The hemochromatosis protein HFE signals predominantly via the BMP type I receptor ALK3 in vivo

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Mutations in HFE, the most common cause of hereditary hemochromatosis, lead to iron overload. The iron overload is characterized by increased iron uptake due to lower levels of the hepatic, iron regulatory hormone hepcidin. HFE was cloned 21 years ago, but the signaling pathway is still unknown. Because bone morphogenetic protein (BMP) signaling is impaired in patients with hereditary hemochromatosis, and the interaction of HFE and the BMP type I receptor ALK3 was suggested in vitro, in vivo experiments were performed. In vivo, hepatocyte-specific Alk3-deficient and control mice were injected with either AAV2/8-Hfe-Flag or PBS. HFE overexpression in control mice results in increased hepatic hepcidin levels, p-Smad1/5 levels, and iron deficiency anemia, whereas overexpression of HFE in hepatocyte-specific Alk3-deficient mice results in no change in hepcidin, p-Smad1/5 levels, or blood parameters. These results indicate that HFE signals predominantly via ALK3 to induce hepcidin in vivo.
**Results**

**HFE interacts with ALK3 but not with ALK2 in vitro.** HFE could affect BMP signaling by directly interacting with the BMP type I receptor. Previous studies demonstrated that ALK3 and to a lesser extent ALK2 were critical to maintain iron homeostasis in mice. Wu et al. showed that HFE co-precipitated with ALK3 suggesting that ALK3 interacts with HFE in vitro. We performed co-immunoprecipitation of tagged receptors and confirmed the interaction of ALK3 with HFE in vitro (Supplementary Fig. 1a). Because the BMP type I receptor ALK2 is also expressed in the liver and is required for optimal hepcidin induction, co-immunoprecipitation of HFE with ALK2 was performed. HFE failed to co-immunoprecipitate with ALK2 in Huh7 cells (Supplementary Fig. 1b). These results indicate that ALK3, but not ALK2, does detectably interact with HFE in vitro.

**HFE is overexpressed in mice injected with AAV2/8-HFE-Flag.** To address whether the effect of HFE on hepcidin expression is dependent on the expression of ALK3 in vivo, mice with hepatocyte-specific Alk3 deficiency (Alk3<sup>fl/fl</sup>; Alb-Cre<sup>18</sup>) and their appropriate controls (Alk3<sup>fl/fl</sup>; Alb-Cre<sup>18</sup>) were injected with an adenovirus (AAV) encoding Hfe-Flag under the control of a liver-specific promotor (AAV2/8-Hfe-Flag) and compared to animals injected with vehicle (PBS). Fourteen days after virus administration, blood and tissues were harvested and analyzed. The absence of inflammation, efficiency of knockdown, and effectiveness of HFE expression were verified. As previously shown, the AAV2/8 virus itself does not cause an inflammatory response, which could result in the induction of hepcidin mRNA independent of the iron status. Consistently, mice expressed similar levels of IL-6 mRNA (Supplementary Fig. 2a). Hepatocyte-specific Alk3-deficient mice presented with a reduction of Alk3 mRNA levels by 90% when compared to control mice (Fig. 1a). In mice with and without hepatocyte-specific Alk3 deficiency injected with AAV2/8-Hfe-Flag, hepatic Hfe mRNA levels were increased to similar extents (Fig. 1b).

HFE-Flag was detected in livers of animals injected with AAV2/8-Hfe-Flag (Fig. 1c) and in membrane-enriched fractions of the liver (Fig. 1d, Supplementary Fig. 2b). The data indicate that Alk3<sup>fl/fl</sup>; Alb-Cre mice were deficient for hepatic Alk3 and that all mice injected with AAV2/8-Hfe-Flag were successfully overexpressing HFE after 14 days.

**HFE overexpression caused anemia in control mice.** Increased HFE expression in wild-type (WT) mice results in increased, phosphorylation of Smad 1/5/8, which induces hepcidin expression. The induction of hepcidin in WT mice leads to anemia. We used mice with and without hepatocyte-specific Alk3 deficiency injected either with AAV2/8-Hfe-Flag or PBS to determine whether HFE induction of hepcidin is dependent on ALK3 expression in vivo. Control mice (Alk3<sup>fl/fl</sup>; Alb-Cre) injected with AAV2/8-Hfe-Flag developed normocytic anemia when compared to PBS-injected animals. Hemoglobin levels, transferrin saturation, and serum iron levels were reduced (Fig. 2a–c). The mean corpuscular volume (MCV) was within a similar range in mice injected with AAV2/8-Hfe-Flag compared to PBS-injected controls (Fig. 2d).

Hepatocyte-specific deficiency of the BMP type I receptor Alk3 (Alk3<sup>fl/fl</sup>; Alb-Cre) causes an imbalance of the systemic iron homeostasis and hence iron overload, as published previously. Mice with hepatocyte-specific Alk3 deficiency injected with PBS presented with higher hemoglobin levels, serum iron levels, and transferrin saturation, and a similar MCV compared to control mice injected with PBS. In contrast to Alk3<sup>fl/fl</sup>; Alb-Cre mice, mice with hepatocyte-specific Alk3 deficiency were unresponsive to the overexpression of Hfe-Flag as they did not develop anemia or present with a reduced iron status. Hemoglobin levels, serum iron levels, and transferrin saturation remained high when compared to mice with hepatocyte-specific Alk3 deficiency injected with PBS (Fig. 2a–c). The data indicate that HFE overexpression leads to the development of anemia in control mice. However, HFE overexpression in mice lacking Alk3 showed no changes in serum iron, Tf-saturation or red blood cell analysis indicating the importance of Alk3 expression on the HFE induced changes in iron homeostasis.
To further test the relationship of HFE and ALK3 in controlling iron homeostasis, non-heme tissue iron levels were measured.

Mice with hepatocyte-specific Alk3 deficiency developed iron overload as indicated by increased hepatic, renal, and cardiac iron content. Splenic iron content was decreased as expected in states of iron overload (Fig. 3a–d). Overexpression of HFE did not change hepatic, renal, cardiac, or splenic iron content in mice with hepatocyte-specific Alk3 deficiency. In contrast, control mice injected with AAV2/8-Hfe-Flag developed anemia, and, as a consequence, retained more iron in the spleen compared to PBS-injected controls (Fig. 3d).

These data reveal that hepatic, renal, and cardiac tissue iron loading were not affected by HFE overexpression for 2 weeks and that ALK3 is required for HFE-mediated iron regulation.

**ALK3 is required for HFE-mediated hepcidin induction.** Next, hepcidin and Id1 mRNA expression were analyzed. Like hepcidin, Id1 expression is increased by BMP signaling. In control mice injected with AAV2/8-Hfe-Flag, hepcidin mRNA expression levels increased fourfold when compared to PBS-injected animals (Fig. 4a). Interestingly, hepcidin mRNA levels in mice with hepatocyte-specific Alk3 deficiency did not respond to the overexpression of HFE: hepcidin mRNA levels remained low at a level of about 4% of that of the control mice (Fig. 4a, inlay). Id1 expression was increased in control animals overexpressing Hfe-Flag compared to PBS-injected animals. The result indicates that HFE overexpression increased BMP signaling. In contrast, hepatocyte-specific Alk3-deficient mice overexpressing HFE did not show an increase in Id1 gene expression (Fig. 4b) indicating that HFE and ALK3 are both required for BMP signaling.

Hepatic BMP6 mRNA levels were increased in hepatocytesspecific Alk3-deficient mice due to increased hepatic iron loading. Overexpression of HFE had no impact on BMP6 mRNA levels in control or hepatocyte-specific Alk3-deficient mice (Fig. 4c). The data show that HFE does not modulate the effective dose of BMP6 to ALK3.

At the protein level, immunoblot analysis revealed that phosphorylated Smad1/5 levels were increased in control animals overexpressing Hfe-Flag compared to animals injected with PBS (Fig. 5a–c, Supplementary Fig. 3a–c). In contrast, hepatocyte-specific Alk3-deficient mice did not increase hepatic pSmad1/5 levels after HFE overexpression (Fig. 5a–c, Supplementary Fig. 3a–c). The data indicate that HFE overexpression induced Sma1/5 phosphorylation, hepcidin mRNA expression, and development of anemia.

In contrast, the parameters in mice with a hepatocyte-specific deficiency of Alk3 remained unchanged indicating the necessity of ALK3 expression for HFE to exert its effects on BMP signaling.

**Discussion**

Mutations in the HFE gene reduce hepcidin expression in the liver, thus causing iron overload. Previous studies suggested that HFE may regulate hepcidin expression through the BMP
Fig. 2 HFE overexpression caused anemia in control mice, but not in mice with hepatocyte-specific Alk3 deficiency. Hematological and iron parameters were analyzed 14 days after AAV2/8-Hfe-Flag or PBS injection in mice with and without hepatocyte-specific Alk3 deficiency. a Hemoglobin levels (Alk3fl; n = 3; Alk3fl; Alb-Cre: n = 6; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 5; *p ≤ 0.0238), b serum iron levels (Alk3fl; n = 6; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 7; **p ≤ 0.0095), c transferrin saturation (Alk3fl; n = 6; Alk3fl; Alb-Cre: n = 6; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 7; **p ≤ 0.0095), d splenic iron content (Alk3fl; n = 3; Alk3fl; Alb-Cre: n = 5; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 5; *p = 0.0159) are shown.

Fig. 3 Hepatic, cardiac, and renal iron content remained similar, while splenic iron content increased in control mice overexpressing HFE. Tissue iron content was determined 14 days after virus or vehicle administration. a Liver iron content (Alk3fl; n = 5; Alk3fl; Alb-Cre: n = 6; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 8; **p ≤ 0.004), b renal iron content (Alk3fl; n = 5; Alk3fl; Alb-Cre: n = 6; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 8; **p ≤ 0.009), c cardiac iron content (Alk3fl; n = 6; Alk3fl; Alb-Cre: n = 6; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 8; *p = 0.0152; **p = 0.004), and d splenic iron content (Alk3fl; n = 6; Alk3fl; Alb-Cre: n = 5; Alk3fl injected with AAV2/8-Hfe-Flag: n = 4; Alk3fl; Alb-Cre injected with AAV2/8-Hfe-Flag: n = 8; *p = 0.038; **p = 0.004) of control mice and hepatocyte-specific Alk3-deficient mice injected with AAV2/8-Hfe-Flag or PBS are shown.
Fig. 4  Hepcidin expression was induced by HFE overexpression in control mice. ** In control animals, overexpression of HFE led to increased hepatic hepcidin mRNA expression, while there was no effect in hepatocyte-specific Alk3 deficient mice (Alk3^{fl/fl}; Alb-Cre: n = 6; Alk3^{fl/fl} injected with AAV2/8-HFe-Flag: n = 4; Alk3^{fl/fl}; Alb-Cre injected with AAV2/8-HFe-Flag: n = 8; **p ≤ 0.0095).  

Fig. 5  Overexpression of HFE leads to an increase in pSmad1/5 levels in control mice. a Hepatic phosphorylation of pSmad1/5, total Smad1 and α-Tubulin protein levels in AAV2/8-HFe-Flag, or PBS-injected mice with and without hepatocyte-specific Alk3 deficiency are shown. b Densitometric analysis of pSMAD1/5/Smad1 of immunoblots depicted in a is shown (Alk3^{fl/fl} mice injected with PBS vs. Alk3^{fl/fl} mice injected with AAV2/8-HFe-Flag: *p = 0.03; n = 3–4). c Densitometric analysis of pSMAD1/5/α-Tubulin of immunoblots depicted in a is shown (Alk3^{fl/fl} mice injected with PBS vs. Alk3^{fl/fl} mice injected with AAV2/8-HFe-Flag: *p = 0.03; n = 3–4)
signaling pathway, but the definitive in vivo evidence was lacking. This manuscript demonstrates for the first time to our knowledge that HFE failed to stimulate hepcidin expression by the liver in the absence of the BMP type I receptor ALK3. The results provide unequivocal evidence in vivo that HFE acts through ALK3 to affect the BMP signaling pathway, which controls hepcidin expression.

Other investigators have previously reported an interaction of HFE and the BMP signaling pathway: Kautz and colleagues speculated that HFE and the BMP receptors may be associated at the membrane and required to induce BMP signaling. \textsuperscript{16} Phosphorylated Smad, hepcidin, and Id1 levels were reduced in Hfe-deficient mice. \textsuperscript{16} Studies performed by Corradi et al. postulated that HFE induces hepcidin expression via an interaction with the BMP6–Smad signaling pathway. \textsuperscript{20,21} Our study shows the inability of mice with hepatocyte-specific Alk3 deficiency to respond to HFE overexpression. The mice are unable to increase hepcidin and Id1 expression or to develop anemia in contrast to control mice. Thus, the HFE-dependent increase in hepcidin expression is dependent on ALK3.

The iron-overload phenotype of mice with hepatocyte-specific Alk3 deficiency is more severe than the iron-overload phenotype of Hfe knockout mice, \textsuperscript{7,18} which indicates a dominant role for ALK3. As HFE signals via ALK3, HFE overexpression could not resolve the iron-overload phenotype in mice with hepatocyte-specific Alk3 deficiency caused by hepcidin deficiency. How HFE interacts with ALK3 to induce hepcidin in vivo remains to be resolved. Our data indicate that ALK3 and HFE interaction is independent of BMP6, because hepatic BMP6 mRNA levels were not affected by HFE overexpression over this time period. Wu et al. suggested that HFE stabilizes ALK3 at the plasma membrane by preventing its degradation and thereby increasing ALK3 cell surface expression in vitro. In vivo, deficiency of Hfe in mice led to a decrease in hepatic ALK3 protein. \textsuperscript{17} Another possibility is that HFE links ALK3 to the iron-sensing complex.

TR2, HFE, and HIV all interact in vitro. HFE could link TR2 to the HIV/BMP/BMPR/Smad signaling pathway. \textsuperscript{15} In conclusion, the data presented here argues for the first time to our knowledge that the effect of HFE on hepcidin expression is dependent on ALK3 expression in vivo, and acts through the BMP signaling pathway.

**Methods**

**Animals.** The current study was performed in accordance with the recommendations and approval of the institutional ethics committee of the North Rhine-Westphalian Agency for Nature, Environment, and Consumer Protection (permit no.: Az. 84-02.04.2014.A015). A Material Transfer Agreement for the use of mice was signed. Mice with homozygous loxP-flanked (“floxed”) Alk3 alleles (Alk3\textsuperscript{flox/flox}) on a C57BL/6 background with or without a Cre recombineer driven by the hepatocyte-specific albumin promoter \textsuperscript{12,23} were held in individually ventilated cages and fed a standard diet (198 ppm iron). Eight-week-old male mice with and without a hepatocyte-specific Alk3 deficiency were injected intravenously with either 5\texttimes10\textsuperscript{11} \textsuperscript{15} particles of an adeno-associated virus (AAV2/8) expressing Hje-Flag under the control of a liver-specific promoter (AAV2/8-Flag\textsuperscript{17}) (Vector Bio-Labs, Malvern, PA, USA) or PBS. PBS was used as a control, as Gao et al. have previously shown that an AAV-expressing GCDH (encoding glutaryl-CoA dehydrogenase, unrelated to iron homeostasis) did not induce hepcidin mRNA expression nor did it affect the iron status. \textsuperscript{19}

Two weeks later, euthanasia was performed in deep anesthesia, and blood and organs were collected for analysis.

**Cell culture.** Cells from the human hepatocellular carcinoma cell line HuH7 were a kind gift from Martina U. Muckenthaler (Heidelberg, Germany). Cells were maintained in high glucose DMEM (Sigma-Aldrich, Hamburg, Germany) supplemented with 10% FCS (heat inactivated, Capricorn, Ebsdorfergrund, Germany), 2 mM glutamine, 100 units mL\textsuperscript{-1} penicillin, and 100 µg mL\textsuperscript{-1} streptomycin (Sigma-Aldrich, Hamburg, Germany) at 37 °C and 5% CO\textsubscript{2}. Cells were tested negative for mycoplasma contamination (GATC, Konstanz, Germany).

**Hematologic and iron parameters.** All efforts were made to minimize suffering. Blood was withdrawn by puncture of the facial vein in deep ketamine/xylazine anesthesia. Complete blood count analysis was performed at the central laboratory of the University Hospital Muenster.

Serum iron concentrations and unsaturated binding capacity (UIBC) were measured using the Iron/UICB Kit from BioLabo (Maizy, France) according to the manufacturer’s instructions. Non-heme tissue iron levels were determined according to Torrance and Bothwell as described previously. \textsuperscript{20}

**Hepatic mRNA levels.** RNA was isolated from tissue samples with TriZol (Sigma-Aldrich, Hamburg, Germany) according to the manufacturer’s instructions. MMLV-reverse transcriptase (Sigma-Aldrich, Hamburg, Germany) was used to synthesize cDNA. Quantitative RT-PCR was performed on a Bio-Rad CFX Connect Real-Time-PCR system using either Taq" Universal SYBR" Green Supermix (BioRad, Munich, Germany) or TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany). Primer pairs used for RT-PCR are listed in Supplementary Table 1. Levels of target genes were normalized to 18S levels using the relative C\textsubscript{T} method.

**Preparation of plasma membrane-enriched fraction.** Samples were prepared as previously described. A volume of 200 mg of liver tissue was homogenized in 2.5 mL of 10 mM HEPES with a pH of 7.4 supplemented with 0.25 M sucrose, 5 mM EDTA and a protease inhibitor cocktail (Roche) for 3 × 10 s in a 6 mm Ultra-turrax. The homogenate was incubated on ice for 30 min and centrifuged at 400 \times g, 4 °C, for 10 min. The supernatant was centrifuged at 3000 \times g for 15 min, the 3000 \times g pellet was homogenized in 1 mL of 2 M NaCl in 10 mM HEPES. Another centrifugation at 3000 \times g for 15 min was performed. The pellet was again homogenized in 0.1 M sodium carbonate and incubated for 1 h while agitating. After centrifugation at 16,000 \times g for 1 h the pellet was homogenized in 1 mL of 10 mM HEPES with 4 M urea and incubated on ice for 30 min. The homogenate was centrifuged at 16,000 \times g. The final pellet was washed with 10 mM HEPES and re-suspended in 125 µL of 25 mM ammonium bicarbonate with 2% SDS. Protein samples were subsequently used for immunoblot analysis.

**Protein analysis.** Tissue samples were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, Hamburg, Germany). Proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany). An equal amount of proteins was separated by electrophoresis using 4–10% bis–tris gels and blotted on nitrocellulose membranes (GE Healthcare, Freiburg, Germany).

Membranes were incubated overnight with antibodies directed against Flag, c-Myc, α-Tubulin (Sigma-Aldrich, Hamburg, Germany), HFE (Santa-Cruz, Heidelberg, Germany), phosphorylated Smad 5 (Abcam, Cambridge, UK; named pSmad1/5 antibody because of cross-reactivity with pSmad1), total Smad1, E-Cadherin, and β-Catenin (Cell Signaling Technology, Leiden, The Netherlands).

Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit- or anti-mouse-IgG (Cell Signaling Technology, Leiden, The Netherlands), and chemiluminescence was detected using ECL-Plus and either the ChemiDoc XRS + system (BioRad, Munich, Germany) or Bio-Rad GS 800 scanner (both BioRad, Munich, Germany). Densitometric analysis was performed with the Image Lab software (BioRad, Munich, Germany) or ImageJ. Full-length and unedited versions of all immunoblots are depicted in Supplementary Figs. 4–9.

**Plasmids.** Eukaryotic expression plasmids encoding ALK2 or ALK3 fused to three copies of the FLAG epitope (3xFLAG-ALK3, 3xFLAG-ALK2; N-terminal) under the control of a CMV promotor were provided by Patricio Leyton and Donald Bloch (Boston, USA). The expression plasmid HFE-cMyc encoding HFE fused to the cMyc epitope at the N-terminus was provided by Martina U. Muckenthaler (Heidelberg, Germany).

**Co-immunoprecipitation analysis.** HuH7 were seeded at a density of 16 × 10\textsuperscript{5} cells per cm\textsuperscript{2}. Transfection was performed 16 h later using 15 µg of plasmid DNA and the TransIT-LT1 transfection reagent (MobiTec, Goettingen, Germany). Twenty-four hours after transfection, cells were harvested and lysed in NET-buffer. Protein samples were immunoprecipitated using ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich, Hamburg, Germany) according to the manufacturers’ instructions and subsequently used for immunoblot analysis.

**Statistical analysis.** Values are expressed at mean ± SD. The corresponding dot plots are overlaid. Data were analyzed with GraphPad Prism (GraphPad Software 6, La Jolla, USA) using non-parametric Mann Whitney U tests with a two-tailed p value. A p value of p ≤ 0.05 was considered statistically significant.

**Data availability.** The authors confirm that any data not included in the paper and its supplementary files are available from the corresponding author upon request.
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Author contributions

A.U.S. designed the research; L.T., C.A.E., J.K., and A.U.S. performed the experiments; L.T. and A.U.S. analyzed the data, prepared the figures, and wrote the manuscript.

Additional information

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