Matriptase-2 (MT2) is a type-II transmembrane, trypsin-like serine protease that is predominantly expressed in the liver. It is a key suppressor for the expression of hepatic hepcidin, an iron-regulatory hormone that is induced via the bone morphogenetic protein signaling pathway. A current model predicts that MT2 suppresses hepcidin expression by cleaving multiple components of the induction pathway. MT2 is synthesized as a zymogen that undergoes autocleavage for activation and shedding. However, the biologically active form of MT2 and, importantly, the contributions of different MT2 domains to its function are largely unknown. Here we examined the activities of truncated MT2 that were generated by site-directed mutagenesis or Gibson assembly master mix, and found that the stem region of MT2 determines the specificity and efficacy for substrate cleavage. The transmembrane domain allowed MT2 activation after reaching the plasma membrane, and the cytoplasmic domain facilitated these processes. Further in vivo rescue studies indicated that the entire extracellular and transmembrane domains of MT2 are required to correct the low-hemoglobin, low-serum iron, and high-hepcidin status in MT2−/− mice. Unlike in cell lines, no autocleavage of MT2 was detected in vivo in the liver, implying that MT2 may also function independently of its proteolytic activity. In conjunction with our previous studies implicating the cytoplasmic domain as an intracellular iron sensor, these observations reveal the importance of each MT2 domain for MT2-mediated substrate cleavage and for its biological function.

Matriptase-2 (MT2) is a member of the matriptase sub-group that belongs to the type II transmembrane serine protease family (1). It is encoded by the TMPRSS6 gene in humans and Tmprss6 gene in mice. For the sake of simplicity, MT2 will be used for the gene and MT2 for the protein throughout the text. MT2 is expressed predominantly in hepatocytes (1, 2), and MT2 acts as an essential suppressor for the expression of hepcidin, an iron regulatory hormone that is also predominantly expressed in hepatocytes under nonpathological conditions (3, 4). Mutations in MT2 in humans result in an inappropriately high-hepcidin expression, which leads to iron-refractory iron-deficiency anemia (IRIDA) (3–5). Similar phenotypes are also reported in mouse models either with a global MT2 knockout (MT2−/−) or with a truncated MT2 that lacks the serine protease (S/P) catalytic domain (mask mice) (6–9). Hepcidin inhibits iron efflux from duodenal epithelial cells, macrophages, and hepatocytes into the circulation by targeting the plasma membrane iron exporter, ferroportin, for degradation (10). Lack of hepcidin causes juvenile hemochromatosis, a severe form of iron overload (11, 12). Under physiological conditions, hepcidin expression is regulated positively by body iron content to maintain iron homeostasis.

Hepatic hepcidin expression is induced via the bone morphogenetic protein (BMP) signaling pathway (13–15). BMP signaling is initiated upon the binding of BMP ligands to type-I and type-II BMP receptors on the cell surface. The BMP2/6 ligands that are responsible for the induction of hepcidin expression in hepatocytes are derived primarily from the adjacent hepatic endothelial cells (16–19). There are multiple type-I BMP receptors (ALK1, ALK2, ALK3, and ALK6) and type-II BMP receptors (BMPR2, ActRIIA, and ActRIIB). Studies in animal models demonstrate that hepatocytes utilize a selective set of BMP receptors, including ALK2, ALK3, BMPR2, and ActRIIA, to induce hepcidin expression (20, 21). Importantly, a normal range of hepcidin expression also requires the involvement of other plasma membrane proteins, including hemojuvelin (HJV), hemochromatosis protein (HFE), transferrin receptor-2 (TfR2), and neogenin (3). In the liver, HJV and TfR2 are exclusively expressed in hepatocytes. HFE is predominantly expressed in hepatocytes, and neogenin is also highly

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2 The abbreviations used are: MT2, matriptase-2; AAV8, adeno-associated virus 8; BMP, bone morphogenetic protein; CM, conditioned medium; CUB, complement protein subcomponents C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1 domain; Cyto, cytoplasmic domain; Ecto, ectodomain; FCI, furin convertase inhibitor; GPI, glycosylphosphatidylinositol; HAI-2, hepcocyte growth factor-activator inhibi-
expressed in hepatocytes (22, 23). Mutations in the HJV gene in humans markedly reduce hepcidin expression in the liver and result in juvenile hemochromatosis (24). Mutations in the HFE and TFR2 genes also decrease hepcidin expression and cause type-I hemochromatosis, the most common form of hereditary iron overload, and type-III hemochromatosis, respectively (12). All these defects have been documented in animal models. Knockout of these genes recapitulate the human mutation phenotypes, indicating that the mutations impart a lack of function.

MT2 is a trypsin-like type-II transmembrane serine protease composed of a short cytoplasmic domain, a transmembrane domain, and a large extracellular domain, which contains a membrane-proximal stem region, a predicted cleavage-activation site, and a catalytic domain (C-terminal S/P domain) (Fig. 1A) (1, 2). Early studies suggested that MT2 suppresses hepcidin expression solely by cleaving HJV (8, 25). Our recent data showed that MT2 suppresses hepcidin expression independently of Hjv in vivo and that it cleaves multiple components of the hepcidin-induction pathway, including BMP receptors (ALK2, ALK3, ActRIIA, and Bmp2), Hfe, and to a lesser extent, Hjv and Tf2, in vitro (9). Consistent with these latter observations, MT2−/− mice have a lower, rather than higher, level of Hjv protein in the liver (26, 27). Thus it is likely that MT2 suppresses hepcidin expression by cleaving and inactivating multiple components of the hepcidin-induction pathway.

MT2 is synthesized as a zymogen in the endoplasmic reticulum. Its activation is predicted to occur on the cell surface by autocleavage (1, 28). MT2 also undergoes ectodomain shedding, and multiple sizes of soluble MT2 are detected in the conditioned medium (CM) of transfected cells (25, 28, 29). At least four functional forms of MT2 have been suggested, including an uncleaved full-length form, an activated form with the cleaved S/P domain attached to membrane-tethered stem region through disulfide bonds, the shed ectodomain where the entire ectodomain is released into the medium and the shed S/P domain (1, 2, 16, 25, 28, 30). However, what forms of MT2 function in vivo and importantly how MT2 functions in vivo are still not known.

We are interested in what roles the different domains of MT2 play in the specificity and efficiency of substrate cleavage. The cytoplasmic domain of MT2 contains an endocytosis motif that mediates the internalization of cell-surface MT2 (31). Our previous studies indicated that this cytoplasmic domain also acts as an intracellular iron sensor to inhibit its degradation upon iron depletion (16, 32). Stabilization of MT2 is expected to suppress hepcidin expression leading to increased iron availability to cells. The precise roles of the transmembrane domain and the stem region for the function of MT2 are less understood. A recent study shows that the CUB, LDLRA, and S/P domains are required for cleavage of HJV (33). MT2 interacts with HJV through its stem region (25). Numerous clinical studies report that the IRIDA-causing mutations in MT2 are located throughout the entire extracellular domain (34–40). They all are loss-of-function mutations. Interestingly, at least three IRIDA-causing mutants in the stem region of MT2, R271Q, T287N, and G442R, are reported to traffic to the cell surface and to cleave HJV similarly to WT MT2 (33, 36, 37). These observations suggest that the nonproteolytic activity mediated through its ectodomain plays an important role for the function of MT2.

In this study, we found that the stem region of MT2 determines the specificity and efficiency of substrate cleavage and that the transmembrane domain was essential for substrate cleavage at specific sites. In vivo studies showed that a functional MT2 required the stem region and transmembrane domain in addition to its S/P domain.

**Results**

**The stem region of MT2 is required for an efficient cleavage of substrates**

We sought to explore the functions of the noncatalytic domains of MT2 by using cultured cells and MT2−/− mice as models. We previously showed that MT2 cleaves multiple components of the hepcidin-induction pathway (9). Our pilot studies on two other membrane-anchored serine proteases, matriptase and prostasin, strongly indicated the critical roles of the nonproteolytic sequence of MT2 in the specific and efficient cleavage of substrates.3 Matriptase is a close family member of MT2. Prostasin is a glycosylphosphatidylinositol (GPI)-anchored serine protease lacking a stem region (Fig. S1) (1, 41–43).

We generated four truncated forms of murine MT2 to determine the contributions of the stem region as well as transmembrane and cytoplasmic domains to the ability of MT2 to cleave its substrates. They are MT2 with deletion of the first 50 amino acids in the cytoplasmic domain (MT2-ΔCyto), the entire MT2 ectodomain (MT2-Ecto), the MT2 S/P domain (MT2-S/P), and MT2 with deletion of the S/P domain (MT2-mask) (Fig. 1A). MT2-Ecto and MT2-S/P are the secreted forms. For the convenience of immunodetection, a FLAG/MYC epitope was added to these constructs as well as to most of other constructs used in this study (Table 1). Addition of a C-terminal FLAG/MYC epitope to MT2 does not affect either its cleavage of substrates in transfected cells or the suppression of hepcidin expression in mice (9, 16, 25). All expressed proteins migrated on SDS-PAGE as predicted by their molecular masses (Fig. 1B). At least two distinct forms of MT2 were detected in the CM of cells expressing either full-length MT2 or MT2-ΔCyto under reducing conditions (Fig. 1B, lanes 2, 3, and 6). The lower band at 34 kDa is consistent with the molecular mass of the S/P domain, because it corresponds to the band observed in the CM of cells expressing MT2-S/P (lanes 8 and 9). The higher molecular weight band is likely a shed fragment of the entire ectodomain. There were three distinct bands detectable in the CM of cells expressing MT2-Ecto, in which the top band corresponds to the uncleaved form (lane 7). Intriguingly, a distinct band was also detected in the CM of cells expressing mask-MT2 (lane 4), which suggests that another protease in the cells can cleave MT2. Together, these data indicate that MT2 undergoes active cleavage in cells.

To elucidate the roles of the MT2 stem region, we first compared the ability of MT2-Ecto and MT2-S/P to cleave BMP receptors. HEK293 cells were co-transfected with a constant

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3 P. Mao, A.M. Wortham, C.A. Enns, and A-S. Zhang, unpublished observations.
amount of BMP receptor construct plasmid and increasing amounts of MT2-Ecto or MT2-S/P plasmid DNA. Full-length MT2 was included as a positive control, and enhanced GFP (EGFP) as a negative control. Because all BMP receptors have short extracellular domains at around 120–150 amino acids in length and because of the lack of appropriate antibodies against their extracellular domains for a direct immunodetection of cleaved products, a decrease in molecular weight in Western blots using an anti-FLAG antibody was adopted as an indicator for MT2 cleavage. As shown in Fig. 2A, MT2-Ecto was able to cleave ALK3 similarly to full-length MT2 (lanes 3–5 versus 9–11). In comparison, cleavage was detected only at the highest concentrations of MT2-S/P (Fig. 2A, lanes 3–5 versus 12–14). Biotinylation of the cell-surface proteins revealed that cells expressing MT2-Ecto had decreased cell-surface full-length ALK3 similarly to full-length MT2, and that the detected ALK3 bands were likely the cleaved products because of the mild band downshift (Fig. 2B, lanes 3 and 5). In contrast, expression of MT2-S/P only led to a small decrease in cell-surface ALK3 (Fig. 2B, lane 2 versus 6; Fig. S2A). No changes in cell-surface TfR1 or NaK-ATPase levels were observed when MT2-Ecto was expressed (Fig. 2B, lane 5 versus 1 and 2), indicating a degree of substrate specificity of MT2-Ecto and MT2. Similarly, we also found that MT2-Ecto but not MT2-S/P, was able to cleave ALK2, ActRIIA, and Bmpr2 in cells and to decrease their levels at the cell surface as efficiently as full-length MT2 (Fig. S2, B–J).

Together these results indicate that an efficient cleavage of BMP receptors by the S/P domain of MT2 requires its stem region.

We next examined the roles of the stem region in the cleavage of Hjv, Hfe, and Tfr2. Hjv was cleaved by both MT2 and the ubiquitously expressed furin (Fig. 3A) (44–47). As predicted, a single furin-cleaved Hjv band migrating at ~45 kDa was detected in the CM when Hjv is expressed alone (Fig. 3B, lanes 1 and 2). Consistent with our previous studies (9), co-expression of full-length MT2 resulted in a decrease in furin-cleaved Hjv in CM with a concurrent detection of at least two MT2-

| Table 1 | Mouse ORF constructs used for transfection studies |
|--------|-----------------------------------------------|
| Mouse ORFs | Encoded proteins | Tag epitope |
| ActRIIA | Type I transmembrane protein | C-terminal FLAG/MYC |
| ALK2 | Type I transmembrane protein | C-terminal FLAG/MYC |
| ALK3 | Type I transmembrane protein | C-terminal FLAG/MYC |
| Bmp2 | Type I transmembrane protein | C-terminal FLAG/MYC |
| HAI-2 | Type I transmembrane protein | C-terminal FLAG/MYC |
| Hfe | Type I transmembrane protein | C-terminal FLAG/MYC |
| Hjv | GPI-anchored membrane protein | N-terminal 3xFLAG |
| MT2 | Type II transmembrane protein | C-terminal FLAG/MYC |
| Tfr2 | Type II transmembrane protein | No tag |

Mechanistic studies of matriptase-2 activity

Figure 1. Generation of truncated MT2 constructs and characterization of their ectodomain shedding. A, diagrams of truncated MT2 constructs with C-terminal FLAG/MYC tag. Cyto: cytoplasmic domain. Catalytic: catalytic domain. TM: transmembrane domain. SEA: sea urchin sperm protein, enteropeptidase agrin. CUB: complement protein subcomponents C1r/C1s, urcin embryonic growth factor, and bone morphogenetic protein 1 domain. L: low-density lipoprotein receptor class A domain (LDLRA). SS: an immunoglobulin κ-chain signal sequence. m: MYC, f: FLAG. The arrow indicates the predicted cleavage-activation site. B, shedding of truncated MT2. HEK293 cells in 12-well plates were transfected with 4 μg of pCMV9-MT2, ΔCyto, mask, Ecto, or S/P construct DNA. At about 30 h post-transfection, medium was changed to Opti-MEM, 1% FCS. Analysis was performed after another 18 h of incubation. About 150 μg of cell lysate proteins and the TCA-precipitated proteins from the CM were subjected to SDS-PAGE and immunodetection. Images with two different exposures for CM were presented. Each panel was cropped from the same image. Ctrl, HEK293 cells with no transfection. All images were derived from the same experiment. Experiments were repeated three times with consistent results.
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To determine the roles of the transmembrane and cytoplasmic domains of MT2 for substrate cleavage, we examined the specificity of cleavage by MT2-ΔCyto in HEK293 cells. In contrast to MT2-Ecto, MT2-ΔCyto retained the capability to cleave Hjv, Hfe, and Tfr2 in a manner similar to MT2. Expression of MT2-ΔCyto resulted in the detection of two distinct Hjv fragments in CM, which were also seen with the co-expression of MT2, but not MT2-Ecto (Fig. 3G, lanes 3–5 versus 9–11). Different from Hfe, MT2-S/P also decreased cellular and cell-surface Tfr2 to a lesser extent than MT2-Ecto (Fig. 3G, lanes 12–14, H, lane 5). Taken together, cleavage studies of MT2-Ecto and MT2-S/P indicate that the stem region of MT2 is indispensable for the proteolytic activity as well as for the efficient substrate cleavage. These results also suggest that membrane tethering is essential for MT2 to cleave substrates at specific residues.

Plasma membrane tethering is required for substrate-specific cleavage by MT2

Similar to the cleavage of Hjv by MT2, MT2-Ecto was able to cleave Hfe and Tfr2 but it did not cleave these proteins with the same specificity/efficiency as full-length MT2. When Hfe was expressed alone, two major Hfe bands were evident (Fig. 3E, lanes 1 and 2). The upper band represents the fully processed mature form that is mainly detected at the cell-surface (9). Expression of MT2 decreased the upper band, and led to the detection of a ~17 kDa Hfe fragment in cell lysates (Fig. 3E, lanes 3–5). In comparison, MT2-Ecto decreased the fully processed Hfe upper band (55 kDa) in cell lysates and abolished cell-surface Hfe more effectively than full-length MT2 (Fig. 3, E, lanes 9–11, and F, lane 6; Fig. S2K). However, compared with the ~17 kDa fragment of Hfe detected in cells expressing MT2, no cleaved products of Hfe were detected in cells that expressed MT2-Ecto (Fig. 3E, lanes 3–5 versus 9–11). MT2-S/P failed to detectably cleave Hfe (Fig. 3, E, lanes 12–14, and F, lane 7). MT2-Ecto also markedly reduced the levels of both cellular and cell-surface Tfr2 (Fig. 3, G, lanes 9–11, and H, lane 5). In contrast to a distinctly cleaved Tfr2 band in CM by full-length MT2, no cleaved Tfr2 products were detectable by MT2-Ecto expression (Fig. 3G, lanes 3–5 versus 9–11). Different from Hfe, MT2-S/P also decreased cellular and cell-surface Tfr2 to a lesser extent than MT2-Ecto (Fig. 3, G, lanes 12–14, H, lane 6; Fig. S2L). Taken together, cleavage studies of MT2-Ecto and MT2-S/P indicate that the stem region of MT2 is indispensable for the proteolytic activity as well as for the efficient substrate cleavage. These results also suggest that membrane tethering is essential for MT2 to cleave substrates at specific residues.

cleaved Hjv fragments (Fig. 3B, lanes 3–5). Interestingly, co-expression of MT2-Ecto led to decreases in furin-cleaved Hjv in CM as full-length MT2. However, there were no detectable MT2-cleaved Hjv bands in CM (Fig. 3B, lanes 9–11). By comparison, MT2-S/P did not detectably cleave Hjv. Different from full-length MT2 and MT2-S/P, MT2-Ecto significantly decreased the cell-surface Hjv (Fig. 3C, lane 6, and D). These data suggest that the stem region is essential for the cleavage of Hjv and that the MT2 ectodomain alone lacks the capability to cleave Hjv at specific residues.
Mechanistic studies of matriptase-2 activity

To seek insight into the mechanisms that cause the differences between MT2 and MT2-Ecto, we analyzed the cellular location of cleavage by taking advantage of two inhibitors, the hepatocyte growth factor-activator inhibitor-2 (HAI-2) and aprotinin. HAI-2 is a membrane-associated serine protease inhibitor that primarily resides intracellularly within the endo-

Figure 3. The transmembrane domain of MT2 is essential for cleavage at specific residues in Hjv, Hfe, and Tfr2 and for the decrease of cell-surface Hfe and Tfr2. A, diagram of N-terminal tagged mouse Hjv with the potential cleavage sites by MT2 and furin, as well as the antibodies used for Western blotting in B. B, cleavage of Hjv by truncated MT2. Co-transfection of pCMV9-Hjv with truncated MT2 and pEGFP-N1 into HEK293 cells was performed as described above in the legends to Fig. 2A. At about 30 h post-transfection, medium was changed to Opti-MEM, 1% FCS. After another 18 h of incubation, about 150 μg of cell lysate proteins and the TCA-precipitated proteins from ~600 μl of the CM were subjected to SDS-PAGE and immunodetection by using anti-FLAG (for Hjv and MT2), β-actin, GFP, and HJV antibodies. Each panel was cropped from the same image. C, effects of truncated MT2 on cell-surface Hjv. HEK293 cells were co-transfected with pCMV9-Hjv, and pEGFP-N1 or pCMV6-MT2 or truncates at 1:1 ratios of plasmid DNA. At about 48 h after transfection, cell-surface proteins were biotinylated at 4 °C, followed by pulldown of the biotinylated proteins using streptavidin-agarose beads. The eluted cell-surface proteins and ~10% of input lysate was subjected to SDS-PAGE and immunodetection by using anti-FLAG antibody. D, quantification of cell-surface Hjv bands in C. E, cleavage of Hfe by truncated MT2. HEK293 cells in 12-well plates were co-transfected with constant amounts of pCMV6-Hfe (1.33 μg) and pJB-1-B2M (1.33 μg), an increasing amount of pCMV6-MT2 or truncated MT2 (0.33, 0.67, and 1.33 μg), and decreasing amount of pEGFP-N1 (1.0, 0.66, and 0 μg). Co-transfection with 1.33 μg of pEGFP-N1 (GFP) was included as negative controls. After 48 h of transfection, ~150 μg of cell lysate proteins was subjected to SDS-PAGE and immunodetection by using anti-FLAG (for Hfe and MT2), β-actin, and GFP antibodies. Each panel was cropped from the same image. F, effects of truncated MT2 on cell-surface Hfe. HEK293 cells were co-transfected with pCMV6-Hfe, pJB-1-B2M, and pEGFP-N1 or pCMV6-MT2 or truncates at 1:1:1 ratios of plasmid DNA. At about 48 h after transfection, cell-surface proteins were biotinylated as described above in C. The eluted cell-surface proteins and ~30% of input lysate were subjected to SDS-PAGE and immunodetection by using anti-FLAG antibody. G, cleavage of Tfr2 by truncated MT2. Co-transfection was performed as described above for Hjv in B. At about 30 h post-transfection, medium was changed to Opti-MEM, 1% FCS. Analysis was performed after another 18 h of incubation. About 150 μg of cell-lysate proteins and the TCA-precipitated proteins from ~600 μl of the CM were subjected to SDS-PAGE and immunodetection using anti-FLAG (for MT2), Tfr2, β-actin, and GFP antibodies. Each panel was cropped from the same image. H, effects of truncated MT2 on cell-surface Tfr2. Co-transfection, biotinylation, and immunodetection were performed essentially the same as described above in C for Hjv, except that Tfr2 was detected by a rabbit antibody against mouse Tfr2 extracellular domain. Ctrl, HEK293 cells with no transfection. n.s., nonspecific bands. All experiments were repeated at least three times with consistent results.

Traffic of MT2 to the plasma membrane is required for MT2-induced cleavage of substrates

To seek insight into the mechanisms that cause the differences between MT2 and MT2-Ecto, we analyzed the cellular location of cleavage by taking advantage of two inhibitors, the hepatocyte growth factor-activator inhibitor-2 (HAI-2) and aprotinin. HAI-2 is a membrane-associated serine protease inhibitor that primarily resides intracellularly within the endo-
plasmic reticulum (48). Aprotinin is a soluble membrane-impermeable serine protease inhibitor consisting of 58 amino acids. Both inhibit the proteolytic activity of MT2 (47, 49, 50). We reasoned that if the substrate cleavage is inhibited by both inhibitors, it would indicate that the cleavage takes place after MT2 reaches the plasma membrane. Conversely, if the cleavage is blocked by HAI-2 but not by aprotinin, it would indicate that the cleavage occurs in the biosynthetic pathway. As expected, co-expression of HAI-2 completely prevented the cleavage of ALK2, ALK3, ActRIIA, Bmpr2, Hfe, Tfr2, and Hjv by MT2 and MT2–H9004 Cyto (Fig. 4, A–D, lane 4 versus 8). These data support HAI-2 as a potent inhibitor of MT2.

We examined the cellular location of substrate cleavage by full-length MT2 and MT2–ΔCyto. Transfected cells were treated with aprotinin (10 μM) for ~18 h. Aprotinin was able to largely block the cleavage of ALK2, ALK3, ActRIIA, Hjv, Hfe, and Tfr2 by full-length MT2 (Fig. 5, A–F, lane 5, and D, lane 7), which indicates that cleavage occurs after the substrates reach the plasma membrane. Consistent with our previous studies (46), the cleavage of Hjv by MT2 was not inhibited by the furin convertase inhibitor (FCI) (Fig. 5D, lane 6). In comparison, only a mild inhibitory effect was detected for MT2 cleavage of

**Figure 4. HAI-2 inhibits the substrate cleavage by MT2 and truncated MT2.** A–D, HAI-2 inhibition of cleavage for ALK2 (A), ALK3 (B), ActRIIA (C), and Bmpr2 (D). pCMV6-ALK2, ALK3, ActRIIA, or Bmpr2 were co-transfected into HEK293 cells with pCMV6-MT2 or truncates and pCMV6-HAI-2 or pEGFP-N1 at 1:1 ratios of plasmid DNA. After 48 h of transfection, ~150 μg of cell lysate proteins were subjected to SDS-PAGE and immunodetection using anti-FLAG (for MT2, HAI-2, ALK2, ALK3, ActRIIA, and Bmpr2), β-actin, and GFP antibodies. E, HAI-2 inhibition of cleavage for Hfe. HEK293 cells were co-transfected with pCMV6-Hfe, pJB-1-B2M, pCMV6-MT2, or truncates, and HAI-2 or pEGFP-N1 at 1:1:1 ratios of plasmid DNA. Analysis was performed at 48 h post-transfection as described above in A–D. F, HAI-2 inhibition of cleavage for Tfr2. Co-transfection of Tfr2 with MT2 truncates and HAI-2 or pEGFP-N1 were performed essentially the same as described above for A–D. At about 30 h post-transfection, medium was changed to Opti-MEM, 1% FCS. Analysis was performed after another 18 h of incubation. About 150 μg of cell-lysate proteins and the TCA-precipitated proteins from ~600 μl of CM were subjected to SDS-PAGE and immunodetection. Tfr2 was detected by using a rabbit anti-Tfr2 antibody. G, HAI-2 inhibition of cleavage for Hjv. Co-transfection of HEK293 cells with Hjv, truncated MT2, and HAI-2 was performed as described above in A–D. At about 30 h post-transfection, medium was changed to Opti-MEM, 1% FCS. After another 18 h of incubation, about 150 μg of cell-lysate proteins and the TCA-precipitated proteins from ~600 μl of CM were subjected to SDS-PAGE and immunodetection using anti-FLAG (for MT2, Hjv, and HAI-2), β-actin, GFP, and HJv antibodies. n.s., nonspecific bands. All experiments were repeated at least three times with consistent results.
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Bmpr2 (Fig. 5G, lane 5), suggesting that its cleavage predominantly takes place in the biosynthetic pathway. When the effects of aprotinin on MT2-ΔCyto were compared with full-length MT2, we detected similar extents of inhibition for the cleavage of ALK2, Hvj, and Hfe, a relatively milder inhibition for ALK3, ActRIIA, and Tfr2, and no obvious effect for Bmpr2 (Fig. 5, A–G; Fig. S3, A–G). These observations suggest that the cytoplasmic domain of MT2 facilitates the cleavage of ALK3, ActRIIA, Tfr2, and Bmpr2 at the cell surface or after retrograde trafficking.

In contrast to full-length MT2 and MT2-ΔCyto, incubation with aprotinin failed to inhibit cleavage by MT2-Ecto for all the tested substrates (Fig. 5, A–G, lane 9, and D, lane 13; Fig. S3, A–G). Similar results were also obtained by using another membrane-impermeable serine protease inhibitor, leupeptin (data not shown). These data indicate that MT2-Ecto cleaves
substrates primarily within cells before reaching the plasma membrane, which is distinct from the membrane-anchored full-length MT2 and MT2-ΔCyto (Table S1). Taken together, these observations suggest that the transmembrane domain is essential for MT2 to cleave substrates after they traffic to the plasma membrane and that the cytoplasmic domain facilitates these processes.

Plasma membrane tethering is essential for MT2 suppression of hepcidin expression in vivo

To determine the contributions of different MT2 domains to its biological function, we expressed MT2-ΔCyto, MT2-Ecto, and MT2-S/P in the liver of MT2−/− mice by AAV8 vector for replacement studies. MT2 is predominantly expressed in the hepatocytes of liver. The AAV8-MT2 constructs contained a hepatocyte-specific promoter to mimic the endogenous expression of MT2. AAV8-MT2 and MT2-mask constructs were used as positive and negative controls, respectively. All MT2−/− mice used in the studies had similar extents of iron deficiency and anemia with no obvious gender-related difference (9). Eight-week-old MT2−/− mice of both genders were injected intraperitoneally with AAV8-MT2 or AAV8-MT2-ΔCyto at ~8 × 10^{11} and ~4 × 10^{12} genome-particles per mouse, and AAV8-MT2-mask, AAV8-MT2-Ecto, or AAV8-MT2-S/P at ~4 × 10^{12} genome-particles per mouse, or PBS vehicle as control. Previous studies indicated that expression of either GFP or glutaryl CoA-dehydrogenase, which are unre-vehicle as control. Previous studies indicated that expression of either GFP or glutaryl CoA-dehydrogenase, which are unre-

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Mechanistic studies of matriptase-2 activity
Mechanistic studies of matriptase-2 activity

indicate that the stem region determines the specificity and efficiency for substrate cleavage. The transmembrane domain allows the cleavage of substrates at specific residues at the cell surface. In vivo studies reveal that suppression of BMP-signaling and hepcidin expression requires both the entire ectodomain and the transmembrane domain of MT2.

By taking advantage of the differences between the MT2 constructs, we demonstrated a determinant role of the nonproteolytic sequence of MT2 for specific cleavage of BMP receptors, Hfe, Tfr2, and Hjv. We showed that the stem region of MT2 is required for efficient substrate cleavage (Figs. 2 and 3; Fig. S2). This is likely due to the possibility that the stem region is

![Figure 6](image-url)
involved in the interaction with all substrates. MT2 interacts with HIV through this region (25). Consistent with this idea, the stem region of MT2 harbors numerous IRIDA-causing mutations (34, 35). Our data also suggest that the shed forms of soluble MT2 lack physiological relevance to iron homeostasis. We detected at least two soluble forms of MT2 from transfected cells, which contributes to the entire ectodomain and S/P domain, respectively (Fig. 1B). A previous study reports two additional cleaved products (28). In this study, we indeed detected a robust activity for soluble MT2 ectodomain and a minor activity for soluble S/P domain (Figs. 2 and 3; Fig. S2), which indicate that all shed forms of MT2 do possess proteolytic activities. However, compared with full-length MT2, the soluble MT2 ectodomain lacks the activity to cleave HJV, Hfe, and Tfr2 at specific residues (Fig. 3), and more importantly it mainly cleaves substrates in the biosynthetic pathway (Figs. 4 and 5). These observations suggest that these soluble forms act differently from full-length MT2. Therefore, it is unlikely that the function of MT2 is mediated through the shed MT2. Rather the shedding of MT2 may represent the pathway of clearance from the cells.

Our studies indicate that MT2 tethering to the plasma membrane is essential for its cleavage specificity (Fig. 3). The different localization of activation between the MT2-Ecto and the full-length MT2 (Figs. 4 and 5) suggests that MT2 mainly cleaves substrates after reaching the plasma membrane, and imply that the tethering of MT2 to the membrane protects MT2zymogen from being activated before it reaches the cell surface. This idea is supported by previous studies suggesting that the cleavage of HJV by MT2 either occurs at cell-surface cells (25) or requires retrograde trafficking from the plasma membrane (52). This specific activation process possesses physiological significance, because it would allow the modulation of MT2 activities by the factors in extracellular environment. These observations support a model that the transmembrane domain acts to ensure the activation and substrate cleavage at the specific sites of cells. Together with findings that the cytoplasmic domain of MT2 facilitates the cleavage processes in this study (Fig. 5), our data indicate that the specific and efficient cleavage of substrates at the precise sites of cells requires the entire sequence of MT2.

Expression of the same constructs of MT2 in the livers of MT2\(^{1−/−}\) mice showed some differences in the behavior of MT2. The \textit{in vivo} studies did not detect MT2 shedding in the liver. In contrast to transfected cells, no soluble MT2 was detectable in the serum when either full-length MT2 or MT2-ΔCyto was expressed in the liver of MT2\(^{1−/−}\) mice (Figs. 1B and 6B). These discrepancies are unlikely due to the use of HEK293 cells, a nonhepatoma cell line, for the \textit{in vitro} study. A previous study shows similar post-translational processing of human MT2 between HEK293 and hepatoma cells (28). We found that the substrate cleavage by mouse MT2 in Hep3B cells (a hepatoma cell line) is similar to that detected in HEK293 cells. Thus, it is likely that other environmental factors may contribute to the differential post-translational processing of MT2 between \textit{in vitro} and \textit{in vivo} systems. Alternatively, the hepatic MT2 may undergo similar autocleavage and shedding as seen in transfected cells. But the levels of cleaved MT2 fragments fall below the limit of immunodetection because of the high dilution into the entire blood volume and/or a rapid clearance from the circulation.

Our \textit{in vivo} studies strongly indicate that the membrane-associated MT2s are the biologically active forms, which suppress BMP signaling and hepcidin expression in the liver (Fig. 6; Table 2). There are two predicted membrane-associated forms, the uncleaved full-length MT2zymogen and the activated form with the cleaved S/P domain attached to the membrane-tethered stem region through a disulfide bond. Previous studies implicate the latter as the most catalytically active form (25, 28). However, our data do not rule out the possibilities that the MT2zymogen is also catalytically active, because both MT1 and prostasinzymogens are functional \textit{in vivo} (41, 42, 53). Nevertheless, our studies did support the idea that soluble forms of MT2 lack the biological activity, because neither MT2-Ecto nor MT2-S/P was able to rescue the defects of MT2\(^{1−/−}\) mice. In transfected cells, we found that soluble MT2-Ecto was able to cleave substrates as robustly as full-length MT2 (Figs. 2 and 3; Fig. S2), but compared with membrane-associated MT2, it lacked specificity and efficiency in cleavage (Figs. 2–5). Thus, we speculate that membrane association would allow MT2 to be activated after reaching the plasma membrane to specifically remove the components of the hepcidin-induction pathway. Alternatively, MT2 may act as a membrane-associatedzymogen to suppress hepcidin expression by binding to the substrates, which inhibits their functions. These hypotheses remain to be tested in future studies.

Finally, the finding that the cytoplasmic domain is dispensable for the suppression of hepcidin in this study (Fig. 6) is not in conflict with our previous observations showing that the cytoplasmic domain acts as an intracellular iron sensor to inhibit its degradation under iron depletion (16, 32). The severe iron deficiency and anemia of MT2\(^{1−/−}\) mice requires a maximal activity of MT2 to suppress hepcidin expression.

### Table 2

| Hematologic parameters for wildtype mice (WT) and MT2\(^{1−/−}\) mice that express full-length or truncated MT2 by AAV8 vectors |
|----------------|----------------|----------------|----------------|
|                | WT             | MT2\(^{+}\)     | MT2\(^{−}\)     |
|                | (n = 17)       | (n = 13)        | (n = 13)        |
| RBC\(^{+}\)    | 8.73 ± 0.43\(^{a}\) | 10.64 ± 0.56\(^{a}\) | 10.81 ± 0.55\(^{a}\) |
| Hb             | 13.04 ± 0.53\(^{a}\) | 8.44 ± 0.74     | 11.86 ± 0.84\(^{a}\) |
| HCT            | 48.71 ± 2.75\(^{a}\) | 36.74 ± 2.11\(^{a}\) | 48.53 ± 3.62\(^{a}\) |
| MCV            | 55.19 ± 2.44\(^{a}\) | 34.54 ± 2.59\(^{a}\) | 45.55 ± 2.92\(^{a}\) |
| MCH            | 14.96 ± 0.25\(^{a}\) | 7.99 ± 0.82     | 11.20 ± 1.11\(^{a}\) |

\(^{a}\) RBC, red blood cells (×10\(^{12}\)/μl); Hb, hemoglobin (g/dl); HCT, hematocrit (%); MCV, mean cell volume (fl); MCH, mean corpuscular hemoglobin (pg). Data are expressed as mean ± S.D.
Mechanistic studies of matriptase-2 activity

In summary, this is the first study, to our knowledge, to systematically investigate the post-translational processing and biological functions of MT2 by using both the in vitro transient expression systems and the in vivo expression systems. Our studies indicate that the specific and efficient cleavage of substrates at the precise site of cells requires the entire sequence of MT2, and that the shed MT2 may represent the products of degradation. In vivo studies demonstrate that suppression of hepcidin expression by MT2 requires its transmembrane domain and entire ectodomain. Our findings have important significance for further elucidating the underlying mechanism by which MT2 suppresses hepcidin expression, and for characterizing the defects of IRIDA-causing mutations in different subdomains of MT2.

Experimental procedures
cDNA constructs

All mouse MT2, ALK2, ALK3, ActRIIA, Bmpr2, Hfe, β2-microglobulin (B2M), Hjv, Tfr2, and pEGFP-N1-expressing constructs were the same as previously described (9). Murine HAI-1 (NM_001082548) ORF with a C-terminal FLAG/MYC epitope in the pCMV6 vector was purchased from Origene (Table 1). The entire coding sequences for all vectors were validated by DNA sequencing.

We generated three truncated murine MT2 constructs (Fig. 1A). MT2-ΔCyto, which lacks the first 50 amino acids in the cytoplasmic domain, was generated by site-directed mutagenesis using pCMV6-MT2 as a template and the QuikChange kit (Stratagene). MT2-Ecto and MT2-S/P constructs were generated by addition of an Ig κ-chain leader sequence (SS) immediately before Phe-77 and Asp-567 residues, respectively, using pCMV6-MT2 as a template and PCR with the Gibson Assembly Master Mix (New England Biolabs). MT2-mask was generated in our previous studies (9). The sequences in all these constructs were verified by DNA sequencing.

AAV8-ΔCyto-MT2, MT2-Ecto, and MT2-S/P constructs were generated by subcloning into an AAV8 construct containing a strong liver-specific promoter as described in our previous study (9, 54). The liver-specific promoter is a combination of two copies of a human α₁-microglobulin/bikunin enhancer and the promoter from the human thyroid hormone-binding globulin gene. AAV8-MT2 and MT2-mask constructs were generated in our previous study (9). AAV8 viral vectors were produced at the Molecular Virology Support Core, OHSU.

Cell lines and transfection

HEK293 cells were obtained from ATCC and maintained in DMEM, 10% fetal calf serum (FCS). We used transient cotransfection with Lipofectamine 2000 (Invitrogen) and polyethyleneimine transfection reagent (Polysciences, Inc., 24765-1) to determine the cleavage of ALK2, ALK3, ActRIIA, Bmpr2, Hjv, Hfe, and Tfr2 by truncated MT2 with or without HAI-1. The detailed procedures were described in the figure legends. All cells were transfected with equal amounts of total plasmid DNA. After 48 h of transfection, cell lysates were collected, and protein concentrations were determined by Pierce BCA protein assay kit (Thermo). Lysate protein (~150 μg) was then subjected to SDS-PAGE. We used Ponceau S staining to ensure equal transfer of proteins onto the nitrocellulose membrane. ALK2, ALK3, ActRIIA, Bmpr2, Hjv, and Hfe were immunodetected by using an HRP-coupled mouse anti-FLAG M2 IgG, chemiluminescence, and the c600 Western blotting imaging system (Azure Biosystems, Inc.). Tfr2, EGFP, Tfr1, and β-actin in cell lysates were probed with rabbit anti-mouse Tfr2 extracellular domain (number 25257), mouse anti-GFP (ProteinTECH), mouse anti-TFR1 (Invitrogen), and mouse anti-β-actin, respectively, immunodetected by using an Alexa Fluor 680 goat anti-rabbit secondary antibody (1:10,000; Invitrogen) or an Alexa Fluor 800 goat anti-mouse secondary antibody (1:10,000; Invitrogen), and visualized by using an Odyssey IR Imaging System (Licor). For most of the transfection studies, images with sequential probing for β-actin and GFP were presented.

To detect the cleaved Hjv and Tfr2 in CM, medium was changed to Opti-MEM, 1% FCS at ~30 h post-transfection. CM was collected after ~18 h of incubation. Proteins in CM were first precipitated by using 6% TCA, followed by resuspending the protein pellets with 1X Laemmli buffer and subjecting to SDS-PAGE. Hjv in CM was probed with both an HRP-coupled mouse anti-FLAG M2 IgG and a rabbit anti-HJV antibody generated against the entire sequence of human HIV (46). The latter was able to detect the cleavage products of Hjv by both furin and MT2. Tfr2 in CM was detected as described above for the cell lysate Tfr2.

To test the effects of aprotinin (G-Biosciences) on MT2 cleavage of all substrates and FCI (Alexis, San Diego, CA) on MT2 cleavage of Hjv, aprotinin at 5 μM and FCI at 1 μM were added to the medium at ~30 h post-transfection. Analysis was performed after ~18 h of incubation as described above. The intensities of ALK2, ALK3, ActRIIA, Bmpr2, Hjv, Hfe, and Tfr2 bands in Western blots were quantified by using either Licor or ImageJ software.

Biotinylation of cell-surface proteins

HEK293 cells were plated in the polylysine-coated 6-well plate and co-transfected with the same amount of pCMV6-ALK2, ALK3, ActRIIA, Bmpr2, Hjv/B2M, pCMV9-Hjv, or pcDNA3-Tfr2 plasmid DNA, and an equal amount of pEGFP-N1, pCMV6-MT2, or MT2 truncate plasmid DNA. After 48 h of transfection, cell-surface proteins were biotinylated and analyzed as described in our previous study (9).

Animal studies

All animal procedures were approved by the Oregon Health & Science University, Department of Comparative Medicine (OHSU DCM). We purchased heterozygous MT2 mutant mice on B6/129 background from Lexion/Mutant Mouse Resource and Research Center (9). Both WT and homozygous MT2−/− mice were generated by breeding heterozygous MT2 mutant mice on mixed background. Animals were genotyped by PCR using genomic DNA from toe clips. All MT2−/− mice used in the studies were within two generations of breeding and had similar extents of iron deficiency and anemia. We previously showed no obvious gender-related difference with respect to iron deficiency, anemia, and high hepatic hepcidin expression in MT2−/− mice (9).
We used MT2−/− mice to examine the function of truncated MT2 in vivo. Eight-week-old MT2−/− mice of both genders were intraperitoneally injected with AAV8-MT2, AAV8-MT2ΔCyto, AAV8-MT2-Ecto, AAV8-MT2-S/P, or AAV8-MT2-mask at dosages indicated in the text and figure legends. Injection of PBS vehicle was included as control. Three weeks later, mice were euthanized for analysis. Blood was collected by cardiac puncture for serum iron and hematology analysis. Livers were rapidly removed, snap-frozen in liquid nitrogen, and then stored at −80 °C for qRT-PCR and Western blotting. Age, gender, and background-matched WT littermates were included as additional controls. Each group consisted of at least 10 animals. The viral vector stocks were handled according to Biohazard Safety Level 2 guidelines published by NIH.

qRT-PCR

qRT-PCR analysis of β-actin, MT2, hepcidin, and Id1 transcripts in the liver were conducted as previously described (9, 54). All primers were verified for linearity of amplification. The results are expressed as the amount relative to that of β-actin for each sample.

Blood parameters and serum iron assays

Blood parameters for MT2−/− mice were measured using Hemavet 950 (Drew Scientific). Serum iron concentrations were measured using a serum iron/TIBC Reagent Set (Teco Diagnostics, Anaheim, CA).

Immunodetection of hepatic MT2 and serum MT2 in mice

About 250 μg of liver extract proteins were separated by SDS-PAGE under reducing conditions. We used Ponceau S staining to ensure equal transfer of proteins onto the nitrocellulose membrane. The AAV8-introduced exogenous MT2 was probed directly by using an HRP-coupled mouse anti-FLAG M2 IgG (1:10,000; Sigma) and chemiluminescence (Super Signal, Pierce). β-Actin was immunodetected with mouse anti-β-actin (1:5,000; Sigma), immunodetected by using the corresponding secondary antibody, and visualized by Licor.

The secreted MT2 in serum (~100 μl) was first pulled down by using anti-FLAG MT2-agarose affinity gel (Sigma; A-1205). The eluates were then subjected to SDS-PAGE and immunodetection using an HRP-coupled mouse anti-FLAG M2 IgG.

Statistical analysis

One-way analysis of variance and Tukey’s post-test were used to analyze the data from animal studies.

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