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RNAi-Mediated Inhibition of *Tmprss6* Elevates *Hamp1* Expression and Reduces Serum Iron Levels in Mice

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## Abstract

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The liver hormone hepcidin (encoded by HAMP) is a central regulator of iron homeostasis<sup>1</sup>. Hepcidin binds to the iron-exporter protein ferroportin (FPN1), which is localized on absorptive enterocytes, hepatocytes and macrophages. Hepcidin binding to the extracellular domain leads to the internalization and degradation of ferroportin thus decreases the absorption of dietary iron from the intestine, the release of iron from macrophages and hepatocytes<sup>2</sup>. Hepcidin expression is regulated by iron, anemia, hypoxia and inflammatory cytokines. The stimulation of hepcidin transcription in response to iron is accomplished through a Bone Morphogenetic Protein (BMP)/Sons of Mothers Against Decapentaplegic (SMAD)-dependent signal transduction cascade that involves the BMP-co-receptor hemojuvelin (HJV)<sup>3</sup>.

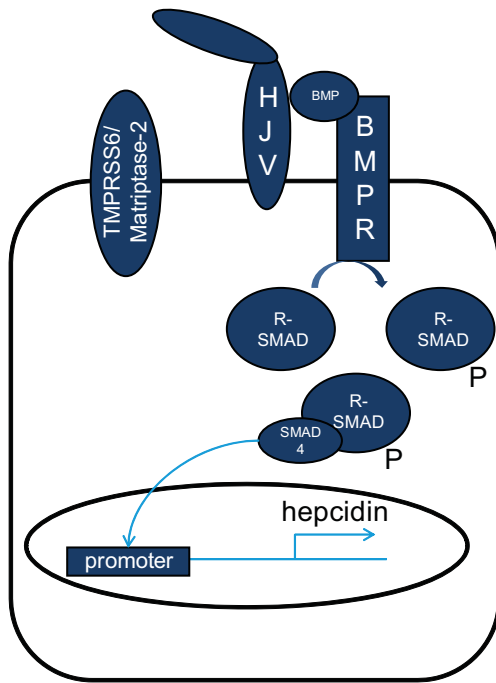
The membrane-bound serine protease Matriptase-2 (encoded by TMPRSS6) inhibits BMP-induced induction of hepcidin by proteolyzing HJV on the hepatocyte cell surface<sup>4</sup>. In humans, loss of function mutations in TMPRSS6 lead to elevated hepcidin levels resulting in iron-resistant iron-deficiency anemia (IRIDA)<sup>5</sup>. In the primary iron overload disorders collectively called hereditary hemochromatosis (HH) and in anemias characterized by massive ineffective hematopoiesis, and iron overload (secondary hemochromatosis), such as  $\beta$ -thalassemia intermedia (TI), patients have the opposite phenotype: hepcidin levels are low despite elevated serum iron concentrations and iron stores. Studies in murine models of TI and HH have shown that stimulating hepcidin production by either genetic inactivation of *Tmprss6*<sup>6</sup> or overexpressing *Hamp1*<sup>7</sup> can prevent iron overload and can correct other aspects of the disease phenotypes. On this basis, therapeutic strategies aimed at specifically inhibiting *Tmprss6* expression could prove efficacious in these, and other, iron overload diseases.

Here we show that systemic administration of a potent lipid nanoparticle (LNP) formulated siRNA directed against *Tmprss6* leads to durable inhibition of *Tmprss6* mRNA in the mouse liver with concomitant elevation of mouse hepcidin mRNA (*Hamp1*) expression. In wild-type C57BL/6 mice, a single dose of 0.3 mg/kg formulated siRNA reduces *Tmprss6* mRNA levels by >90% within 24h of administration. Silencing of *Tmprss6* is maintained at >80% for 14 days. Levels of liver *Hamp1* mRNA are increased 2-3 fold from 24h through 7 days post administration. This leads to ~50% decreases in serum iron concentration and Transferrin saturation through 14 days, as well as decreases in Hemoglobin levels and changes in other hematologic parameters between 14 and 28 days.

In Th3/+ mice, a model of  $\beta$ -thalassemia intermedia, a single dose of 1 mg/kg formulated siRNA reduces tissue iron levels and improves erythropoiesis. Silencing of *Tmprss6* dramatically reduces splenomegaly, a measure of extramedullary erythropoiesis, and results in substantial improvements in hematological parameters, including a significant decrease in the number of reticulocytes and an improvement in the blood hemoglobin levels.

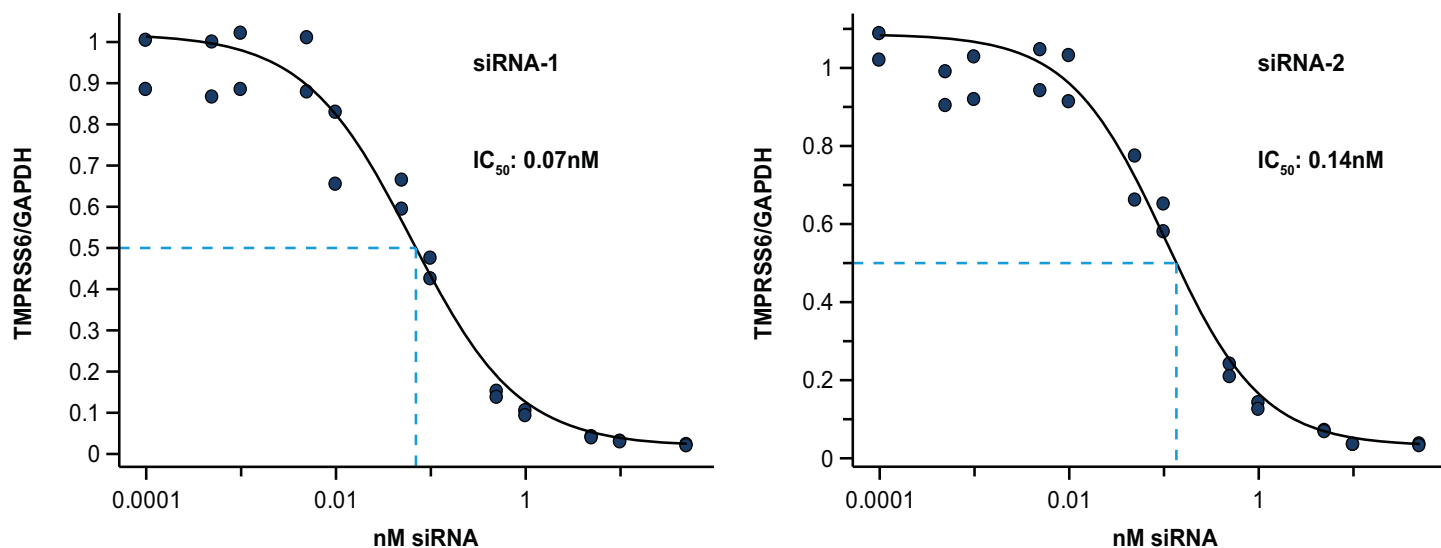
These results demonstrate that silencing of *Tmprss6* by systemic administration of formulated siRNA increases *Hamp1* expression to levels sufficient to ameliorate the phenotype in a mouse model of thalassemia intermedia. Further testing in mouse genetic models of  $\beta$ -thalassemia Intermedia and hereditary hemochromatosis will support the rationale for developing LNP formulated TMPRSS6 siRNA as a novel treatment modality for these conditions.

**Figure 1. Model of Hepcidin Regulation by TMPRSS6**



TMPRSS6 inhibits BMP-mediated HAMP upregulation by cleaving the BMP co-receptor HJV, preventing BMP signaling, SMAD translocation to the nucleus and HAMP transcriptional activation. Loss of TMPRSS6 function leads to IRIDA due to elevated Hepcidin levels.

Figure 2. siRNA Lead Selection

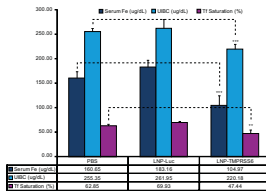


Chemically-modified siRNAs were screened by transfection in HEP3B human hepatoma cells. Two highly potent siRNAs with multi-species reactivity (human, cynomolgus monkey, rat, mouse) and minimal predicted off-target potential were selected for evaluation *in vivo*. Confirmation of the potency in primary mouse hepatocytes demonstrates 70 pM for siRNA-1 and 140 pM for siRNA-2.

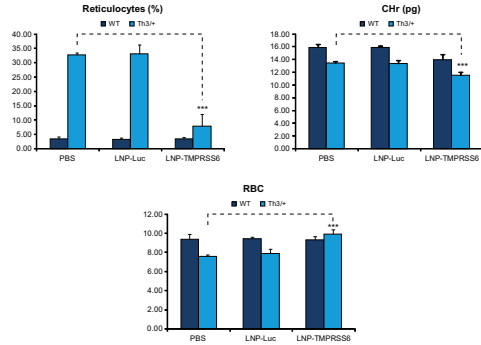


**Fig 4. Effect of *Tmprss6* Silencing in Th3/+ Mice**

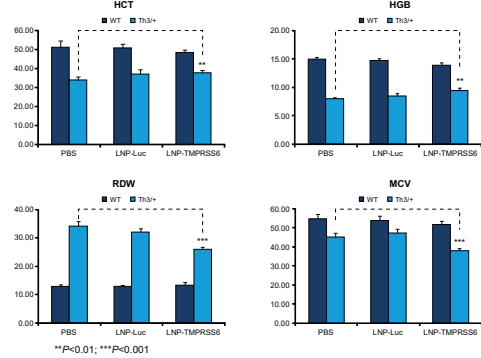
**A Reduction of *Tmprss6* leads to reduction of serum parameters**



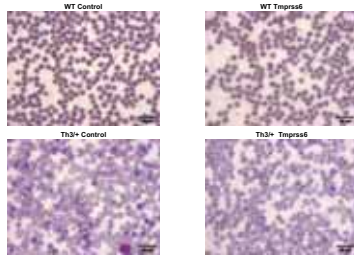
**B Reduction of *Tmprss6* leads to reduction of ineffective erythropoiesis**



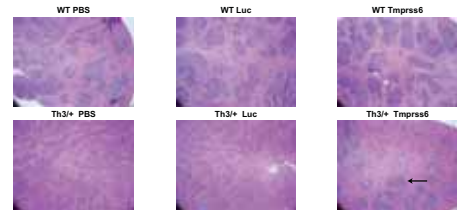
**C Hematological parameters improvement**



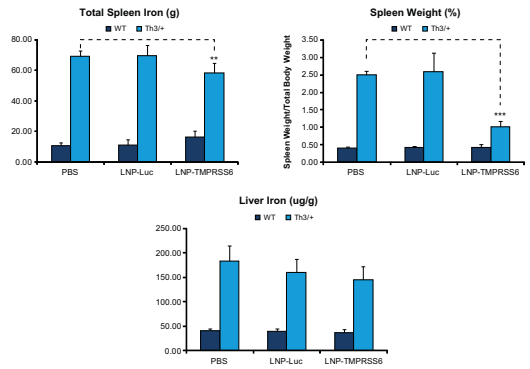
**D Impovement of peripheral blood morphology**



**E Normalization of splenic architecture**



**F Significant reduction of splenic iron and splenomegaly**



(A) Silencing of *Tmprss6* in Th3/+ mice leads to significant reduction of serum iron, UIBC and Tf saturation. 6 week old Th3/+ mice were injected via tail vein with 1 mg/kg LNP-*Tmprss6*-1 siRNA or LNP-Luc control or PBS. Animals were sacrificed 2 weeks post dose and serum iron parameters analyzed. N=5 per group. Data represent mean  $\pm$  s.d. \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  (B) Single dose of LNP-TMPRSS6 dramatically reduced the number and the hemoglobin content of reticulocytes in Th3/+ mice and increased the number of mature erythrocytes- dramatic improvement in ineffective erythropoiesis. (C) Hematological Parameters of TMPRSS6 Treated Mice Improve After a Single Dose. (D) Peripheral blood morphology from wild-type and Th3/+ mice treated with a control siRNA or *Tmprss6* siRNA. Note the very slight anisocytosis induced by the *Tmprss6* siRNA in the wild-type animal. In Th3/+ animals treated with the *Tmprss6* siRNA there is a marked decrease in polychromasia (bluish RBCs) representative of the decreased reticulocyte count as well as an overall trend toward normalization of the mature RBC morphology. May-Grunwald/Gimsa stain at 100X magnification. (E) Reduction of *Tmprss6* in the Th3/+ mice led to normalization of splenic architecture including reduction in sinusoidal extramedullary erythropoiesis and reappearance of white pulp nodules (arrow head) H&E stain, 10X magnification. (F) Reduction in TMPRSS6 Leads to Significant Reduction of Iron Content in Spleen and Dramatic Reduction in Splenomegaly. Some non-significant trend toward reduction in liver iron.

**Conclusions**

- LNP-TMPRSS6 is a novel siRNA lipid nanoparticle formulation being developed for congenital iron overload disorders characterized by abnormally low Hcpidin levels
  - eg,  $\beta$ -thalassaemia intermedia and hereditary hemochromatosis
- Efficacy has been demonstrated in wild-type and  $\beta$ -thalassaemia intermedia (Th3/+) mice
  - Dose dependent silencing of *Tmprss6* leads to concomitant induction of *Hamp1*
  - Increased *Hamp1* expression leads to decreases in serum iron and Transferrin saturation
  - In a  $\beta$ -thalassaemia intermedia model, decreased serum iron leads to improved hematological parameters and reduced extra-medullary erythropoiesis
- These results demonstrate that silencing of *Tmprss6* by systemic administration of formulated siRNA increases *Hamp1* expression to levels sufficient to ameliorate the phenotype in a mouse model of  $\beta$ -thalassaemia intermedia
- Further studies using multi-dose administration in  $\beta$ -thalassaemia and hereditary hemochromatosis mouse models are in progress

**References**

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**Conflict of Interest**

- IT, TR, SM, BRB, JH, DWYS, DB are employees and/or stockowners of Ainyam Pharmaceuticals
- Mark Fleming receives research funding from Ainyam; Consultant in Experimental Pathology, Millennium Pharmaceuticals
- ZC and PS have no relevant conflict of interest