



HAL
open science

Hepcidin upregulation by inflammation is independent of Smad1/5/8 signaling by activin B Supplementary data

Céline Besson-Fournier, Aurélie Gineste, Chloé Latour, Ophélie Gourbeyre, Delphine Meynard, Patricia Martin, Eric Oswald, Hélène Coppin, Marie-Paule Roth

► To cite this version:

Céline Besson-Fournier, Aurélie Gineste, Chloé Latour, Ophélie Gourbeyre, Delphine Meynard, et al.. Hepcidin upregulation by inflammation is independent of Smad1/5/8 signaling by activin B Supplementary data. *Blood*, 2016, 129 (4), [Epub ahead of print]. 10.1182/blood-2016-10-748541 . inserm-01430303

HAL Id: inserm-01430303

<https://inserm.hal.science/inserm-01430303>

Submitted on 9 Jan 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Hepcidin upregulation by inflammation is independent of Smad1/5/8 signaling by activin B

Céline Besson-Fournier*¹, Aurélie Gineste*¹, Chloé Latour¹, Ophélie Gourbeyre¹,
Delphine Meynard¹, Patricia Martin^{1,2}, Eric Oswald^{1,2}, Hélène Coppin^{§1}, Marie-Paule Roth^{§1}

¹ IRSD, Université de Toulouse, INSERM, INRA, ENVT, UPS, Toulouse, France

² Laboratoire de Bactériologie-Hygiène, CHU Toulouse, Toulouse, France

*CBF and AG contributed equally to this manuscript

§HC and MPR contributed equally to this manuscript

Correspondence to: Hélène COPPIN & Marie-Paule ROTH
Institut de Recherche en Santé Digestive (IRSD)
Inserm U1220 Bat B
CHU Purpan
Place du Docteur Baylac
CS 60039
F-31024 Toulouse Cedex 3

e-mail: helene.coppin@inserm.fr & marie-paule.roth@inserm.fr
tel: (33) 5 62 74 45 08
fax: (33) 5 62 74 45 58

Word count: 1160

Number of figures: 2

Number of tables: 0

Number of references: 14

Scientific category: Red cells, iron and erythropoiesis

To the Editor:

Activin B, which is strongly induced by inflammatory stimuli in the mouse liver, has recently appeared as a potent inducer of hepcidin *in vitro*, via the crossactivation of non-canonical SMAD1/5/8 signaling^{1,2}. To confirm the cause and effect relationship between activin B, Smad1/5/8 phosphorylation, and hepcidin *in vivo*, we challenged *Inhbb*^{-/-} mice³ (deficient in activin B) with LPS or infected them with a *E. coli* septicemic strain, as indicated in Supplementary Methods.

To examine whether, as observed in human hepatoma cell lines and in mouse primary hepatocytes, activin B also stimulates canonical SMAD2/3 and non-canonical SMAD1/5/8 signaling *in vivo*, we compared by western-blot analysis Smad2 and Smad5 phosphorylation levels in the liver of wild-type and *Inhbb*^{-/-} mice four hours after LPS stimulation (Fig. 1A) or *E. coli* infection (Fig. 1B). Whereas these inflammatory stimuli strongly induce Smad2 and Smad5 phosphorylation in wild-type mice, none of these Smad effectors is activated in mice deficient for activin B. These data demonstrate that activation of Smad1/5/8 signaling in these mice directly results from the strong induction of activin B mRNA seen after LPS administration (Fig. 1C) or after *E. coli* infection (Fig. 1D).

In parallel to activating SMAD1/5/8 phosphorylation *in vitro*, activin B was also shown to induce hepcidin expression^{1,2}. Remarkably, pretreatment of hepatocytes with the BMP type I receptor inhibitor LDN-193189 prevented both the induction of SMAD1/5/8 phosphorylation and the upregulation of the hepcidin (*HAMP*) gene expression by activin B¹, suggesting that, at least *in vitro*, the effect of activin B on hepcidin expression was entirely attributable to the activation of non-canonical SMAD signaling. Since activin B also activates non-canonical Smad1/5/8 signaling *in vivo*, we expected that induction of hepcidin expression in response to LPS administration or to *E. coli* infection would be impaired in *Inhbb*^{-/-} mice. However, the magnitude of hepcidin mRNA induction and its evolution over time was unexpectedly similar in wild-type and in *Inhbb*^{-/-} mice challenged with LPS (Fig. 1E) or infected with *E. coli* (Fig. 1F), pointing out the limitations of *in vitro* studies. To confirm the data at the protein level, serum hepcidin was quantified by competitive ELISA in wild-type and in *Inhbb*^{-/-} mice before and 4 hours after a LPS-challenge. As suggested by the quantitative PCR data, and similarly to wild-type mice, *Inhbb*^{-/-} mice produce on average three times more hepcidin after endotoxin administration (Fig. 1G). These results show that neither activation of Smad1/5/8 signaling nor activin B induction are necessary for upregulation of hepcidin production by inflammatory stimuli such as LPS administration or *E. coli* infection. Hepcidin induction during inflammation was previously shown to be due at least in part to direct transcriptional regulation by the IL6/STAT3 pathway⁴⁻⁶. Four hours after challenge with LPS or infection with *E. coli*, Stat3 phosphorylation was similarly induced in wild-type

and in *Inhbb*^{-/-} mice (Fig. 1A & B), suggesting a preponderant role of Stat3 activation in hepcidin induction by these inflammatory stimuli.

Mice lacking the iron-inducible Smad1/5/8-activating ligand *Bmp6* have very low basal hepcidin levels⁷. However, they respond to LPS by inducing liver expression of *Inhbb* as much as do wild-type mice¹ and, as shown on Fig. 2A, this leads to a similar induction of Smad1/5/8 phosphorylation in the two categories of mice. Importantly, despite a marginally significant increase in the amount of circulating hepcidin 4 hours after a LPS challenge in *Bmp6*^{-/-} mice, the level reached after stimulation remains similar to that in unchallenged wild-type animals and about 3 times lower than that in LPS-challenged wild-type mice (Fig. 2B). These data highlight a lack of proportionality between Smad1/5/8 signaling and hepcidin production in the inflammatory context.

Circulating iron and tissue iron both activate the Smad1/5/8 signaling cascade in the hepatocyte, which leads to the induction not only of hepcidin mRNA but also of the mRNA of other targets such as *Id1* and *Smad7*^{8,9}. We therefore examined whether activation of Smad1/5/8 phosphorylation by inflammation was also accompanied by an induction of *Id1* and *Smad7* gene expression. As shown on Fig. 2C-F, neither LPS nor *E. coli* infection led to the induction of *Id1* or *Smad7* mRNA in the liver of either wild-type or *Inhbb*^{-/-} mice. *Id1* mRNA expression was strongly repressed at the earliest time point (2 hours) but returned to baseline at 4 hours (Fig. 2C & D). *Smad7* mRNA expression was also strongly repressed at 2 hours post LPS challenge or post *E. coli* infection, and remained below baseline for the whole timing (Fig. 2E & F). Notably, there were no differences in the kinetics of expression of these two genes between wild-type and *Inhbb*^{-/-} mice. These data point out major differences between Smad1/5/8 activation by iron and by inflammation. Indeed, whereas there is a good correlation between Smad1/5/8 activation by iron and induction of *Hamp*, *Id1*, and *Smad7* gene expression^{8,9}, such a concordance is totally lacking in the inflammatory context, which suggests that activation of Smad1/5/8 signaling by inflammatory stimuli could take place in non-parenchymal cells rather than in hepatocytes.

To determine whether activin B gene expression is induced in any type of inflammation, we infected mice with different pathogens (bacteria, parasite) and injected them with turpentine to cause sterile tissue abscess. As shown on Supplementary Fig. 1, liver *Inhbb* mRNA expression was induced not only in a mouse model of infection with the Gram-negative extracellular pathogenic bacteria *E. coli* but also in mice infected with the Gram-negative intracellular pathogenic bacteria *Salmonella enterica* serovar Typhimurium or the Gram-positive *Staphylococcus aureus*. However, despite notable induction of hepcidin (*Hamp*) mRNA, liver *Inhbb* mRNA expression was not significantly increased in the malaria

model induced by *Plasmodium berghei* K173-infected red blood cells nor in the sterile inflammation induced by turpentine. These data suggest that production of activin B by the liver is a biomarker of bacterial infection rather than a key player in anemia of inflammation.

Bmp6^{-/-} mice have strong impairment of Bmp signaling, likely similar to the one achieved when treating wild-type animals with the BMP type I receptor inhibitor LDN-193189 or the BMP ligand antagonist ALK3-Fc. When challenged with LPS, they hardly increase their hepcidin production to the level seen in unchallenged wild-type mice. Much higher circulating hepcidin levels would be expected if the inflammation and the iron (BMP) signals had additive transcriptional effects on hepcidin promoter. Rather, our *in vivo* observations suggest transcriptional synergy between these two signals and explain why lowering one of them using LDN-193189 or ALK3-Fc is sufficient to attenuate the induction of hepcidin gene expression by various inflammatory stimuli¹⁰⁻¹². A similar attenuation is observed when BMP signaling is genetically impaired, as here in *Bmp6*^{-/-} mice or in mice with liver-specific deletion of Alk3¹³. The present data are compatible with the previously proposed synergy between IL6/STAT3 and BMP/SMAD signaling in regulating hepcidin¹⁴. They show that full induction of hepcidin expression by inflammatory stimuli requires a functional BMP6-activated signaling pathway in the hepatocyte but is independent of activin B and its activation of Smad1/5/8 signaling that likely occurs in other cells of the liver.

Acknowledgements. The authors thank Cindy Moriceau, Rachel Balouzat and Yara Barreira (US006 ANEXPLO, Toulouse) for their technical assistance and help in the mouse breeding. This work was supported by grants from FRM (DEQ2000326528) and ANR (ANR-13-BSV3-0015-01).

Authorship Contributions. C.B.F. challenged the mice with LPS and performed RT-PCR and western-blotting experiments. A.G. infected the mice with the different pathogens and performed RT-PCR experiments. C.L. was in charge of mouse genotyping, challenged the mice with plasmodium and turpentine and performed hepcidin ELISAs. O.G. helped with mouse experiments. D.M. helped with data analysis and interpretation. P.M. and E.O. were involved in the design of infection experiments. H.C. and M.P.R. led and supervised the project through all stages, helped in data analyses and wrote the manuscript with suggestions and comments from all authors.

Disclosure of Conflicts of Interest. The authors have no conflict of interest to declare.

References

1. Besson-Fournier C, Latour C, Kautz L, et al. Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling. *Blood*. 2012;120(2):431-439.
2. Canali S, Core AB, Zumbrennen-Bullough KB, et al. Activin B Induces Noncanonical SMAD1/5/8 Signaling via BMP Type I Receptors in Hepatocytes: Evidence for a Role in Hepcidin Induction by Inflammation in Male Mice. *Endocrinology*. 2016;157(3):1146-1162.
3. Vassalli A, Matzuk MM, Gardner HA, Lee KF, Jaenisch R. Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes Dev*. 1994;8(4):414-427.
4. Pietrangelo A, Dierssen U, Valli L, et al. STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. *Gastroenterology*. 2007;132(1):294-300.
5. Verga Falzacappa MV, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU. STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood*. 2007;109(1):353-358.
6. Wrighting DM, Andrews NC. Interleukin-6 induces hepcidin expression through STAT3. *Blood*. 2006;108(9):3204-3209.
7. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet*. 2009;41(4):478-481.
8. Kautz L, Meynard D, Monnier A, et al. Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. *Blood*. 2008;112(4):1503-1509.
9. Corradini E, Meynard D, Wu Q, et al. Serum and liver iron differently regulate the bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway in mice. *Hepatology*. 2011;54(1):273-284.
10. Steinbicker AU, Sachidanandan C, Vonner AJ, et al. Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. *Blood*. 2011;117(18):4915-4923.
11. Theurl I, Schroll A, Sonnweber T, et al. Pharmacologic inhibition of hepcidin expression reverses anemia of chronic inflammation in rats. *Blood*. 2011;118(18):4977-4984.
12. Mayeur C, Kolodziej SA, Wang A, et al. Oral administration of a bone morphogenetic protein type I receptor inhibitor prevents the development of anemia of inflammation. *Haematologica*. 2015;100(2):e68-71.
13. Mayeur C, Lohmeyer LK, Leyton P, et al. The type I BMP receptor Alk3 is required for the induction of hepatic hepcidin gene expression by interleukin-6. *Blood*. 2014;123(14):2261-2268.
14. Verga Falzacappa MV, Casanovas G, Hentze MW, Muckenthaler MU. A bone morphogenetic protein (BMP)-responsive element in the hepcidin promoter controls HFE2-mediated hepatic hepcidin expression and its response to IL-6 in cultured cells. *J Mol Med*. 2008;86(5):531-540.

Figure legends

Fig. 1. Although, in the absence of activin B, Smad1/5/8 signaling is not activated by inflammatory stimuli, this has no impact on the induction of hepcidin expression. (A&B) Fresh protein extracts were prepared from livers of wild-type (WT) and activin B (*Inhbb*)-deficient mice 4 hours after a LPS challenge or infection with *E. coli*. Phospho-Smad2, phospho-Smad5, phospho-Stat3, total Smad2, total Smad5, and total Stat3 were detected by immunoblot techniques on a Chemidoc MP Imaging System (Bio-Rad). **(C&D)** Evolution over time of the activin B (*Inhbb*) and **(E&F)** hepcidin (*Hamp*) mRNA expression was examined in wild-type mice and (for hepcidin) *Inhbb*^{-/-} mice challenged with LPS or infected with *E. coli*. Point estimates of the fold changes in gene expression relative to baseline ($2^{-\Delta\Delta Ct}$) are shown on the graphs, together with their 95% confidence intervals (CIs). When the lower limit of the fold-change CI exceeds 1, gene expression is significantly induced relative to baseline. **(G)** Serum hepcidin levels were measured by competitive ELISA on wild-type and *Inhbb*^{-/-} mice at baseline and 4 hours after LPS challenge. Values shown are geometric means \pm 95% CIs. Comparisons of log-transformed serum hepcidin levels were made by two-way ANOVA followed by Sidak's multiple comparison tests of planned contrasts. Results of comparisons with baseline levels in mice of the same genotype are shown above the bars. ****, $p < 0.0001$.

Fig. 2. In contrast to Smad1/5/8 activation by iron, activation by inflammatory stimuli does not lead to the expected induction in hepcidin and has no impact on the expression of its hepatocyte targets Id1 and Smad7. (A) Fresh protein extracts were prepared from livers of wild-type and *Bmp6*^{-/-} mice. Phospho-Smad5 and total Smad5 were detected by immunoblot techniques on a Chemidoc MP Imaging System (Bio-Rad). **(B)** Serum hepcidin levels were measured by competitive ELISA on wild-type and *Bmp6*-deficient mice at baseline and 4 hours after LPS challenge. Values shown are geometric means \pm 95% confidence intervals (CIs). Comparisons of log-transformed serum hepcidin levels were made by two-way ANOVA followed by Sidak's multiple comparison tests of planned contrasts. Results of comparisons with baseline levels in mice of the same genotype are shown above the bars. Results of comparison between wild-type and *Bmp6*^{-/-} mice are indicated by connecting lines. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. **(C&D)** Evolution over time of the *Id1* and **(E&F)** *Smad7* mRNA expression was examined in wild-type and *Inhbb*^{-/-} mice challenged with LPS or infected with *E. coli*. Point estimates of the fold changes in gene expression relative to baseline ($2^{-\Delta\Delta Ct}$) are shown on the graphs, together with their 95% confidence intervals (CIs). When the upper limit of the fold-change CI is below 1, gene expression is significantly repressed relative to baseline.