Alb-Cre* mice but not *Alk2<sup>fl/fl</sup>; Alb-Cre* mice (Figure 2F,H), consistent with the reduced splenic iron content that we observed in the former. Prussian blue staining also detected iron accumulation in the proximal renal tubules, as well as in the heart and pancreas of *Alk3<sup>fl/fl</sup>; Alb-Cre* mice (supplemental Figure 2B), but not in the tissues of *Alk2<sup>fl/fl</sup>; Alb-Cre* mice (data not shown).

### Impact of BMP type I receptor deletion on hepcidin, transferrin receptor 1 (*Tfrc*), BMP6, and *Id1* mRNA expression levels

Hepatocellular iron accumulation and macrophage iron depletion are the cellular hallmarks of systemic hepcidin deficiency. A mild reduction in hepatic hepcidin expression was observed in *Alk2<sup>fl/fl</sup>; Alb-Cre* mice, and a more marked reduction was seen in *Alk3<sup>fl/fl</sup>; Alb-Cre* mice (Figure 3A). As is typical in hepatic iron overload states,<sup>35,36</sup> we found reduced *Tfrc* and increased *Bmp6* mRNA levels in the livers of *Alk3<sup>fl/fl</sup>; Alb-Cre* mice with similar trends seen in *Alk2<sup>fl/fl</sup>; Alb-Cre* mice (Figure 3B-C). The expression of *Id1* was also reduced in *Alk3<sup>fl/fl</sup>; Alb-Cre* animals (Figure 3D). Taken



**Figure 1. Serum and tissue iron levels are increased in *Alk2*<sup>fl/fl</sup>, *Alb-Cre* and *Alk3*<sup>fl/fl</sup>, *Alb-Cre* mice.** (A) Hepatic *Alk2* mRNA levels were measured in *Alk2*<sup>fl/fl</sup> and *Alk2*<sup>fl/fl</sup>; *Alb-Cre* mice, and *Alk3*<sup>fl/fl</sup> and *Alk3*<sup>fl/fl</sup>; *Alb-Cre* mice. Mice carrying the Cre recombinase transgene are indicated by "Cre." \**P* < .0001, *Alk2*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk2*<sup>fl/fl</sup>. (B) Hepatic *Alk3* mRNA levels were measured in *Alk2*<sup>fl/fl</sup>, *Alk2*<sup>fl/fl</sup>; *Alb-Cre*, *Alk3*<sup>fl/fl</sup> and *Alk3*<sup>fl/fl</sup>; *Alb-Cre* mice. \**P* = .001, *Alk3*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk3*<sup>fl/fl</sup>. (C) Serum iron concentration measured in all 4 genotypes (*n* ≥ 8 per group). \**P* = .04, *Alk2*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk2*<sup>fl/fl</sup>. #*P* = .04, *Alk3*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk3*<sup>fl/fl</sup>. (D) Transferrin saturation measured in all 4 genotypes (*n* ≥ 8 per group). \**P* = .054, *Alk2*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk2*<sup>fl/fl</sup>. \**P* = .049, *Alk3*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk3*<sup>fl/fl</sup>. (E) Liver iron content measured in all 4 genotypes (*n* ≥ 8 per group). \**P* = .0025, *Alk2*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk2*<sup>fl/fl</sup>. #*P* = .0005, *Alk3*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk3*<sup>fl/fl</sup>. (F) Spleen iron content measured in all 4 genotypes (*n* ≥ 8 per group). \**P* = .04; *Alk3*<sup>fl/fl</sup>; *Alb-Cre* versus *Alk3*<sup>fl/fl</sup>.

together, the observations that mice with liver-specific deficiency of BMP type I receptors have reduced hepcidin expression and iron overload in the context of increased *Bmp6* gene expression suggest that the ability to respond to BMP signaling is impaired in mice with hepatic *Alk2* or *Alk3* deficiency.

#### Impact of BMP type 1 receptor deficiency on the ability of BMP signaling to modulate hepatic hepcidin gene expression

To further evaluate the impact of deleting *Alk2* or *Alk3* on the regulation of hepcidin gene expression, we measured basal hepcidin expression, as well as the ability of BMP ligands to induce hepcidin gene expression, in primary hepatocytes isolated from animals of each of the 4 genotypes. In the absence of exogenous BMP ligand, hepcidin mRNA levels did not differ in *Alk2*<sup>fl/fl</sup>; *Alb-Cre* and *Alk2*<sup>fl/fl</sup> hepatocytes (Figure 4A). However, basal hepcidin expression was substantially reduced in *Alk3*<sup>fl/fl</sup>; *Alb-Cre* hepatocytes compared with *Alk3*<sup>fl/fl</sup> control hepatocytes (Figure 4B). Taken together with our *in vivo* data, these results suggest that *Alk3* is the type I BMP receptor responsible for basal hepcidin expression in hepatocytes.

In isolated control hepatocytes, BMP2 more robustly increased hepcidin mRNA levels than did BMP6 (data not shown). We therefore used BMP2 to investigate the effect of hepatic *Alk2* and *Alk3* deficiency on the regulation of hepcidin gene expression by BMP signaling. BMP2 stimulated hepcidin expression in *Alk2*<sup>fl/fl</sup> and *Alk3*<sup>fl/fl</sup> control hepatocytes, but not in hepatocytes of either *Alk2*<sup>fl/fl</sup>; *Alb-Cre* or *Alk3*<sup>fl/fl</sup>; *Alb-Cre* mice. These results suggest that the presence of both *Alk2* and *Alk3* is required for the induction of hepcidin gene expression by BMP2.

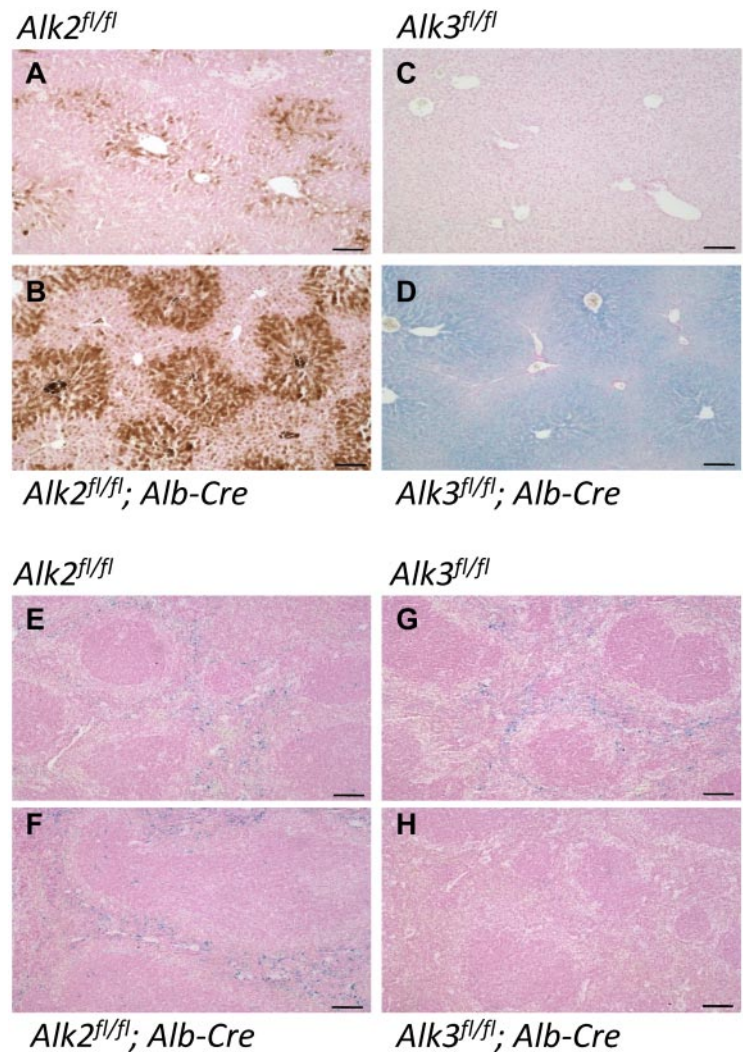
#### Influence of BMP type I receptor deficiency on the ability of iron challenge to induce hepatic hepcidin gene expression

We<sup>11</sup> and others<sup>8,9,10,15</sup> have previously reported that iron challenge induces hepcidin gene expression in a BMP-dependent manner. To test whether iron requires *Alk2*, *Alk3*, or both to increase hepcidin expression, we measured hepatic hepcidin mRNA levels in mice challenged with iron dextran (Figure 5). After iron administration, serum iron levels and transferrin saturations were similar in mice of all 4 genotypes (supplemental Figure 5A-D), and iron challenge did not alter hepatic *Alk2* or *Alk3* mRNA levels (supplemental Figure 5E-H). In both *Alk2*<sup>fl/fl</sup> and *Alk3*<sup>fl/fl</sup> animals, iron challenge increased hepcidin mRNA levels ~3-fold (Figure 5). Similarly, we observed that iron challenge induced *Id1* gene expression (supplemental Figure 5I-J). Iron challenge did not increase hepcidin or *Id1* mRNA levels in mice with hepatocyte-specific *Alk2* or *Alk3* deficiency. These studies of iron-challenged mice, like our studies of BMP2-stimulated hepatocytes, suggest that both of these BMP type I receptors are required for induction of hepcidin gene expression in response to BMP signaling.

## Discussion

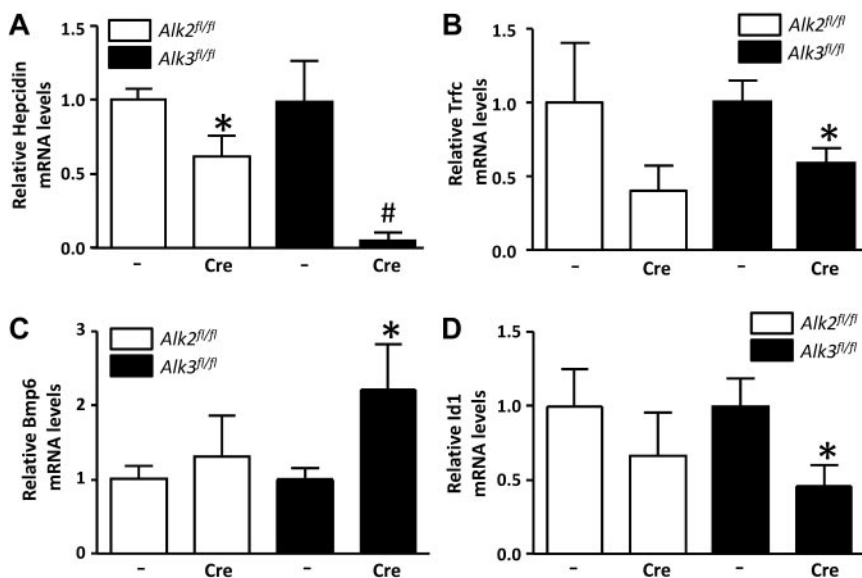
The study of hereditary hemochromatosis and iron overload disorders in humans and mice has identified a critical role for BMP signaling in the regulation of systemic iron homeostasis. However, the specific type I BMP receptors required for iron homeostasis were unknown. In the present study, we report that hepatocyte-specific deficiency of either *Alk2* or *Alk3* causes systemic iron

**Figure 2.** Iron deposition in liver and spleen in *Alk2<sup>fl/fl</sup>*, *Alk2<sup>fl/fl</sup>*; *Alb-Cre*, *Alk3<sup>fl/fl</sup>* and *Alk3<sup>fl/fl</sup>*; *Alb-Cre* mice. Formalin-fixed, paraffin-embedded liver (A-D) and spleen (E-H) sections prepared from *Alk2<sup>fl/fl</sup>*, *Alk2<sup>fl/fl</sup>*; *Alb-Cre*, *Alk3<sup>fl/fl</sup>*, and *Alk3<sup>fl/fl</sup>*; *Alb-Cre* mice were stained with Prussian blue and diaminobenzidine (A-B) or Prussian blue alone (C-H). Sections were prepared from at least 4 animals in each group; representative photomicrographs are shown. Scale bars represent 100  $\mu$ m.

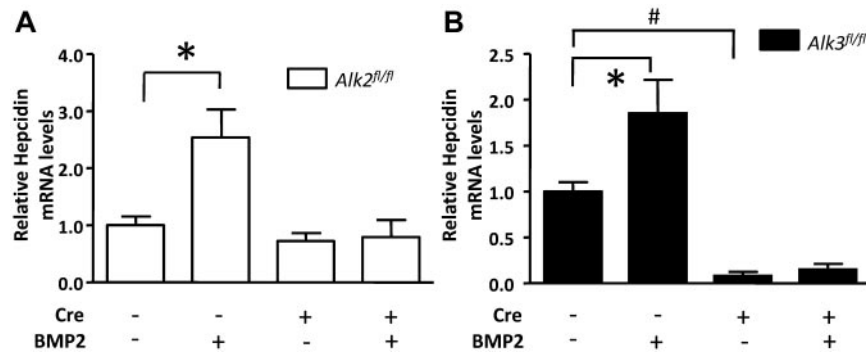


overload and blocks the ability of BMP ligands and iron to induce hepatic hepcidin expression. The iron overload phenotype was more severe in *Alk3<sup>fl/fl</sup>*; *Alb-Cre* than in *Alk2<sup>fl/fl</sup>*; *Alb-Cre* mice,

probably because of a marked reduction in basal BMP signaling and hepcidin expression in *Alk3<sup>fl/fl</sup>*; *Alb-Cre* mice. These findings suggest that Alk3 is the type I BMP receptor responsible for basal



**Figure 3.** Hepatic hepcidin, transferrin receptor 1 (Tfrc), Bmp6, and Id1 mRNA levels are decreased in *Alk3<sup>fl/fl</sup>*; *Alb-Cre* mice at the age of 12 weeks. (A) Hepatic hepcidin mRNA levels were determined by quantitative RT-PCR in *Alk2<sup>fl/fl</sup>*, *Alk2<sup>fl/fl</sup>*; *Alb-Cre*, *Alk3<sup>fl/fl</sup>*, and *Alk3<sup>fl/fl</sup>*; *Alb-Cre* mice ( $n \geq 7$  per group). \* $P = .03$ , *Alk2<sup>fl/fl</sup>*; *Alb-Cre* versus *Alk2<sup>fl/fl</sup>*. # $P = .02$ , *Alk3<sup>fl/fl</sup>*; *Alb-Cre* versus *Alk3<sup>fl/fl</sup>*. (B) Hepatic Tfrc ( $n \geq 7$  per group). \* $P = .04$ , *Alk3<sup>fl/fl</sup>*; *Alb-Cre* versus *Alk3<sup>fl/fl</sup>*. (C) Bmp6 ( $n \geq 7$  per group). \* $P = .049$ , *Alk3<sup>fl/fl</sup>*; *Alb-Cre* versus *Alk3<sup>fl/fl</sup>*. (D) Id1 ( $n \geq 7$  per group). \* $P = .04$ , *Alk3<sup>fl/fl</sup>*; *Alb-Cre* versus *Alk3<sup>fl/fl</sup>*. mRNA levels were also measured.



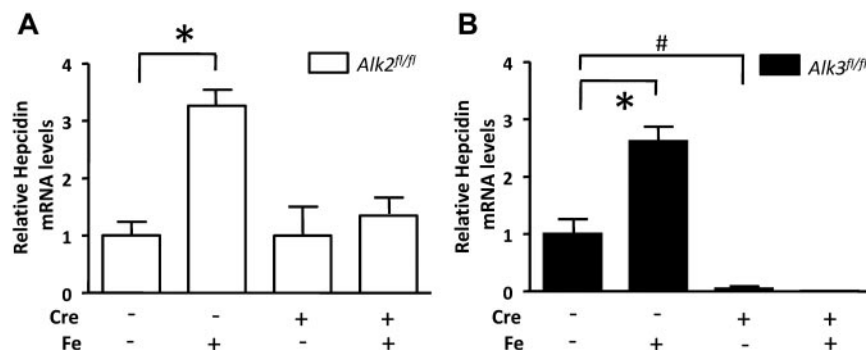
**Figure 4. Alk2 and Alk3 are required for BMP2-mediated induction of hepcidin gene expression and loss of Alk3 suppresses basal hepcidin mRNA levels in isolated hepatocytes.** Isolated hepatocytes were treated with and without BMP2 (100 ng/mL) for 2 hours ( $n = 5$  hepatocyte isolations from *Alk2*<sup>fl/fl</sup> mice;  $n = 3$  hepatocyte isolations from *Alk2*<sup>fl/fl</sup>; *Alb-Cre*, *Alk3*<sup>fl/fl</sup> and *Alk3*<sup>fl/fl</sup>; *Alb-Cre* mice, each). (A) Hepcidin mRNA levels in *Alk2*<sup>fl/fl</sup> and *Alk2*<sup>fl/fl</sup>; *Alb-Cre* hepatocytes. \* $P = .0075$ , *Alk2*<sup>fl/fl</sup> without versus with BMP2. (B) Hepcidin mRNA levels in similarly treated *Alk3*<sup>fl/fl</sup> and *Alk3*<sup>fl/fl</sup>; *Alb-Cre* hepatocytes. \* $P < .05$ , *Alk3*<sup>fl/fl</sup> without versus with BMP2. # $P < .0001$  *Alk3*<sup>fl/fl</sup>; *Alb-Cre* without BMP2 versus *Alk3*<sup>fl/fl</sup> without BMP2.

hepcidin expression in hepatocytes. In contrast, both Alk2 and Alk3 were required for the induction of hepcidin by BMP and iron. These results demonstrate that Alk2 and Alk3 have nonredundant roles in iron homeostasis.

The iron overload phenotype and reduction in basal hepcidin mRNA levels were more marked in *Alk3*<sup>fl/fl</sup>; *Alb-Cre* mice than in *Alk2*<sup>fl/fl</sup>; *Alb-Cre* mice. An increase in hepatic BMP6 mRNA levels was detected in mice with hepatic-specific Alk3 deficiency, but not in mice with hepatocyte-specific Alk2 deficiency. Potential explanations for the difference in severity of iron overload in Alk2- versus Alk3-deficient livers include the possibility that Alk3 is more highly expressed in hepatocytes than is Alk2 or that Alk3 is more sensitive to the BMPs present in the hepatic milieu.

Experiments in isolated hepatocytes and in intact mice revealed that both BMP type I receptors, Alk2 and Alk3, are required for the induction of hepatic hepcidin gene expression by BMP2 or iron. Intriguingly, Little and Mullins<sup>37</sup> have recently demonstrated that the zebrafish orthologs of the Alk2 and Alk3 receptors have nonredundant roles in dorsal-ventral axis specification. Moreover, the 2 BMP type I receptors in zebrafish assemble into heteromeric BMP receptor complexes, which appear to be more sensitive to BMP ligand heterodimers. It is conceivable that similar heteromeric receptor complexes containing both Alk2 and Alk3 are required for BMP ligands and iron to induce hepcidin gene expression in hepatocytes. Additional studies will be necessary to investigate the possibility that Alk2 and Alk3 can form heteromeric complexes in mammalian hepatocytes.

The pattern of iron deposition varies in the different types of hemochromatosis and experimental iron overload models. For example, in hemochromatosis resulting from autosomal dominant mutations in the gene encoding Fpn1, iron is preferentially deposited in Kupffer cells. In contrast, in the more common autosomal recessive forms of hemochromatosis, including those attributable to mutations in the *Hfe* and *Tfr2* genes, iron is preferentially deposited in periportal hepatocytes.<sup>1,38,39</sup> It has been hypothesized that the periportal distribution of iron seen in most autosomal recessive forms of hemochromatosis is attributable to a first pass effect of intestinally-absorbed iron carried by the portal venous system.<sup>40</sup> In contrast, in hepcidin<sup>4-</sup> and BMP6<sup>16-</sup>deficient mice, iron deposition is predominantly centrilobular. Moreover, Kautz et al<sup>41,42</sup> reported that BMP6 immunoreactivity was abundant in centrilobular hepatocytes of mice with iron overload induced by an iron-rich diet or *Hfe* deficiency, both of which are characterized by periportal iron deposition. We observed modest iron deposition in the periportal region of mice with liver-specific Alk2 deficiency. In contrast, abundant centrilobular iron deposition was found in mice with liver-specific Alk3 deficiency. Our findings of centrilobular iron deposition in mice with hepatocyte-specific Alk3 deficiency, together with those made in hepcidin<sup>4-</sup> and BMP6<sup>16-</sup>deficient mice, strongly suggest that the distribution of hepatic iron in overload states is not the result of a “passive” first-pass effect. Instead, it appears that hepatic iron deposition may be an active process that is regulated, at least in part, by BMP signaling.



**Figure 5. Alk2 and Alk3 are essential for iron-dependent hepcidin regulation in vivo.** Eight-week-old mice were challenged intravenously with dextran (as a control) or iron-dextran (Fe; 0.2 mg/kg). (A) Hepatic hepcidin mRNA levels in *Alk2*<sup>fl/fl</sup> and *Alk2*<sup>fl/fl</sup>; *Alb-Cre* mice ( $n \geq 6$  per group). \* $P < .0001$ , *Alk2*<sup>fl/fl</sup> mice challenged with dextran versus *Alk2*<sup>fl/fl</sup> mice challenged with iron-dextran. (B) Hepatic hepcidin mRNA levels in similarly challenged *Alk3*<sup>fl/fl</sup> and *Alk3*<sup>fl/fl</sup>; *Alb-Cre* mice ( $n \geq 3$  per group). \* $P < .0045$ , versus *Alk3*<sup>fl/fl</sup> mice challenged with dextran versus *Alk3*<sup>fl/fl</sup> mice challenged with iron-dextran. # $P < .005$ , *Alk2*<sup>fl/fl</sup>; *Alb-Cre* mice challenged with dextran versus *Alk3*<sup>fl/fl</sup> mice challenged with dextran.

Under physiologic conditions, hepcidin degrades Fpn1, the sole iron exporter in the body. Interestingly, Zhang et al<sup>43</sup> and Ramey et al<sup>44</sup> showed that Fpn1 is predominantly localized in the periportal region. Ramey et al hypothesized that in iron overload models associated with very low or absent hepcidin levels, high periportal Fpn1 expression protects the periportal region from iron overload, resulting in a centrilobular distribution of iron deposition.<sup>44</sup> In contrast, in iron overload states associated with relatively preserved hepcidin expression, lower Fpn1 levels are less able to protect the periportal region from iron deposition.

Our observations appear to be consistent with the hypothesis put forward by Ramey et al.<sup>44</sup> Hepcidin levels are very low at baseline in Alk3-deficient hepatocytes, probably leading to abundant periportal Fpn1 levels and relative sparing of the periportal region and accumulation of iron in the centrilobular region. In contrast, hepcidin levels are normal or only modestly reduced in Alk2-deficient hepatocytes, probably reducing periportal Fpn1 levels and leading to periportal iron accumulation.

Our findings have several therapeutic implications. First, BMP signaling inhibitors that can increase serum iron levels represent an attractive therapeutic strategy for the treatment of ACD.<sup>11,20,45</sup> The observation that Alk3 plays a critical role in basal hepcidin gene expression suggests that strategies that target Alk3 will reduce hepcidin expression and increase serum iron levels. Alk3-specific inhibitors may be superior to nonselective BMP signaling inhibitors for treating ACD because the former would not be expected to inhibit the diverse effects mediated by other type I BMP receptors.

In conclusion, we report that hepatocyte-specific deficiency of either Alk2 or Alk3 caused systemic iron overload and blocked the ability of iron and BMP ligands to induce hepatic hepcidin expression in mice. The iron overload phenotype was more severe in *Alk3<sup>fl/fl</sup>; Alb-Cre* than in *Alk2<sup>fl/fl</sup>; Alb-Cre* mice, probably because of a marked reduction in basal BMP signaling and hepcidin gene expression in the former. In contrast, both Alk2 and Alk3 were required for the induction of hepcidin by BMP and iron. Taken together, these data indicate partially overlapping but nonredundant

functions for these BMP type I receptors in hepatocellular regulation of systemic iron metabolism.

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## Authorship

Contribution: A.U.S., L.K.L., P.L., C.M., S.M.K., and A.E.P. performed experiments; A.U.S., T.B.B., L.K.L., P.L., C.M., S.M.K., A.E.P., M.D.F., and K.D.B. analyzed data; A.U.S., T.B.B., R.T.P., P.B.Y., D.B.B., M.D.F., and K.D.B. supervised the experimental design; and A.U.S., T.B.B., M.D.F., D.B.B., and K.D.B. wrote the manuscript.

Conflict-of-interest disclosure: The Massachusetts General Hospital has filed patents related to the use of small molecule inhibitors of BMP signaling to modulate iron metabolism, and P.B.Y., R.T.P., and K.D.B. may be eligible to receive royalties. The remaining authors declare no competing financial interests.

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