Functional analysis of matriptase-2 mutations and domains: insights into the molecular basis of iron-refractory iron deficiency anemia

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McDonald CJ, Ostini L, Bennett N, Subramaniam N, Hooper J, Velasco G, Wallace DF, Subramaniam VN. Functional analysis of matriptase-2 mutations and domains: insights into the molecular basis of iron-refractory iron deficiency anemia. Am J Physiol Cell Physiol 308: C539–C547, 2015. First published January 14, 2015; doi:10.1152/ajpcell.00264.2014.—Mutations in the TMPRSS6 gene are associated with severe iron-refractory iron deficiency anemia resulting from an overexpression of hepcidin, the key regulator of iron homeostasis. The matriptase (MT)-2 protein (encoded by the TMPRSS6 gene) regulates hepcidin expression by cleaving hemojuvelin [HJV/hemochromatosis type 2 (HFE2)], a bone morphogenetic protein (BMP) coreceptor in the hepcidin regulatory pathway. We investigated the functional consequences of five clinically associated TMPRSS6 variants and the role of MT-2 protein domains by generating epitope-tagged mutant and domain-swapped MT-2-MT-1 (encoded by the ST14 gene) chimeric constructs and expressing them in HepG2/C3A cells. We developed a novel cell culture immunofluorescence assay to assess the effect of MT-2 on cell surface BMP expression levels, compatible with HIV cleavage. The TMPRSS6 variants Y141C, I212T, G442R, and C510S were retained intracellularly and were unable to inhibit BMP6 induction of hepcidin. The R271Q variant, although it has been associated with iron-refractory iron deficiency anemia, appears to remain functional. Analysis of the chimeric constructs showed that replacement of sperm protein, enterokinase, and agrin (SEA), low-density-lipoprotein receptor class A (LDLRA), and protease (PROT) domains from MT-2 with those from MT-1 resulted in limited cell surface localization, while the complement C1r/C1s, Uegf, Bmpr1 (CUB) domain chimera retained localization at the cell surface. The SEA domain chimera was able to reduce cell surface HIV expression, while the CUB, LDLRA, and PROT domain chimeras were not. These studies suggest that the SEA and LDLRA domains of MT-2 are important for trafficking to the cell surface and that the CUB, LDLRA, and PROT domains are required for cleavage of HIV.

of absorption and recycling: increased expression of hepcidin leads to decreased duodenal iron absorption and recycling of iron by macrophages (22), which reduces iron availability within the body, lowering iron status. The growth factor BMP6 is upregulated in response to increased iron status and signals through SMAD1/5/8 to induce hepcidin expression (21). MT-2 exerts its influence as a regulator of this pathway by cleaving hemojuvelin [HJV/hemochromatosis type 2 (HFE2)], a cell surface BMP coreceptor required for transmission of the BMP signaling cascade through phosphorylation of SMAD1/5/8 to hepcidin (28). Loss-of-function mutations, in which MT-2 is rendered unable to cleave HJV, allow constitutive signaling through the BMP6-SMAD1/5/8 pathway, leading to excessive expression of hepcidin, thus restricting iron absorption and recycling and resulting in iron-refractory iron deficiency anemia (IRIDA) (9, 24).

The role of MT-2 as a key negative regulator of hepcidin and the impact of loss-of-function mutations have led to the proposal that MT-2 is a target for the pharmaceutical treatment of iron overload due to low hepcidin (25). Two studies have shown that inhibition of MT-2 function, through mRNA degradation techniques, can reduce iron overload in mouse models of HFE-associated hereditary hemochromatosis and β-thalassemia (11, 26). Both approaches resulted in restoration of normal hepcidin levels and demonstrate the potential of MT-2 as a target in the treatment of iron overload. Both of these studies targeted mRNA degradation, effectively reducing the overall levels of MT-2 protein; however, an understanding of the roles of the separate domains of the protein may provide targets for alternate methods of inhibition in cases of iron overload or increased activity in cases of IRIDA. While the role of the trypsin-like serine peptidase [protease (PROT)] domain and key functional amino acids involved in HIV cleavage have been clarified (2, 28), the functional roles of the remaining domains, sperm protein, enterokinase, and agrin (SEA), complement C1r/C1s, Uegf, Bmpr1 (CUB), and low-density-lipoprotein receptor class A (LDLRA), of the MT-2 protein are less clear.

In this study we examined the functional consequences of a series of mutations that have been reported to be associated with IRIDA and are distributed throughout the MT-2 protein. We further investigated the contribution of the four major MT-2 protein domains to its function through domain-swap experiments, in which each of the SEA, CUB, LDLRA, and PROT domains of the MT-2 protein were substituted with the homologous domains from the closely related MT-1 protein (encoded by the ST14 gene). These studies suggest that there
are two potential factors that affect functionality of MT-2, appropriate localization and enzymatic capacity, and that the domain localization of mutations may provide a good basis for the expected functional consequence of an identified mutation.

**MATERIALS AND METHODS**

Generation of TMPRSS6 mutant constructs. The wild-type (WT) TMPRSS6 construct, which is described elsewhere (30), contains the TMPRSS6 coding sequence with a COOH-terminal FLAG tag in the pcDNA3.1 vector. Site-directed mutagenesis was used to introduce the TMPRSS6 disease, causing the following mutations: Y141C, I212T, R271Q, G442R, and C510S. The primers listed in Table 1 were used to introduce the mutations according to the method of Scott et al. (27). The presence of mutations was confirmed by the Sanger method of DNA sequencing.

Generation of TMPRSS6-ST14 domain-swap constructs. The WT ST14 construct, which is described elsewhere (13), contains the ST14 coding sequence in the pcDNA3.1 vector. Recombination cloning using an In-Fusion HD cloning kit (Clontech, Mountain View, CA) was used to replace the SEA, CUB, LDLRA, and PROT domains of TMPRSS6 with the homologous domains of ST14. The primers listed in Table 2 were used to effect recombination of the desired domains according to the manufacturer’s protocol. Correct recombination of the domain-swap constructs was confirmed by the Sanger method of DNA sequencing.

Generation of HJV construct. WT HJV (transcript variant A; gene ID no. NM_213653.3) minus the signal peptide was amplified from human skeletal muscle cDNA using the primer pairs listed in Table 1 and cloned into the Mlu I site of the pEF-IRE5-P vector (14), engineered to contain the IL-3 signal peptide followed in-frame with a Myc tag, to create NH2-terminal Myc-tagged mature HJV.

**Transfections and BMP6 treatment.** A clonal colony of HepG2/C3A cells (catalog no. CRL-10741, American Type Culture Collection, Manassas, VA) stably expressing HJV (designated C3A/HJV) was generated by transfection with WT HJV using TransIT-LT1 (Minus Bio, Madison, WI). Cells were placed under selection using MEM + GlutaMAX, supplemented with 10% fetal calf serum and puromycin at 5 μg/ml. A colony originating from a single cell was selected for expansion. Positive selection was maintained with 1 μg/ml puromycin. Transient transfection of the various constructs was accomplished by reverse transfection in collagen-coated 6- or 12-well plates using Lipofectamine 3000 (Invitrogen, Mount Waverley, VIC, Australia) according to the manufacturer’s instructions. For BMP6 treatment, cells were serum-deprived for 6 h in Opti-MEM (Invitrogen) before addition of recombinant human BMP6 (R & D Systems, Minneapolis, MN) at 10 ng/ml for 1 h. HepG2/C3A cells were selected for this study, as the impact of mutated MT-2 protein on hepcidin antimicrobial peptide (HAMP) regulation could be studied endogenously without the need for reporter assays. Furthermore, these cells endogenously express the hepatocyte growth factor activator inhibitor (HAI)-1 protein, which is required for correct surface localization of the MT-1 protein and is important for the MT-2/MT-1 domain-swap studies (12).

**Immunofluorescence analysis.** Transfected cells were grown on collagen-coated glass coverslips for 48 h. For permeabilized labeling, cells were treated as previously described (20) using the primary antibodies anti-Myc (1:100 dilution; catalog no. 71D10, Cell Signaling Technology, Danvers, MA) and anti-FLAG (1:1000 dilution; catalog no. M2, Sigma-Aldrich, Castle Hill, NSW, Australia). Cells were then incubated with secondary antibody [donkey anti-mouse Alexa 488 or donkey anti-rabbit Alexa 594 (Invitrogen) diluted in fluorescein dilution buffer (5% fetal bovine serum, 5% normal donkey serum, and 2% bovine serum albumin in PBS, pH 7.6)] for 45 min. For surface-specific labeling, cells were treated, as previously described (31), with the antibodies described above. After final washes, coverslips were mounted using ProLong Gold Antifade mounting medium with 4',6-diamidino-2-phenylindole (Invitrogen).

**Table 1. Mutagenesis, cloning, and quantitative PCR primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Y141C</td>
<td>ACTCCAGCTCGTCGTCGTTCTTGGGGAGGG</td>
<td>CTCGCCAGAAGAGAGGACAGGAGGTGGAGT</td>
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<tr>
<td>I212T</td>
<td>GCCAGTGGGAAGACAGACCTGGTAAATCCCAG</td>
<td>GTGGTATTCTAGCCGATGCTGTCTTCACTGTCG</td>
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<tr>
<td>R271Q</td>
<td>CGAGTCGCCGGCGACAGCTGCGATGTAGTA</td>
<td>TCTATAGCGCGTGCTTGGCCGACAGTTG</td>
</tr>
<tr>
<td>G442R</td>
<td>CAGATCTCGCTCAGAGCGCGGCGGCGGCG</td>
<td>CCGGACAGCGCGGTGCTGAGGAGATCTG</td>
</tr>
<tr>
<td>C510S</td>
<td>CTCAACTGCCAAGCTGTCGATGCGGGCGG</td>
<td>TCCAGGGGCAAGGGTTACTGGAAGAAAG</td>
</tr>
<tr>
<td>HJV</td>
<td>CACAAAACGCCTAATGGAAGAATCCTGCA</td>
<td>CGACGGCAGCCTTTACTGAAGAAGAGAAG</td>
</tr>
<tr>
<td>ACTB</td>
<td>GCCAGCAGGGGGCGTG</td>
<td>GCCCATAGGAATCTCTTGGTA</td>
</tr>
<tr>
<td>HAMP</td>
<td>CCAACAGAACGCGGGAAC</td>
<td>AAAATGCAGATGGGAGAAGT</td>
</tr>
<tr>
<td>HPRT</td>
<td>GAAAAGGGTTGTATTCTTCCATC</td>
<td>CCACATCTCCTCATGACAT</td>
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**Table 2. MT-2-MT-1 domain-swap recombination primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Vector/MT-2 F</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>MT-2 SEA 5' F</td>
<td>GACCATCAGCTGCTGCTGCTGAA</td>
</tr>
<tr>
<td>Vector/MT-2 R</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>MT-2 SEA 3' R</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>Vector/MT-2 F</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>MT-2 CUB 5' F</td>
<td>GACCATCAGCTGCTGCTGCTGAA</td>
</tr>
<tr>
<td>Vector/MT-2 R</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>MT-2 CUB 3' R</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>Vector/MT-2 F</td>
<td>GACCATCAGCTGCTGCTGCTGAA</td>
</tr>
<tr>
<td>MT-2 LDLRA 5' F</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>Vector/MT-2 R</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
<tr>
<td>MT-2 LDLRA 3' R</td>
<td>TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA</td>
</tr>
</tbody>
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**Domain**

- **SEA domain**
  - Vector/MT-2 F: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 SEA 5' F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 SEA 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 CUB 5' F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 CUB 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 LDLRA 5' F: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 LDLRA 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA

- **CUB domain**
  - Vector/MT-2 F: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 SEA 5' F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 SEA 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 CUB 5' F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 CUB 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 LDLRA 5' F: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 LDLRA 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA

- **PROT domain**
  - Vector/MT-2 F: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 SEA 5' F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 SEA 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 CUB 5' F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 CUB 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 F: GACCATCAGCTGCTGCTGCTGAA
  - MT-2 LDLRA 5' F: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - Vector/MT-2 R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA
  - MT-2 LDLRA 3' R: TGGAGGATGCTAGAGGCAGGTGGCGTGCTGACCCA

**MT, matriptase; F, forward; R, reverse; SEA, sperm protein, enterokinase; and agrin; CUB, complement C1r/C1s, Uegf, Bmp1; LDLRA, low-density-lipoprotein receptor class A; PROT, protease.**

**HJV, hepcidin; ACTB, β-actin; HAMP, hepcidin antimicrobial peptide; HPRT, hypoxanthine phosphoribosyltransferase.** Boldface indicates the nucleotide substituted to introduce the relevant point mutation. 
Western blotting. Cells were lysed in SDS sample buffer [50 mm Tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol], electrophoresed on a 7.5% or 10% SDS-polyacrylamide gel, and transferred onto Hybond-C+ membrane. Blots were blocked in 10% skim milk powder in 0.1% Tween 20 in Tris-buffered saline (blocking buffer) for 2.5 h at room temperature. Primary antibodies [anti-FLAG (1:2,000 dilution; Sigma-Aldrich), anti-Myc (1:10,000 dilution; catalog no. 9B11, Cell Signaling Technology), anti-MT/ST14 (1:8,000 dilution; Bethyl Laboratories, Montgomery, TX), and anti-actin (1:40,000 dilution; Sigma-Aldrich)] were diluted in blocking buffer and applied to the blot overnight at 4°C. The blots were washed in 0.1% Tween 20 in Tris-buffered saline and then incubated with anti-rabbit or anti-mouse horseradish peroxidase (In-vitrogen) for 1 h at room temperature. The blots were washed again, chemiluminescence substrate (catalog no. WBLUF0500, Lumina Forte, Merck Millipore) was applied, and the blots were exposed to film. The film was scanned on a ScanMaker 9800XL (Microtek, Taipei, Taiwan) with ScanWizard Pro v7.0.22 in positive-film-density mode. Band volume and density were quantified using SynGene GeneTools v4.0 (Synoptics, Cambridge, UK).

RESULTS

MT-2 mutations affect surface localization. To investigate the effect of the IRIDA-causing mutations Y141C, I212T, R271Q, G442R, and C510S on trafficking of the MT-2 protein, we transiently transfected HepG2/C3A cells with WT or mutant expression constructs. Expression of the correctly sized proteins was confirmed by Western blotting (data not shown). All six constructs expressed appropriately and showed predominantly intracellular localization when visualized by whole cell immunofluorescence (IF; Fig. 1A). As MT-2 is functional at the cell surface, we also assessed localization using cell surface-specific IF. While protein expressed by all constructs was identifiable at the cell surface, the various mutations led to significant differences in the percentage of cells showing surface expression. To investigate this further, the number of cells with detectable protein expression were counted within the whole cell and cell surface-specific IF (Fig. 1B). This process identified more WT MT-2-expressing cells in the cell surface-specific than whole cell IF, indicating that the protein is effectively transported to the surface. For standardization of surface localization, WT was normalized to 100%. Far fewer cells expressing the Y141C mutant where identified by cell surface-specific than whole cell IF (14%), indicating a significant reduction in the surface localization of this mutant protein compared with WT (P < 0.0001, by 1-way ANOVA with Tukey’s multiple comparison test). Similarly, the I212T (33%, P < 0.0001), G442R (41%, P = 0.0002), and C510S (16%, P < 0.0001) mutants showed significantly reduced localization at the cell surface compared with WT. The R271Q mutant showed a 94% surface localization, similar to that of cells transfected with WT (P = 0.87), suggesting normal trafficking of the R271Q mutant protein.

Some MT-2 mutants retain the capacity to affect surface HJV expression when they achieve cell surface localization. WT and mutant TMPRSS6 constructs were transfected into C3A/HJV cells, and the capacity of the overexpressed MT-2 proteins to affect cell surface HJV was investigated. In cells expressing WT MT-2, staining of cell surface HJV was undetectable, while its expression was not affected in surrounding untransfected cells, which is compatible with cleavage of HJV by WT MT-2 (Fig. 2). The Y141C mutant failed to effectively achieve cell surface localization (Fig. 1) to any significant extent and, as such, failed to show any reduction in cell surface HJV. In the limited number of cells for which the I212T and G442R mutants achieved surface localization, the mutant proteins retained some capacity to affect HJV, with a clear reduction in the amount detectable on the cell surface. The C510S mutant again showed very limited surface expression; however, unlike the I212T and G442R mutants, it showed no capacity to alter HJV surface expression, even when localized at the cell surface. The R271Q mutant again appeared more similar to WT than the other mutants, with significant surface localization and effective reduction of surface HJV in the cells in which it was expressed. To confirm that a loss of surface HJV in cells transfected with WT MT-2 correlated with a reduction in full-length HJV, compatible with cleavage of HJV, whole culture samples were subjected to Western blot analysis (Fig. 3A). Quantification of the Western blots showed that expression of WT MT-2 correlated with reduced levels of full-length cellular HJV protein (Fig. 3B). Cells transfected with the C510S mutant did not show reduced levels of cellular full-length HJV.

Functional mutations fail to inhibit hepcidin induction. As MT-2 cleaves HJV, which in turn affects hepcidin expression, we used quantitative PCR to assess the functional impact of these mutations on the regulation of HAMP expression. C3A/HJV cells were again transfected with WT or mutant TMPRSS6 constructs. At 17 h after transfection, the cells were serum-starved for 6 h to reduce the basal level of HAMP expression due to serum factors and then treated with 10 ng/ml recombinant BMP6 for 1 h. After serum starvation, all vehicle-treated cells showed low levels of HAMP mRNA, which were unaffected by transfection with WT or mutant TMPRSS6 constructs (Fig. 4). In control mock-transfected cells, treatment with BMP6 induced a 37-fold induction of HAMP mRNA expression. BMP6 treatment of cells transfected with WT MT-2 induced only a 24-fold induction of HAMP mRNA expression, 36% lower than in mock-transfected cells (P = 0.006, by 2-way ANOVA with Tukey’s multiple comparison test). HAMP induction was inhibited to a similar extent by the
R271Q construct, with a 25-fold induction following BMP6 treatment, 33% lower than in mock-transfected cells ($P = 0.01$). Comparatively, the Y141C, I212T, and C510S mutants failed to significantly inhibit the induction of $HAMP$ in response to BMP6, showing 35-, 33-, and 35-fold induction, respectively, and no significant difference compared with mock-transfected cells ($P = 0.956$, $P = 0.778$, and $P = 0.993$, respectively). The G442R mutant induced a 29-fold induction in $HAMP$ mRNA expression in response to BMP6 treatment, placing it midway between mock-transfected and WT MT-2-transfected cells and showing no statistical difference from either group ($P = 0.11$ vs. mock-transfected cells, $P = 0.65$ vs. WT). This suggests that the Y141C, I212T, and C510S MT-2 mutants show significantly impaired function, as they failed to inhibit $HAMP$ induction by BMP6; however, the G442R mutant shows a partial inhibition of function, and the R271Q mutant appears to retain WT-equivalent levels of function.

Replacement of MT-2 protein domains with homologous MT-1 domains affects localization and ability to reduce $HJV$ surface expression. We next investigated the contribution of various MT-2 protein domains to its localization and function. We generated four constructs, each containing the MT-2 protein with a single domain [SEA, amino acids (aa) 84–162; CUB, aa 243–452; LDLRA, aa 457–569; and PROT, aa 558–811] replaced by its homologous domain from the closely related MT-1 protein [SEA, aa 85–162 (23.3% identity, 36.6% similarity); CUB, aa 214–447 (16.7% identity, 30.8% similarity); LDLRA, aa 452–560 (35.7% identity, 44.4% similarity); and PROT, aa 615–854 (38.2% identity, 51.5% similarity); Fig. 5A]. Correct assembly of these DNA constructs was confirmed by the Sanger sequencing method, and expression of the correctly sized proteins was confirmed by Western blotting (Fig. 5B). After transfection into C3A/HJV cells, the cellular localization of the domain-swapped constructs was determined by intracellular and cell surface IF. There was significant intracellular expression of the WT MT-2 construct (Fig. 5C).

Replacement of the MT-2 SEA domain with the MT-1 SEA domain resulted in lower surface localization of the protein, as seen by cell surface-specific IF (Fig. 6A). However, where the protein did reach the cell surface, it retained the ability to affect $HJV$, as seen by a loss of surface expression specifically in MT-2 construct-expressing cells (Fig. 6B; $P = 0.398$ vs. WT, by 1-way ANOVA with Dunnett’s multiple comparison test). MT-2 protein containing both of the MT-1 CUB domains showed predominant surface localization. However, despite the
correct localization, the capacity of MT-2 protein to affect HJV surface expression was significantly reduced relative to WT cells \((P = 0.016)\). The LDLRA domain-swap protein showed only limited cell surface localization and a significantly reduced capacity to affect HJV surface expression, even when its surface localization was achieved \((P = 0.001)\). Both the PROT domain-swap construct and the WT MT-1 construct achieved cell surface localization and reduced HJV surface expression in \(\sim 20\%\) of cells, significantly less than WT MT-2 \((P = 0.005\) and \(P = 0.027\), respectively).

Fig. 2. Coexpression of MT-2 and hemjuvelin (HJV) indicates reduced HJV cleavage by some mutants. Cell surface-specific IF was performed on HepG2/C3A cells stably expressing HJV and transiently transfected (48 h) with TMPRSS6 constructs expressing WT protein or the Y141C, I212T, R271Q, G442R, or C510S mutation. Cells expressing WT MT-2 showed a loss of cell surface HJV, as did cells expressing the R271Q protein. I212T and G442R showed reduced catalytic efficiency, with retention of some HJV on the surface of coexpressing cells; C510S showed a complete loss of catalytic capacity, as indicated by the presence of surface HJV on all MT-2-expressing cells. Scale bar = 50\,\mu\text{m}.

Fig. 3. Expression of WT MT-2, but not mutant C510S, leads to a reduction in cellular HJV. A: Western blotting of stably expressed HJV protein. Expression of the WT MT-2 in transfected HepG2/C3A cells reduced the level of detectable cellular HJV protein, whereas expression of the C510S mutant did not. Cont, control. B: quantification of Western blot in A. Experiments were repeated 3 times; representative results are shown. UT, untransfected.

Fig. 4. Y141C, I212T, and C510S mutations fail to inhibit hepcidin anti-microbial peptide (HAMP) upregulation in response to treatment with bone morphogenetic protein-6 (BMP6). HJV-expressing HepG2/C3A cells were transiently transfected (17 h) with TMPRSS6 constructs expressing WT protein or the Y141C, I212T, R271Q, G442R, or C510S mutation. Cells were then serum-starved for 6 h and treated with 10 ng/ml BMP6 for 1 h to induce HAMP expression. Resulting mRNA expression of HAMP was measured by quantitative PCR. Values are means \(\pm\) SE. Statistical significance was assessed by 2-way ANOVA with Tukey’s multiple comparison posttests: \(*P < 0.001\) vs. UT.
We have performed a systematic analysis of the functional effects of IRIDA-causing mutations in MT-2, and we have examined the roles of the various MT-2 protein domains in the trafficking and function of the protein. Our data demonstrate that mutations in MT-2 can cause intracellular retention of the protein or an impaired ability to decrease HJV expression at the cell surface, a function compatible with proteolytic cleavage. Recently, Jiang et al. (16) reported that N-linked glycosylation played an important role in MT-2 activation. We have shown that, at lower levels of expression, the Y141C, I212T, G442R, and C510S mutants are predominantly retained intracellularly, probably in the endoplasmic reticulum (ER), and are not transported to the cell surface, whereas the R271Q mutant shows WT-like cell surface localization. Furthermore, our studies suggest that an ER retention threshold appears to be reached in some cells, resulting in protein escape from the ER and trafficking to the cell surface. Our novel cell culture IF assay for HJV and MT-2 shows that when this occurs, even at low levels of surface localization, a reduction in surface HJV, compatible with HJV cleavage, can still be achieved by some of these mutant proteins. This is the first time that the loss of cell surface HJV has been shown directly in cells expressing MT-2. Of the MT-2 mutants that reached the surface in a limited number of cells, most likely due to ER overload, I212T and G442R retained the ability to
reduce HJV surface expression. This result for the I212T mutant is in contrast to an earlier report by De Falco et al. (5) of a deficiency in proteolytic activity of the I212T mutant. A notable difference between these results, however, is that, in our study, functional capacity is assessed only in cells in which there is surface expression of the protein. Cell surface localization for the Y141C mutation was minimal, and it was not possible to definitively determine whether the Y141C mutation retained the ability to reduce HJV surface expression. In the original case report by Altamura et al. (1), however, where a strong overexpression system drove significant surface localization, the Y141C protein failed to cleave HJV. One mutation that has been associated with IRIDA (R271Q) appeared to have no defects in its ability to traffic to the cell surface or its ability to reduce HJV surface expression, a finding that confirms an earlier functional assessment of this mutation by De Falco et al. This suggests that 1) any functional consequences of the R271Q mutation are beyond the detection thresholds of the experiments we performed or 2) the R271Q mutation has a minimal effect or no effect on MT-2 trafficking or function. In IRIDA patients, the R271Q mutation has only been reported in the compound heterozygous state with the I212T mutation (5); hence, whether it can cause a profound IRIDA phenotype when it is in the homozygous state is not known.

A potentially therapeutically relevant outcome from our MT-2 overexpression studies is that some mutant proteins that ordinarily do not reach the cell surface retain their catalytic activity and ability to affect HJV surface expression when they do reach the surface. This situation is similar to that in some forms of cystic fibrosis. The common ΔF508 mutation in CFTR results in ER retention and subsequent degradation of the protein, but the ability of the mutant CFTR protein to transport chloride is retained (6). Several studies have shown that in cell and mouse models the transport defect of the ΔF508 mutant can be overcome to increase surface localization of the protein and restore function (3, 8). It is possible that rescue of transport-defective MT-2 mutants that retain catalytic function could be achieved in a similar way.

Across the type II transmembrane serine protease (TTSP) family, 11 different domains with various functions are contained in various combinations within their stem regions. Specifically of interest here is a subgroup of three proteins, MT-1, MT-2, and MT-3 (encoded by the TMPRSS7 gene), which contain a SEA domain followed by 2 CUB domains, multiple LDLRA domains, and, finally, the peptidase S1 domain. Despite the conserved structure of this group and the presence of these domains across the TTSP family, little is known about the specific functional contributions of the SEA, CUB, and LDLRA domains in these proteins.

Fig. 6. Domain replacement differentially affects protein localization and cleavage ability. A: cell surface-specific IF was performed on HepG2/C3A cells stably expressing HJV and transiently transfected (48 h) with TMPRSS6 constructs expressing WT protein or the SEA-, CUB-, LDLRA-, or PROT-domain swap. Scale bar = 50 μm. B: cell surface IF was used to assess for expression of HJV in TMPRSS6-expressing cells. Surface-expressing cells were counted, and the presence or absence of HJV on the cell surface was determined, with absence compatible with cleavage. Data are shown as box-and-whisker plots, showing median values, 5th–95th percentile, and range. Statistical significance was assessed by 1-way ANOVA with Dunnett’s multiple comparison posttests; *P < 0.05 vs. WT MT-2.
In some cases, the SEA domain has been shown to be required for autoproteolysis, producing a soluble protein, although there is no evidence of this within the MT proteins (17). Reports on clinically associated IRIDA-causing mutations within the SEA domain indicate reduced autoactivation of the MT-2 protein, which leads to a failure to cleave HJV (1, 24).

Our experiments with complete replacement of the SEA domain of MT-2 with the SEA domain of MT-1 led to reduced surface localization; however, this domain swap did not appear to have a significant effect on the protein’s ability to reduce HJV surface expression. This suggests that a structurally correct SEA domain, rather than specifically the MT-2 SEA domain, is important for autoactivation and indicates that the SEA domain is not likely involved in the MT-2-HJV association.

In contrast to the SEA domain, replacement of the CUB, LDLRA, and PROT domains with the homologous domains of MT-1 reduced the ability of the enzyme to decrease HJV surface expression to the same minimal levels as seen with WT MT-1 protein (only 20% of that seen with WT MT-2). The reduction in HJV surface expression mediated by the MT-1 protein provides further evidence of the capacity for nonspecific cleavage of HJV by peptidase S1-containing proteins in an overexpression system, further supporting our observation that some point mutations, which have reduced surface localization with lower levels of expression, retain their ability to cleave if overexpression allows escape from the ER. This failure of these domain swaps to cleave HJV above the nonspecific level of MT-1 indicates that these domains are required for autocatalytic cleavage of thezymogen or provide a degree of specificity for HJV. Interestingly, replacement of the CUB domain did not significantly affect surface localization of the protein, and given that the critical residues for autocatalytic activation fall significantly outside the CUB domains, we suggest that the CUB domain is required for the MT-2-HJV interaction. Indeed, the proteolytic activity of MT-1 is inhibited by HAI-1 and HAI-2, which have been shown to bind the CUB domains of the MT-1 protein (15, 29). HAI-2 has also recently been shown to inhibit the proteolytic activity of MT-2, demonstrating the requirement of an available CUB domain for effective cleavage of HJV (18).

The stem regions of MT-2, including the SEA, CUB, and LDLRA domains, are expected to contribute to the proteolytic cleavage of HJV through localization, substrate association, and autocatalytic cleavage, to produce proteolytically active protein. The limited, although identifiable, capacity of MT-1 to cleave HJV demonstrates a proteolytic capacity of its protease S1 domain to cleave HJV, although with limited efficiency. It is interesting then that, with the construct combining the MT-2 stem region with the MT-1 protease S1 domain (PROT), HJV cleavage capability, as assessed by the ability to reduce HJV surface expression, remains only equal to WT MT-1 cleavage capacity. This suggests that the PROT domains of the MT family confer specificity for their substrates.

In conclusion, we have shown that IRIDA-causing mutations in MT-2 can affect the localization of the protein, the ability to reduce surface expression of its substrate HJV, or both. The net effect of these structural changes is the reduced ability of MT-2 to suppress signaling through the BMP-SMAD pathway to downregulate hepcidin. Similar effects were seen when the various MT-2 domains were replaced with the homologous domains of MT-1 and suggest that the CUB domains of MT-2 are important for the interaction and specificity for its substrate HJV. This information will be important for understanding the biology of MT-2 and developing potential therapeutics for the targeting of the MT-2-HJV interaction for the treatment of iron disorders such as hereditary hemochromatosis and thalassemia.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
C.J.M., J.H., D.F.W., and V.N.S. developed the concept and designed the research; C.J.M., L.O., N.C.B., N.S., and G.V. performed the experiments; C.J.M., L.O., N.C.B., N.S., D.F.W., and V.N.S. analyzed the data; C.J.M., L.O., N.S., J.H., D.F.W., and V.N.S. interpreted the results of the experiments; C.J.M. prepared the figures; C.J.M. drafted the manuscript; C.J.M., L.O., N.C.B., N.S., J.H., D.F.W., and V.N.S. edited and revised the manuscript; C.J.M., L.O., N.C.B., N.S., J.H., G.V., D.F.W., and V.N.S. approved the final version of the manuscript.

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