Identification and characterization of a novel murine allele of *Tmprss6*

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ABSTRACT

Mutagenesis screens can establish mouse models of utility for the study of critical biological processes such as iron metabolism. Such screens can produce mutations in novel genes or establish novel alleles of known genes, both of which can be useful tools for study. In order to identify genes of relevance to hematologic as well as other phenotypes, we performed N-ethyl-N-nitrosourea mutagenesis in C57BL/6J mice. An anemic mouse was identified and a putative mutation was characterized by mapping, sequencing and *in vitro* activity analysis. The mouse strain was backcrossed for ten generations then phenotypically characterized with respect to a previously established null mouse strain. Potential modifying loci were identified by quantitative trait locus analysis. Mapping and sequencing in an anemic mouse termed *hem8* identified an I286F substitution in Tmprss6, a serine protease essential for iron metabolism; this substitution impaired *in vitro* protease activity. After backcrossing to C57BL6/J for ten generations, the *hem8*$^{-/-}$ strain exhibited a phenotype similar in some but not all aspects to that of *Tmprss6*$^{-/-}$ mice. The hem8 and Tmprss6-null mutations were allelic. Both *hem8*$^{-/-}$ and *Tmprss6*$^{-/-}$ mice responded similarly to pharmacological modulators of bone morphogenetic protein signaling, a key regulator of iron metabolism. Quantitative trait locus analysis in the *hem8* strain identified potential modifying loci on chromosomes 2, 4, 7 and 10. In conclusion, the *hem8* mouse model carries a novel allele of *Tmprss6*. Potential uses for this strain in the study of iron metabolism are discussed.

Introduction

Chemical mutagenesis screens can be highly advantageous in the study of physiological processes.[1] These screens have little to no bias towards genes of specific function and isolated mutants by definition harbor defects of functional relevance. The use of chemical mutagenesis in the study of anemia is further enabled by the relatively straightforward screen for mutants in quantitative hematologic parameters. Although anemia can result from defects in a variety of different pathways, mutations in genes associated with hemoglobin production, specifically those that encode or regulate the globin genes themselves or those that are involved in heme or iron metabolism, give rise to a class of anemias characterized by small, pale red blood cells, the so-called hypochromic, microcytic anemias.

Systemic iron metabolism is largely regulated by hepcidin, a peptide hormone secreted predominantly by the liver which inhibits dietary iron absorption and macrophage iron efflux.[2] Hepcidin expression is regulated by multiple factors including anemia, hypoxia, iron levels and inflammation.[3] The regulation of hepcidin expression by iron levels is mediated by a complex signaling pathway in which soluble factors diferric transferrin and bone morphogenetic protein (BMP)-6 stimulate hepcidin expression in a pathway dependent upon hepatocyte membrane-bound factors HFE, transferrin receptor 2 and hemojuvelin. This pathway itself is subject to further regulation, as the BMP co-receptor hemojuvelin can be cleaved from the cell membrane by the transmembrane serine protease Tmprss6.[4] Patients with mutations in Tmprss6 develop a condition referred to as iron-refractory iron-deficiency anemia (IRIDA).[5-16] Decreased Tmprss6 activity leads to increased hepcidin expression through the hemojuvelin-dependent pathway; increased hepcidin expression leads to decreased iron absorption and iron-deficiency anemia. Mouse models of Tmprss6 deficiency recapitulate this phenotype.[17-19]

Here we report the results of an N-ethyl-N-nitrosurea (ENU) mutagenesis screen in which we identified a mouse strain termed *hem8*. Using multiple experimental approaches, we have assembled evidence that the *hem8* phenotype results from a partial loss-of-function amino acid substitution in Tmprss6. Potential uses of this mouse strain in the study of iron biology are discussed.

Design and Methods

Animal procedures were approved by the Animal Care and Use Committee, Children's Hospital Boston. The *hem8* strain was identified in an ENU mutagenesis screen for anemic mice and was propagated from a single ENU-mutagenized C57BL/6J (B6) male.[20,21] Initial mapping studies were performed using 22 anemic 129S6/SvEvTac (129S6)

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x B6 F_2 intercross animals and polymerase chain reaction genotyping as previously described.[21] Secondary mapping studies were performed by crossing an anemic 129S6B6F1 female mouse with a *Mus musculus castaneus* CAST/Ei (CAST) male mouse and intercrossing the progeny; F_2 progeny were genotyped at the Center for Applied Genomics, The Hospital for Sick Children, Toronto, Ontario, Canada. Mice were genotyped for 1,449 markers using Mouse Medium Density Linkage Panels (Illumina Inc., San Diego, CA, USA), GoldenGate Genotyping Assays and universal 1,536-plex 12-sample BeadChip microarrays. Arrays were scanned using Illumina iScan with analysis and intra-chip normalization performed by Illumina GenomeStudio Genotyping Module software v.2011 and genotype calls generated by clustering project samples with a manual review of each single nucleotide polymorphism plot. Primary sequence alignments and molecular modeling were performed as previously described.[22] Tmprss6 proteolytic activity was determined using conditioned media from transfected HEK293T cells and the chromogenic substrate Boc-Gln-Ala-Arg-para-nitroanilide as previously described.[23,24] Tmprss6 protein levels were analyzed by immunoblot using an antibody kindly provided by Caroline Enns. Samples were collected and hematologic, iron and gene and protein expression analyses were performed as previously described.[19,25] The *Tmprss6* polymorphism was backcrossed for ten generations onto B6 prior to full characterization. *Tmprss6*$^{-/-}$ mice were maintained on B6 and have been described elsewhere.[19] Quantitative trait locus (QTL) analysis was performed using R/QTL according to the software's instructions.[26] Dorsomorphin and LDN-193189 were administered intraperitoneally at 10 mg/kg and 3 mg/kg, respectively, as previously described;[27,28] LDN-193189 solutions were adjusted to neutral pH prior to injection.

Results

Identification of Tmprss6 *as the gene mutated in* hem8

The *hem8* strain was identified by complete blood counts in an ENU mutagenesis screen for hematologic abnormalities in B6 mice; it was named *hem8* as this strain represents the eighth hematologic mutant noted in the screen.[20,21] Initial mapping of the *hem8* allele was performed by intercrossing the *hem8* strain with a 129S6 mouse; analysis of 129S6B6F_2 mice demonstrated linkage to chromosome 15 (*data not shown*). To refine the mapping of the *hem8* mutation, we intercrossed an affected 129S6B6F_1 female mouse to a CAST male mouse, the latter strain chosen for its genetic heterogeneity relative to 129S6 or B6 mice. F_2 mice were characterized by complete blood counts. Forty-seven anemic female and 57 anemic male F_2 mice were genotyped for approximately 1500 single nucleotide polymorphisms, 577 of which were polymorphic between founders (*data not shown*). In anemic mice, the 75.1-88.9 Mb region on chromosome 15 was conserved from the *hem8* founder strain (Figure 1). A strong candidate gene residing within this region was *Tmprss6* at 78.27-78.30 Mb encoding a transmembrane serine protease essential for iron metabolism. Patients and mice with Tmprss6 deficiency develop IRIDA. In this condition, patients are refractory to enteral and parenteral iron administration. Tmprss6 down-regulates hepcidin expression by cleaving and liberating hemojuvelin, a membrane-bound BMP co-receptor essential for hepcidin expression. Tmprss6 deficiency leads to hepcidin excess, which in turn results in impaired dietary iron absorption, sequestration of iron stores within macrophages, iron deficiency and anemia.

Sequencing of exons and exon/intron junctions in *Tmprss6* revealed an A856T transversion that resulted in an Ile286Phe (I286F) substitution. Ile286 is a highly conserved residue in the first CUB domain of Tmprss6 and is situated within the vicinity of several other IRIDA-associated residues (Figure 2A-C).[5-16] Genotyping of all mice from the *hem8* x CAST intercross described above revealed that F286/F286 mice had decreased mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) levels, total hemoglobin levels and hematocrit relative to I286/I286 and I286/F286 mice (Figure 2D,E; *data not shown*). To assess the *in vitro* significance of the I286F substitution on Tmprss6 function, we transfected mouse Tmprss6 cDNA expression constructs encoding the I286 or F286 variant into HEK293T cells. Immunoblots with a Tmprss6-specific antibody revealed similar expression levels for I286 and F286 variants (*data not shown*). Incubation of conditioned media from these transfections with the chromogenic protease substrate Boc-Gln-Ala-Arg-para-nitroanilide[23,24] demonstrated that I286F Tmprss6 and the protease-inactive R774C variant had 48% and 9% of wild-type activity, respectively, indicating that the I286F allele is likely a Tmprss6 hypomorph (Figure 2F).

To determine the role of the I286F polymorphism in the *hem8* phenotype, we backcrossed the *hem8* strain to B6

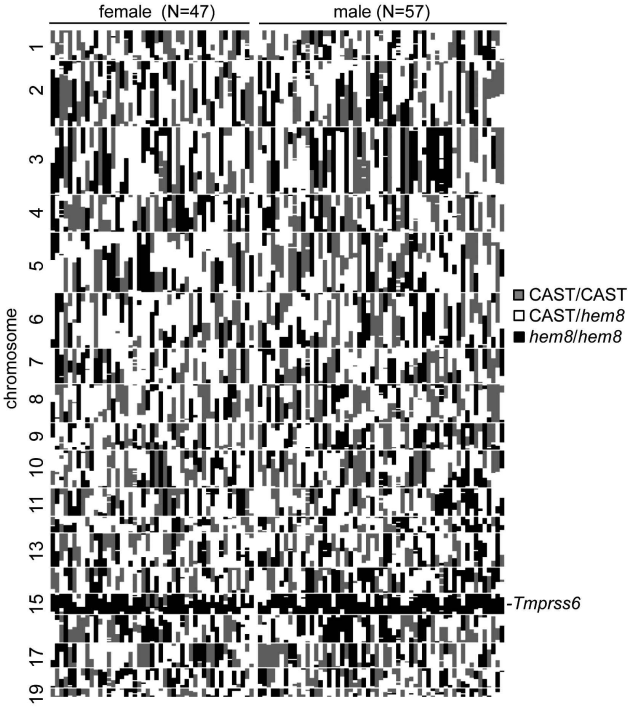

Figure 1. Genotype of anemic F_2 progeny used for *hem8* mapping and QTL analysis. A 129S6B6F_1 *hem8* female mouse was mated to a CAST male mouse and 47 female and 57 male anemic F_2 progeny were genotyped at 1 month of age using Illumina Mouse Medium Density Linkage Panel for approximately 1500 single nucleotide polymorphisms, 577 of which were polymorphic between founders. Each row represents an individual single nucleotide polymorphism with chromosomes arranged from 1 to 19 and separated by blank rows; each column represents an anemic female or male mouse. Squares indicate that an individual mouse carries the same genotype at a specific single nucleotide polymorphism as the CAST founder (dark gray), *hem8* founder (black) or that an individual mouse is heterozygous for CAST and *hem8* founder alleles (white). The genomic location of *Tmprss6* is indicated.

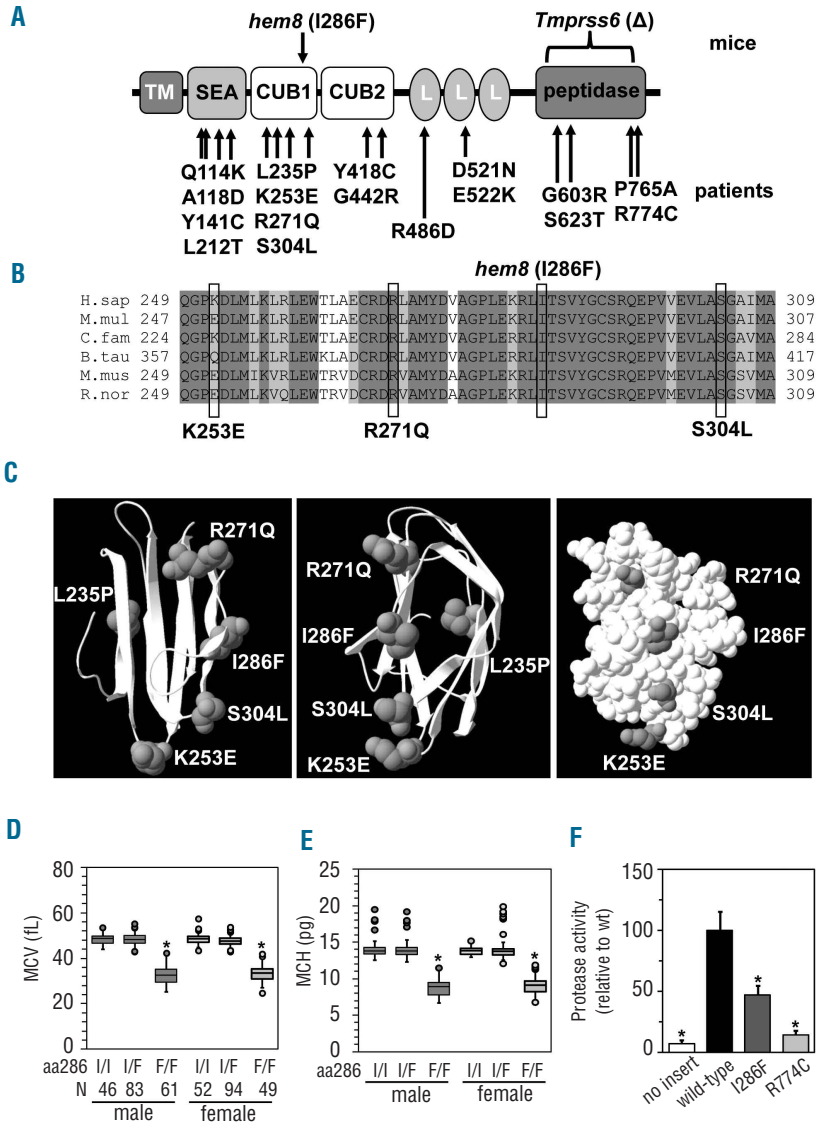

Figure 2. Characterization of the Tmprss6 I286F substitution. (A) Schematic of Tmprss6 structure with known protein domains, location of mouse mutations (*hem8* and null; indicated above) studied in this paper and patients' mutations associated with IRIDA (indicated below). TM stands for transmembrane domain and L for LDLR domains. (B) Primary sequence alignment of a segment of the CUB1 domain from Tmprss6 sequence homologues from *Homo sapiens* (H.sap), *Macaca mulatta* (M.mul), *Canis familaris* (C.fam), *Bos taurus* (B.tau), *Mus musculus* (M.mus) and *Rattus norvegicus* (R.nor). Residues affected by the I286F polymorphism in *hem8* mice and by disease-associated mutations in IRIDA patients are bracketed. (C) Structural prediction of the CUB1 domain and locations of residues indicated in (B), as determined using HHPred and SwissPDB Viewer. The two leftmost panels represent the same structure from different viewing angles; the two rightmost panels represent the same structure using ribbon or space-filling representations from similar viewing angles. Residues of interest are indicated as gray space-filling. (D-E) Mean corpuscular volumes (MCV) (D) and mean corpuscular hemoglobin (E) levels are indicated in box plots for male and female F_2 mice resulting from mating between a 129S6B6F$_1$ *hem8* female mouse and a CAST male mouse. Mice were sacrificed at one month of age. Mice are represented based upon genotype at Tmprss6 amino acid residue 286 (CAST genotype or I/I; *hem8* genotype or F/F; heterozygous genotype or I/F). Asterisks indicate that F/F data sets differ significantly from I/I and I/F data sets by *P*-value less than 10^{-34} (Student's t-test; unpaired; unequal variance). Error bars represent standard deviation. (F) Concentrated conditioned media from HEK293T cells transfected with expression vectors carrying no insert, wild-type mouse Tmprss6 or I286F or R774C variants were incubated with the chromogenic protease substrate Boc-Gln-Ala-Arg-para-nitroanilide; cleavage was measured as increased absorbance at 405 nm. Absorbance levels were arbitrarily set at 100% for wild-type Tmprss6 and all other values expressed relative to that. Asterisks indicate that values differ significantly (t-test, $P<0.05$) from wild-type. Each bar represents n=6. Error bars represent standard deviations.

mice for ten generations, selecting for the F286 allele in each generation, and then characterized the phenotype of *hem8*$^{-/-}$ mice relative to *Tmprss6*$^{-/-}$ mice on the B6 background. Like *Tmprss6*$^{-/-}$ mice, *hem8*$^{-/-}$ mice had body hair loss sparing the face (*data not shown*) and decreased MCV, MCH, serum iron levels, transferrin saturations and liver iron levels although *hem8*$^{-/-}$ mice did not have reticulocytosis (Figure 3A-G). *Hem8*$^{-/-}$ mice also displayed increased liver hepcidin and Id1 RNA levels but, in contrast to *Tmprss6*$^{-/-}$ mice, had unchanged liver Bmp6 RNA levels and increased liver Tmprss6 RNA levels (Figure 3H-K). To determine whether the *hem8* and *Tmprss6*-null mutations were allelic, we next intercrossed *Tmprss6*$^{+/-}$ and *hem8*$^{+/-}$ mice and characterized the phenotype of *Tmprss6*$^{+/-}$ *hem8*$^{+/-}$ mice. Relative to *Tmprss6*$^{+/+}$ *hem8*$^{+/+}$ mice, *Tmprss6*$^{+/-}$ *hem8*$^{+/-}$ mice had decreased MCV, MCH, serum iron levels, transferrin saturations and liver iron levels and increased reticulocyte counts and liver hepcidin RNA levels (Figure 4). This indicated that the *hem8* and *Tmprss6*-null mutation are allelic and that the *hem8* allele should be regarded as *Tmprss6*^{hem8}.

Mapping genetic modifiers of hem8

To identify modifying loci, we performed QTL analysis using open-source software R/QTL, single nucleotide polymorphism genotype data obtained from the *hem8* x CAST mapping intercrosses (Figure 1) and phenotype data for multiple parameters including complete blood counts, body, liver and spleen mass, liver and spleen iron levels, liver RNA levels of hepcidin, Id1 and Bmp6 normalized to β-actin levels and liver hepcidin:β-actin, Id1:β-actin and Bmp6:β-actin RNA levels relative to liver iron levels. Males and females were analyzed separately and as a combined group. Four QTL were identified for which LOD scores exceeded genome-wide significance thresholds and *P*-values were less than 0.05 (Figure 5). A QTL associated with decreased hemoglobin level in male *hem8* mice was noted on chromosome 2 and resides near activin receptor genes *Acvr2a* (48.7 Mb) and *Acvr1* and *Acvr1c* (58.2 Mb); we recently demonstrated that liver-specific deficiency in BMP receptor type I genes *Acvr1* (*Alk2*) or *Bmpr1a* (*Alk3*) induces iron overload in mice.[29] A QTL associated with increased reticu-

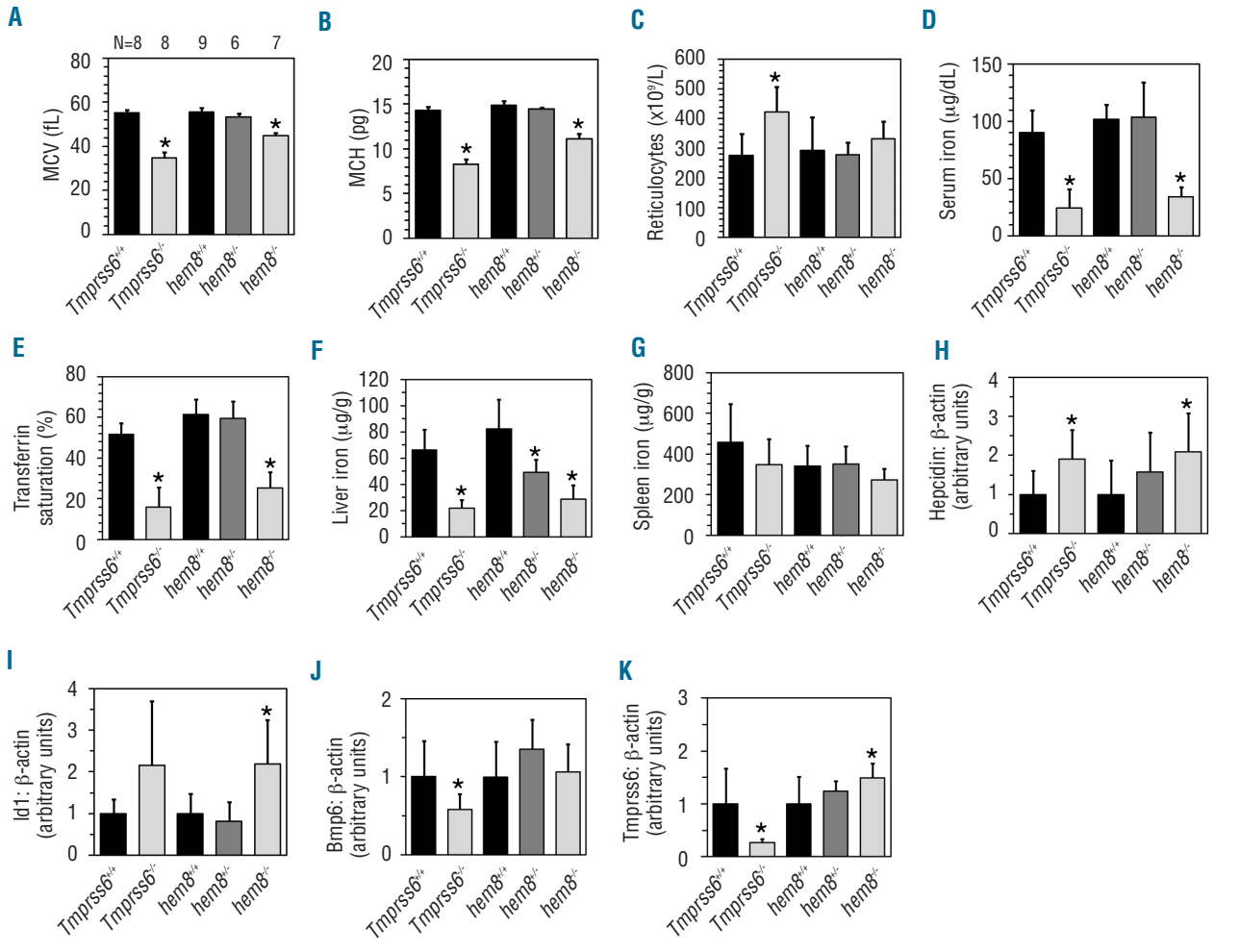

Figure 3. Phenotype of *hem8*$^{-/-}$ mice relative to *Tmprss6*$^{-/-}$ mice. At least six female *Tmprss6*$^{+/+}$, *Tmprss6*$^{-/-}$, *hem8*$^{+/+}$, *hem8*$^{+/-}$ and *hem8*$^{-/-}$ mice were characterized at 2 months of age for mean corpuscular volumes (MCV) (A), mean corpuscular hemoglobin (MCH) levels (B), reticulocyte counts (C), serum iron levels (D), transferrin saturation (E), liver iron levels (F), spleen iron levels (G), liver RNA levels of hepcidin (H), Id1 (I), Bmp6 (J) and Tmprss6 (K) relative to β-actin levels as measured by quantitative polymerase chain reaction. Error bars indicate standard deviations.

locyte count in the combined male and female analysis was noted on chromosome 4 and resides near the Na^+/H^+ exchanger *Slc9a1* (132.9 Mb) and blood group antigen *Rhd* (134.4 Mb) on chromosome 4. Slc9a1 plays a central role in the regulation of red cell volume and pH,[30] while the Rh proteins, including Rhd, form a multi-protein complex in the erythrocyte membrane with a possible role in sequestration of blood ammonia.[31] A QTL associated with decreased corpuscular hemoglobin concentration mean (CHCM) in male mice was noted on chromosome 7 and resides in the vicinity of the β-globin gene cluster at 111 Mb. A QTL associated with increased CHCM in male mice was noted on chromosome 10 and resides near nuclear receptor *Nr2c1* (93.6 Mb) and Kit receptor ligand *Kitl* (99.5 Mb). Nr2c1, or TR2 orphan nuclear receptor, modulates gene expression during erythroid development,[32,33] while Kit ligand, or stem cell factor, is a cytokine essential for hematopoiesis.[34]

Pharmacological intervention in Tmprss6 deficiency

To characterize the effect of pharmacological modulation of BMP signaling in Tmprss6 deficiency, we employed LDN-193189, a small molecule that inhibits SMAD1/5/8 phosphorylation by BMP type I receptors Alk2, 3 and 6.[35,36] We first characterized the response of B6 mice to single intraperitoneal doses of LDN-193189 at 3 mg/kg. Serum iron levels and transferrin saturation were decreased at 1.5 h and increased at 7 h post-injection (Figure 6A,B). No significant changes were noted in liver iron levels or BMP6 RNA levels at any time point (*data not shown*). Hepcidin and Id1 RNA levels were decreased at 1.5 and 5 h post-injection (Figure 6 C, D). While the increased serum iron levels and transferrin saturation at 7 h post-injection most likely reflect the decreased hepcidin RNA levels at 1.5 h and 5 h post-injection, the etiology of the decreased serum iron levels and transferrin saturation at 1.5 h post-injection is unclear.

To determine whether pharmacological modulation of BMP signaling altered the phenotype of *Tmprss6*$^{-/-}$ mice, we injected mice with a single 3 mg/kg intraperitoneal dose of LDN-193189. While hepcidin levels decreased at 1.5 h and serum iron levels and transferrin saturation increased at 7 h post-injection in wild-type littermates, only hepcidin levels decreased at 1.5 h in *Tmprss6*$^{-/-}$ mice (Figure 6E,F). Similarly, treatment of *Tmprss6*$^{-/-}$ or *hem8*$^{-/-}$ mice with dorsomorphin, the parent molecule from which LDN-193189 was derived, produced no change in serum iron levels (*data not shown*).

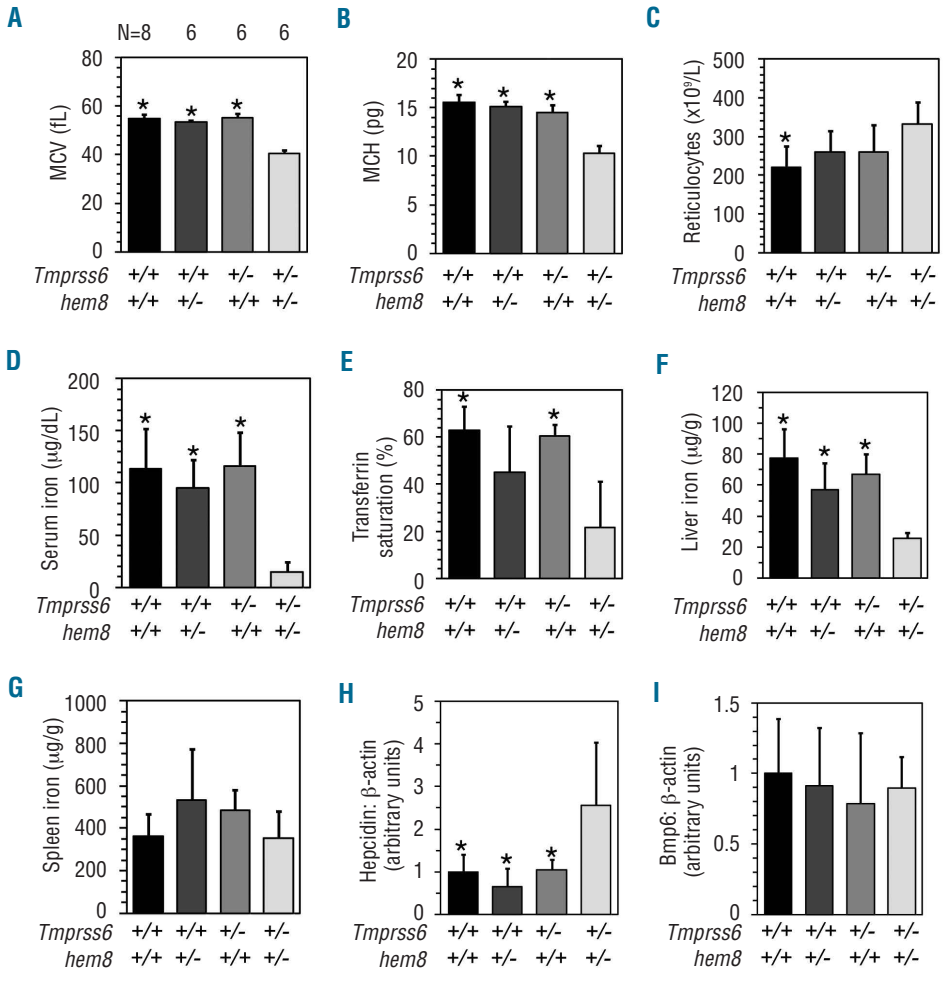

Figure 4. Phenotype of *Tmprss6*$^{+/-}$ *hem8*$^{+/-}$ mice. At least six female *Tmprss6*$^{+/+}$ *hem8*$^{+/+}$, *Tmprss6*$^{+/+}$ *hem8*$^{+/-}$, *Tmprss6*$^{+/-}$ *hem8*$^{+/+}$ and *Tmprss6*$^{+/-}$ *hem8*$^{+/-}$ mice were characterized at 2 months of age for mean corpuscular volumes (MCV) (A), mean corpuscular hemoglobin (MCH) levels (B), reticulocyte counts (C), serum iron levels (D), transferrin saturation (E), liver iron levels (F), spleen iron levels (G) and hepatic RNA levels of hepcidin (H) and Bmp6 (I) relative to β-actin levels as measured by quantitative polymerase chain reaction. Error bars indicate standard deviations.

Discussion

While many advances in our understanding of mammalian iron metabolism have come from the construction and characterization of knock-out and transgenic mice, chemical mutagenesis screens have contributed significantly. ENU-based screens have identified a variety of factors as key players in iron metabolism including the gastric hydrogen-potassium ATPase α subunit[37] and the phosphatidylinositol-binding clathrin assembly protein (Picalm).[38] These screens have also led to the identification of an endosomal targeting motif in the ferrireductase Steap3[39] and the establishment of *hem6*, a novel anemic mouse strain with possible defects in heme biosynthesis.[21]

ENU mutagenesis also produced the *mask* mouse strain. These mice carry a splice-site mutation leading to a truncated Tmprss6 transcript lacking the C-terminal catalytic domain. Characterization of these mice established Tmprss6 as an essential regulator of hepcidin, the peptide hormone that inhibits dietary iron absorption and macrophage iron efflux.[17] Soon after, the role of Tmprss6 in hepcidin suppression was confirmed by characterization of mice with genetically engineered deficiency in Tmprss6.[18] The mechanism of action of Tmprss6 was elucidated by a combination of *in vitro* and *in vivo* experiments demonstrating that Tmprss6 proteolytically cleaves the BMP co-receptor hemojuvelin from the cell membrane, thereby limiting hepcidin expression.[4,19] The relevance of TMPRSS6 to human iron metabolism was demonstrated concomitantly with the report of the *mask* mouse, as mutations in TMPRSS6 were identified in patients with IRIDA;[5] later, several genome-wide association studies established variants in TMPRSS6 as significant contributors to natural variation in red cell parameters and iron status in human populations.[40-46]

Here we report the establishment, characterization and study of the *hem8* mouse. We present evidence that the *Tmprss6* allele is hypomorphic, based on our tissue culture studies of Tmprss6 F286 activity (Figure 2F) and the milder defects in red cell parameters – MCV, MCH and reticulocyte counts – in *hem8* mice compared to *Tmprss6*$^{-/-}$ mice (Figure 3A-C). Given this, the *hem8* mouse has the first hypomorphic *Tmprss6* allele reported. We believe that the hypomorphic nature of this strain renders it potentially useful in ways not possible with other Tmprss6-null strains.

First, the *hem8* strain may prove useful in the identification of genes that modify the *Tmprss6* phenotype. Given the severity of the Tmprss6-null phenotype, QTL analysis in Tmprss6-null mice may only identify loci that attenuate the underlying phenotype. QTL analysis in a hypomorphic *Tmprss6* strain could identify loci that attenuate or worsen the *hem8* phenotype given its milder nature, as we demonstrated in our QTL analysis presented above. For example, QTL on chromosome 7 and 10 were associated with

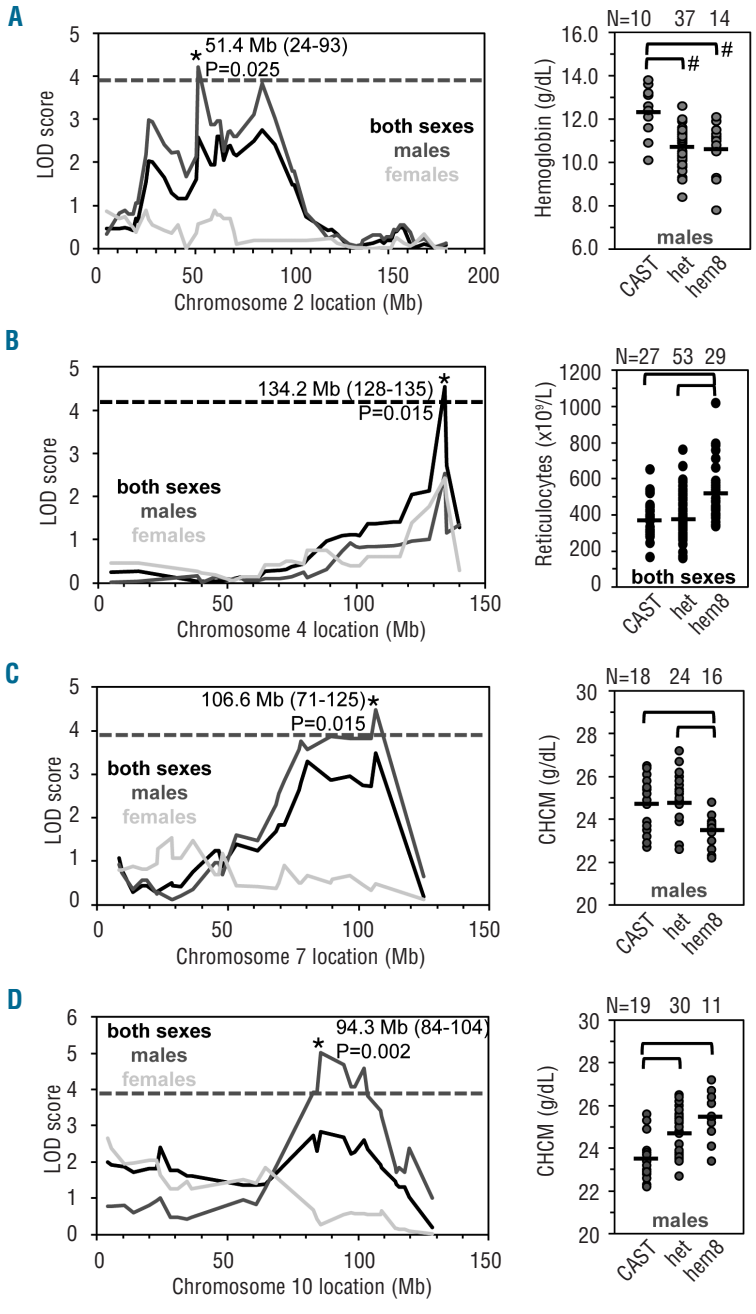

Figure 5. Quantitative trait locus (QTL) analysis in *hem8* mice. A 129S6B6F$_1$ *hem8* female mouse was mated to a CAST male mouse and anemic F$_2$ progeny were genotyped at 1 month of age using Illumina Mouse Medium Density Linkage Panel. Data were analyzed using open-source software R/QTL, single nucleotide polymorphism genotype data and phenotype data obtained from mice sacrificed at 1 month of age for: red cell parameters measured by complete blood count; body mass; liver and spleen mass; liver and spleen iron concentrations; liver RNA levels of hepcidin, Id1 and Bmp6 normalized to β-actin levels; liver RNA levels relative to liver iron levels. Males and females were analyzed as separate and combined groups. Results are shown for chromosome 2 hemoglobin QTL in male mice (A), chromosome 4 reticulocyte count QTL in all mice (B) and chromosome 7 (C) and 10 (D) corpuscular hemoglobin concentration mean (CHCM) QTL in male mice. For all panels on the left, chromosome location in Mb is plotted *versus* LOD score for both sexes (black line), male mice (dark gray line) and female mice (light gray line). Asterisks mark the peak at which LOD scores exceed genome-wide significance thresholds (dashed line); text adjacent to asterisks indicate LOD peak and 1.5-LOD interval in parentheses along with *P*-values. For all panels on the right, phenotypic data are plotted for mice of specific genotype at the single nucleotide polymorphism location indicated by asterisks in the graphs on the left: "CAST" and "hem8" refer respectively to mice with the same genotype as the CAST and *hem8* founders; "het" refers to mice carrying one allele from the CAST founder and one from the *hem8* founder. Circles indicate individual mice; horizontal bars indicate average values; brackets indicate statistical significance between groups, as measured by Student's t-test (unpaired; unequal variance). '#' indicates *P*-values of 0.002; all other brackets indicate *P*-values less than 0.001. Numbers above graphs on the right indicate number of individuals per genotype.

increased and decreased CHCM, respectively (Figure 5C,D). Second, the study of mice harboring Tmprss6 missense mutations typically provides more concrete evidence of the physiological significance of particular amino acid residues and protein domains than the study of patients with TMPRSS6 polymorphisms. In patients with IRIDA, it can be difficult to state with confidence that any particular polymorphism leads to functional Tmprss6 deficiency and IRIDA. A recent report indicates that the results of *in vitro* cleavage assays and hepcidin repression assays for Tmprss6 activity do not always agree,[16] further highlighting the need for caution when deciding whether a polymorphism is simply a normal variant or whether it alters Tmprss6 function significantly. With mice, a polymorphism of interest can be backcrossed for multiple generations as we did in this study. Tissue gene expression can be analyzed with relative ease to confirm that a linked mutation affecting gene expression levels is not responsible for the observed phenotype. A mouse harboring a missense mutation can be mated to a knock-out mouse line to test allelism. These and other studies provide a more in-depth analysis than is possible with patients' mutations.

Third, the study of mice harboring Tmprss6 missense mutations can provide unique data that studying mice harboring Tmprss6 deletions cannot. While splice-site or deletion mutations in mice are useful in that they typically result in loss-of-function alleles, amino acid substitutions such as the I286F studied here provide complementary information on the function of particular motifs or domains with a protein. Based on *in vitro* studies, we know that the N-terminal cytoplasmic domain is essential for Tmprss6 endocytosis[47] and that the extracytoplasmic SEA domain is required for catalytic activity of Tmprss6 but not membrane localization[13] while the LDL and serine protease

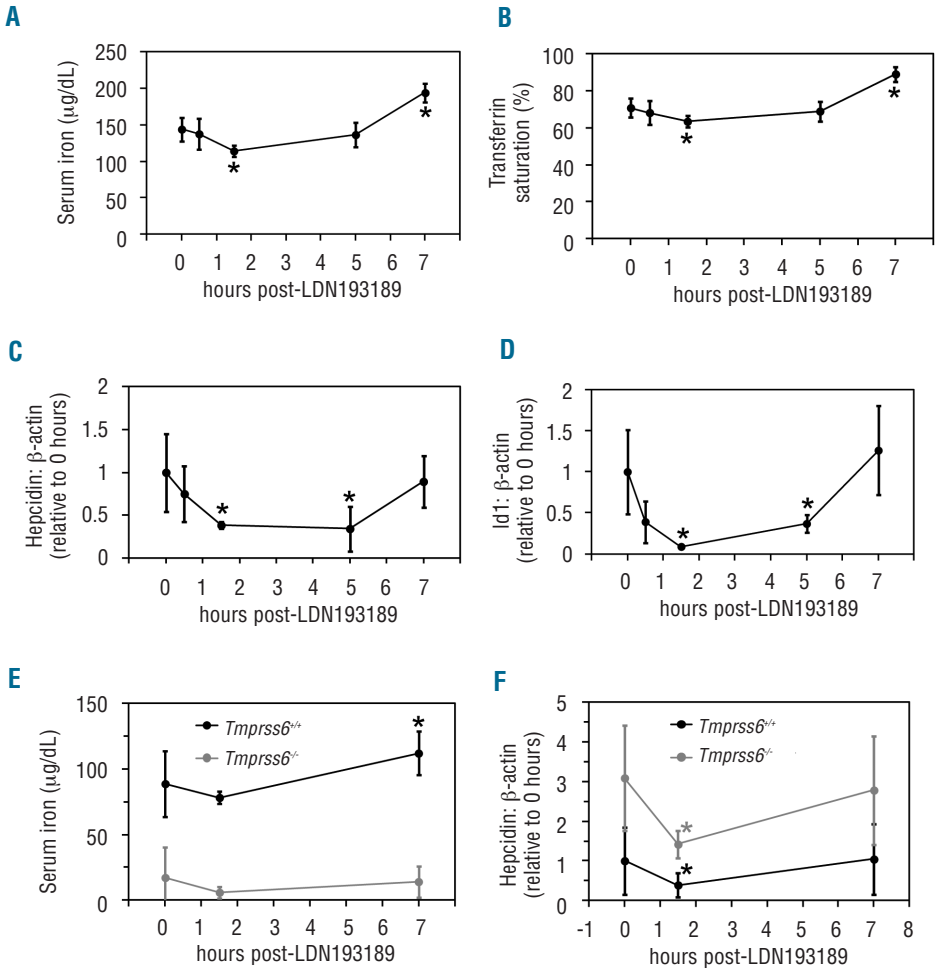

Figure 6. Pharmacological modulation of bone morphogenetic protein (BMP) signaling in mutant mice. (A-D) Wild-type female B6 mice at 2 months of age were treated with intraperitoneal injections of LDN-193189 at a dose of 3 mg/kg then harvested at 0-7 h post-injection and characterized for serum iron levels (A), transferrin saturation (B) and liver hepcidin (C) and Id1 (D) RNA levels relative to β-actin levels as measured by quantitative polymerase chain reaction. Each point represents five mice. Asterisks indicate that values differ significantly (Student's t-test; unpaired; unequal variance) from values at 0 h. Bars indicate standard deviations. (E-F) *Tmprss6*$^{+/+}$ and *Tmprss6*$^{-/-}$ mice at 2 months of age were treated similarly as mice in (A-D) and characterized for serum iron levels (E) and liver hepcidin RNA levels (F). Each point represents at least five female mice.

domains are required for membrane localization and cleavage activity.[4,8] The *hem8* mouse is essentially an *in vivo* structure-function model, demonstrating that the CUB1 domain is essential for Tmprss6 function.

Based on our *in vitro* studies and the milder change in red cell parameters in *hem8* mice relative to Tmprss6-null mice, we hypothesized above that the I286F substitution creates a hypomorphic allele of *Tmprss6*. However, there are features of *hem8* mice that are not strictly hypomorphic. For example, liver hepcidin RNA levels are significantly increased, and serum and liver iron levels significantly decreased, in both *hem8* and Tmprss6-null mice, yet liver Bmp6 RNA levels are decreased in Tmprss6-null but not in *hem8* mice. The reason for the unchanged Bmp6 levels in *hem8* mice is not clear at present. Other groups have shown that the increase in hepcidin levels in Tmprss6-null mice is mediated by Bmp6 *in vivo*[48] and that Bmp6 stimulates Tmprss6 expression in an Id1-dependent manner *in vitro*.[23] This led Meynard *et al.* to suggest that Tmprss6 is a component of a regulatory loop in which Bmp6 stimulates not only hepcidin expression but also Tmprss6 expression, thereby indirectly attenuating further hepcidin expression.[23] Perhaps Tmprss6 is also required for stimulation of Bmp6 and the *hem8* mutation presented here inhibits Tmprss6-dependent hepcidin expression but not Tmprss6-dependent Bmp6 expression; however, without supporting data, this point is purely speculative at this time.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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