SUPPLEMENTAL INFORMATION

The Ectodomain of Matriptase-2 Plays an Important Non-Proteolytic Role in Suppressing Hepcidin Expression in Mice

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Supplemental Materials and Methods

cDNA constructs. We purchased mouse Mt2 (NM_027902.1), Alk2 (NM_007394.1), Alk3 (NM_009758.3), ActRIIA (NM_007396.1), Bmpr2 (NM_007561.3), Hfe (NM_010424.1), and the hepatocyte growth factor activator inhibitor-2 (Hai-2; NM_011464.2) ORFs with a C-terminal FLAG/MYC epitope in pCMV6 vector (pCMV6-Mt2, Alk2, Alk3, ActRIIA, Bmpr2, Hfe, and Hai-2) from Origene (Table 1). pcDNA3-Tfr2 construct was generated by subcloning mouse Tfr2 ORF into pcDNA3. Mouse Hjv ORF with a N-terminal 3xFLAG epitope in pCMV9 vector (pCMV9-Hjv) was generated in our previous study.[1] Mouse β2-microglobulin (B2M) and pEGFP-N1-expressing constructs were the same as previously described.[2,3] We generated pCMV6-Mt2^{I286F}, pCMV6-Mt2^{S762A}, and pCMV6-Mt2^{mask} constructs by site-directed mutagenesis in this and previous studies.[2] All the sequences were verified by DNA sequencing.

The Mt2, Mt2^{I286F}, Mt2^{S762A}, Mt2^{mask}, and Hai-2 constructs were subcloned into an AAV8 construct containing a strong liver-specific promoter (LSP) as described in our previous study.[4] The LSP 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, Mt2^{I286F}, Mt2^{S762A}, Mt2^{mask}, and Hai-2 vectors were generated at the Molecular Virology Support Core, OHSU.

Animal studies. All animal procedures were approved by OHSU DCM. We purchased heterozygous *Tmprss6*$^{+/-}$ mutant mice on B6/129 background.[2] Both wild-type and homozygous *Tmprss6*$^{-/-}$ mice were generated by breeding *Tmprss6*$^{+/-}$ mice on mixed background. All *Tmprss6*$^{-/-}$ mice used in the studies were within two generations of breeding and had similar degree of iron deficiency and anemia with no obvious gender-related difference.[2] Wild-type 129S6 mice were bred and maintained in the DCM of OHSU. All animals were fed a PicoLab Laboratory Rodent Diet 5L0D containing 240 ppm iron (LabDiet).

Eight-week old *Tmprss6*$^{-/-}$ mice were intraperitoneally injected with AAV8-Mt2, AAV8-Mt2^{S762A}, or AAV8-Mt2^{I286F} viral vectors at the doses indicated in the figure legends. The AAV8 vector alone had no effects on iron homeostasis in mice.[1,5] Injection of PBS was included as a control. Mice were euthanized for analysis three weeks after injection. Blood was collected by cardia puncture for serum iron assay using a serum iron/TIBC Reagent Set (Teco Diagnostics, Anaheim, CA) and hematology analysis using Hemavet 950 (Drew Scientific). Livers were snap-frozen for qRT-PCR and western blot. Age, gender, and background-matched wild-type littermates were included as additional controls. Injection of AAV8-Mt2^{mask} into *Tmprss6*$^{-/-}$ mice was performed in our previous studies.[3]

Eight-week old wild-type 129S6 mice were intraperitoneally injected with AAV8-Hai-2 viral vectors at the doses indicated in the figure legends. Injection of PBS was included as a control. Animals were euthanized for analysis three weeks after injection.

qRT-PCR. qRT-PCR analysis of *Tmprss6*, hepcidin, Inhibitor of DNA binding-1 (*Id1*), *Hai-2*, and β-actin transcripts in the liver were conducted as previously described.[2,4] All primers (Supplemental Table S1) were verified for linearity of amplification. The results are expressed as the amount relative to that of β-actin for each sample.

Immunodetection of hepatic Mt2, Hjv, and Hai-2. Protein extracts from whole liver tissues, liver membrane fractions, as well as the concentrated FLAG-tagged Hai-2 by using anti-FLAG affinity gel, were separated by using SDS-PAGE under reducing conditions. The liver tissues from *Tmprss6*$^{-/-}$ mice that express exogenous Mt2^{mask}, wild-type or *Hjv*$^{-/-}$ mice on 129S6 background, and wild-type or *Hjv*$^{-/-}$ mice that express exogenous FLAG-Hjv were from our previous studies.[1,2] Liver membrane fractions were prepared as previously described.[6] FLAG-tagged Hai-2 from ~2 mg liver extract proteins was pulled down by using anti-FLAG affinity gel (Sigma #A2220), followed by elution with 3xFLAG peptide at ~200 μg/ml (Sigma F4799). The transduced exogenous Mt2, Hai-2, and Hjv in the liver were probed directly by using an HRP-coupled mouse anti-FLAG M2 IgG (Sigma) and chemiluminescence. The endogenous Hjv and β-actin were detected by using purified mouse anti-Hjv, mouse anti-β-actin (Sigma), and the corresponding secondary antibodies. The intensities of specific bands were quantified by using the ImageJ software.

Serum HGF assay. Serum HGF concentrations were detected by using a Mouse HGF ELISA kit (#RAB0214; Sigma-Aldrich).

Cell lines and transfection. Hep3B, HEK293, and CHO cells were obtained from ATCC. We used transient co-transfection with Lipofectamine-2000 (Invitrogen) to determine the cleavage of Alk2, Alk3, ActRIIA, Bmpr2, Hjv, Hfe, and Tfr2 by wild-type, mutated, or truncated Mt2. To detect the cleaved Hjv and Tfr2 fragments in conditioned medium (CM), medium was changed to Opti-MEM/1% FCS with or without aprotinin (G Biosciences) or furin convertase inhibitor (FCI; Alexis, San Diego, CA) at ~30 hr post-transfection. CM was collected after ~24 hr of incubation for immunodetection as previously described.[3]

Biotinylation of cell surface proteins. Cell surface proteins of transfected cells were biotinylated on ice and analyzed as described in our previous studies.[2,3]

Immunoprecipitation. HEK293 cells were transfected with individual plasmid. Cell lysates were collected and mixed in the presence of protease inhibitors. The cell lysates were pre-cleared, and co-immunoprecipitation was performed by using protein-A agarose beads (Invitrogen) coated with either rabbit anti-MT2 antibody[7] or rabbit anti-HJV 18745 antibody[8].

References

1. Zhao N, Maxson JE, Zhang RH, Wahedi M, Enns CA, Zhang AS. Neogenin Facilitates the Induction of Hepcidin Expression by Hemojuvelin in the Liver. *J Biol Chem*. 2016;291(23):12322-12335.
2. Wahedi M, Wortham AM, Kleven MD, et al. Matriptase-2 suppresses hepcidin expression by cleaving multiple components of the hepcidin induction pathway. *J Biol Chem*. 2017;292(44):18354-18371.
3. Mao P, Wortham AM, Enns CA, Zhang AS. The catalytic, stem, and transmembrane portions of matriptase-2 are required for suppressing the expression of the iron-regulatory hormone hepcidin. *J Biol Chem*. 2019;294(6):2060-2073.
4. Zhang AS, Gao J, Koeberl DD, Enns CA. The role of hepatocyte hemojuvelin in the regulation of bone morphogenic protein-6 and hepcidin expression in vivo. *J Biol Chem*. 2010;285(22):16416-16423.
5. Gao J, Chen J, De Domenico I, et al. Hepatocyte-targeted HFE and TfR2 control hepcidin expression in mice. *Blood*. 2010;115(16):3374-3381.
6. Frydlova J, Fujikura Y, Vokurka M, Necas E, Krijt J. Decreased hemojuvelin protein levels in mask mice lacking matriptase-2-dependent proteolytic activity. *Physiol Res*. 2013;62(4):405-411.

7. Enns CA, Ahmed R, Zhang AS. Neogenin Interacts with Matriptase-2 to Facilitate Hemojuvelin Cleavage. *J Biol Chem*. 2012;287(42):35104-35117.
8. Zhang AS, West AP, Jr., Wyman AE, Bjorkman PJ, Enns CA. Interaction of hemojuvelin with neogenin results in iron accumulation in human embryonic kidney 293 cells. *J Biol Chem*. 2005;280(40):33885-33894.

Supplemental Table S1. List of mouse-specific primers used for qRT-PCR analysis

Gene	Forward primer	Reverse primer
β-actin	5'-CTGCCTGACGGCCAGGT-3'	5'-TGGATGCCACAGGATTCCAT-3'
Hai-2	5'-TATGGAGGCTGTGAAGGCAAT-3'	5'-AGAGTCGGCTCCATTCCTGTT-3'
Hepcidin	5'-CACCAACTTCCCCATCTGCATCTT-3'	5'-GAGGGGCTGCAGGGGTGTAGAG-3'
Id1	5'-ACCCTGAACGGCGAGATCA-3'	5'-TCGTCGGCTGGAACACATG-3'
Tmprss6 (1)	5'-TTGCTGGTCTTGGCTGCGCT-3'	5'-AATGACGGTTGAGCACCCGGAG-3'
Tmprss6 (2)	5'-GCCAGTGGATGATCCAGAACA-3'	5'-TGAAGTTGATGGTGACACCATCT-3'

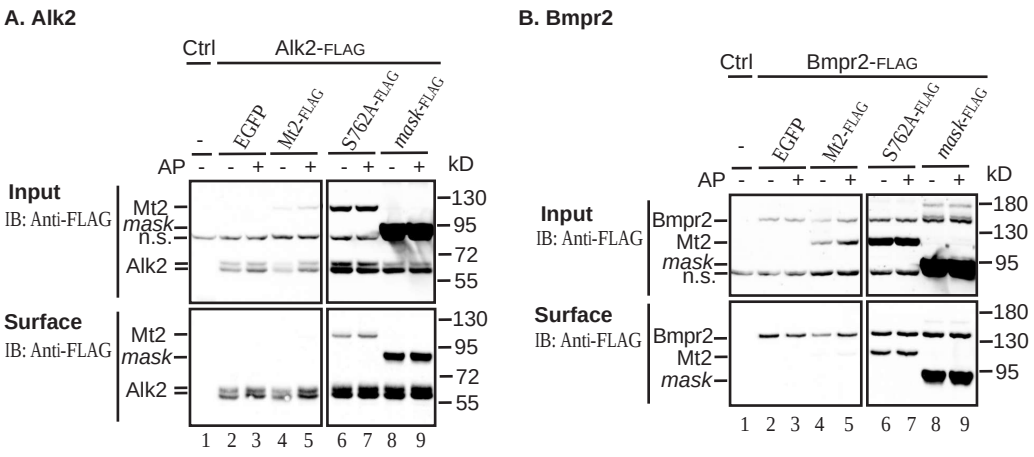

Figure S1. *Cleavage of Alk2* (**A**) *and Bmpr2* (**B**) *by Mt2, the protease-dead Mt2*^{S762A}*, and Mt2*^{mask} *in Hep3B cells.* The co-transfection, biotinylation, and immunodetection were performed essentially the same as described in the legend to **Figure 2A.** Each panel was cropped from the same image. Alk2, Bmpr2, Mt2, Mt2^{S762A}, and Mt2^{mask} were immunodetected by using an anti-FLAG antibody. IB: immunoblotting. All experiments were repeated three times with consistent results.

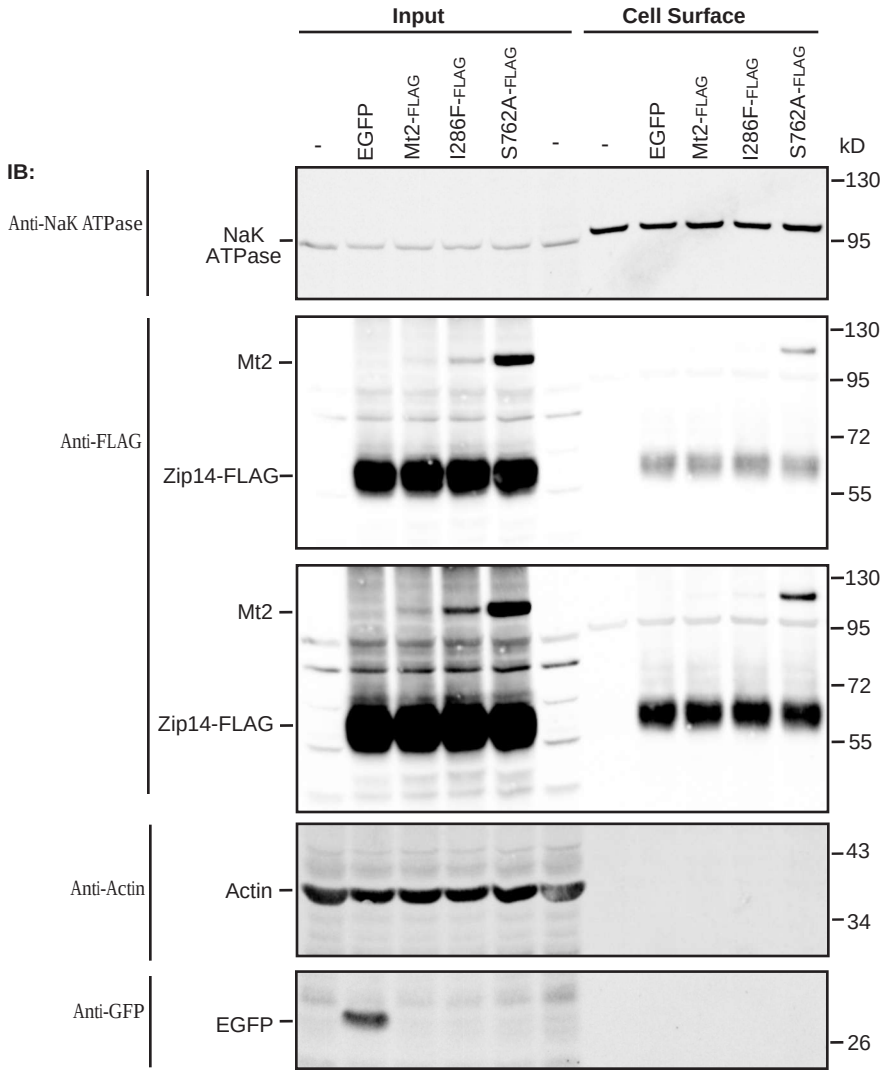

Figure S2. *Mt2 does not cleave Zip14 and display no evident effect on cell surface Zip14 in Hep3B cells.* The co-transfection, biotinylation, and immunodetection were performed essentially the same as described in the legend to **Figure 2A.** Zip14, Mt2, $Mt2^{I286F}$, and $Mt2^{S762A}$ were immunodetected by using an anti-FLAG antibody. Experiments were repeated three times with consistent results.

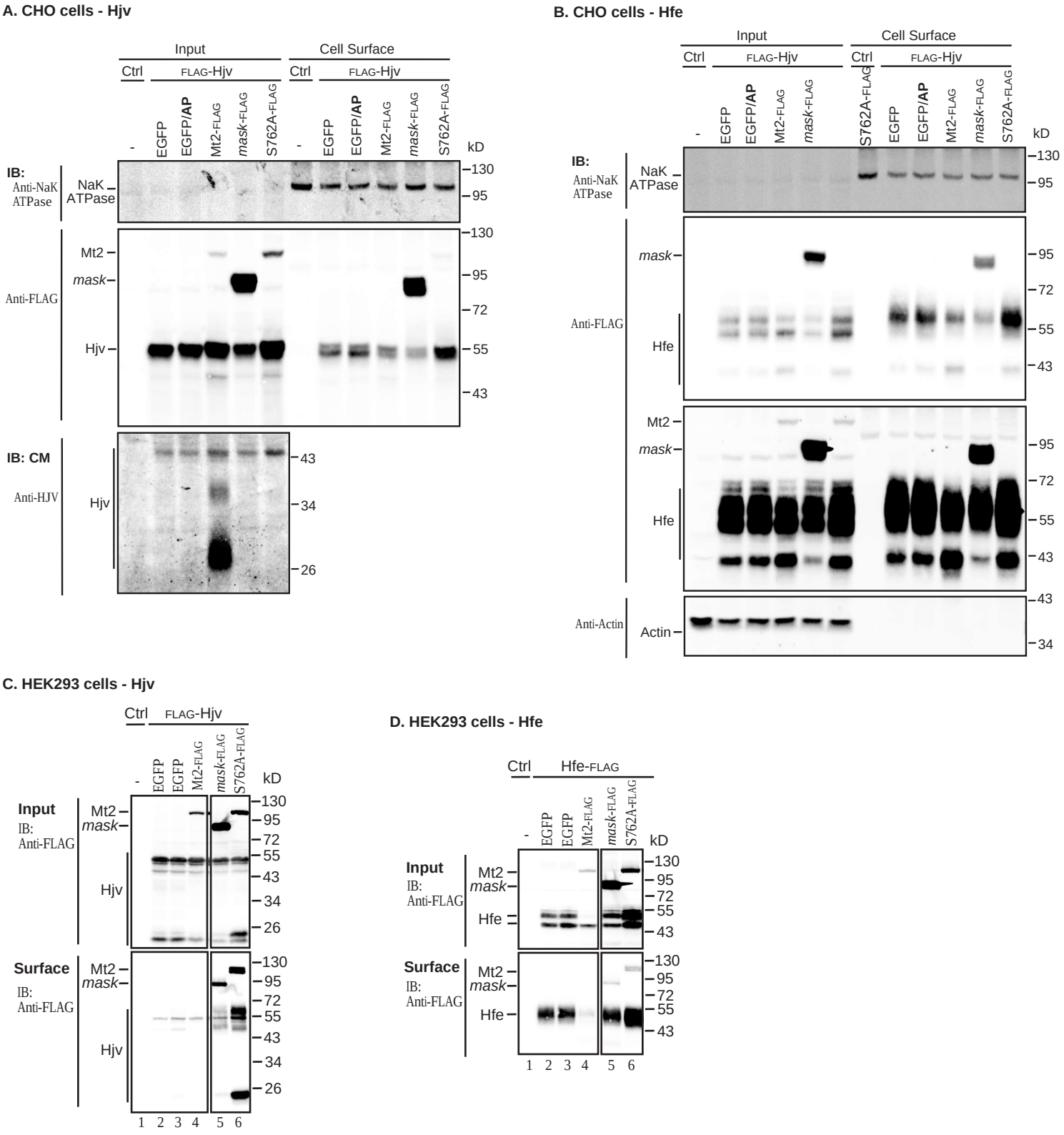

Figure S3. *Effects of Mt2, Mt2^{S762A} and Mt2^{mask} on Hjv and Hfe in CHO cells (**A/B**) and HEK293 cells (**C/D**).* Transfection of Hjv and Hfe, biotinylation, and immunodetection were performed as described in the legends to **Figure 2A/E**. Two images for Mt2 and Hfe with different exposure were presented in **B**. Cellular and cell surface Mt2, Mt2^{S762A}, Mt2^{mask}, Hjv, and Hfe were immunodetected by using an anti-FLAG antibody. Hjv in the CM was immunodetected by using an anti-HJV antibody. All experiments were repeated at least three times with consistent results.

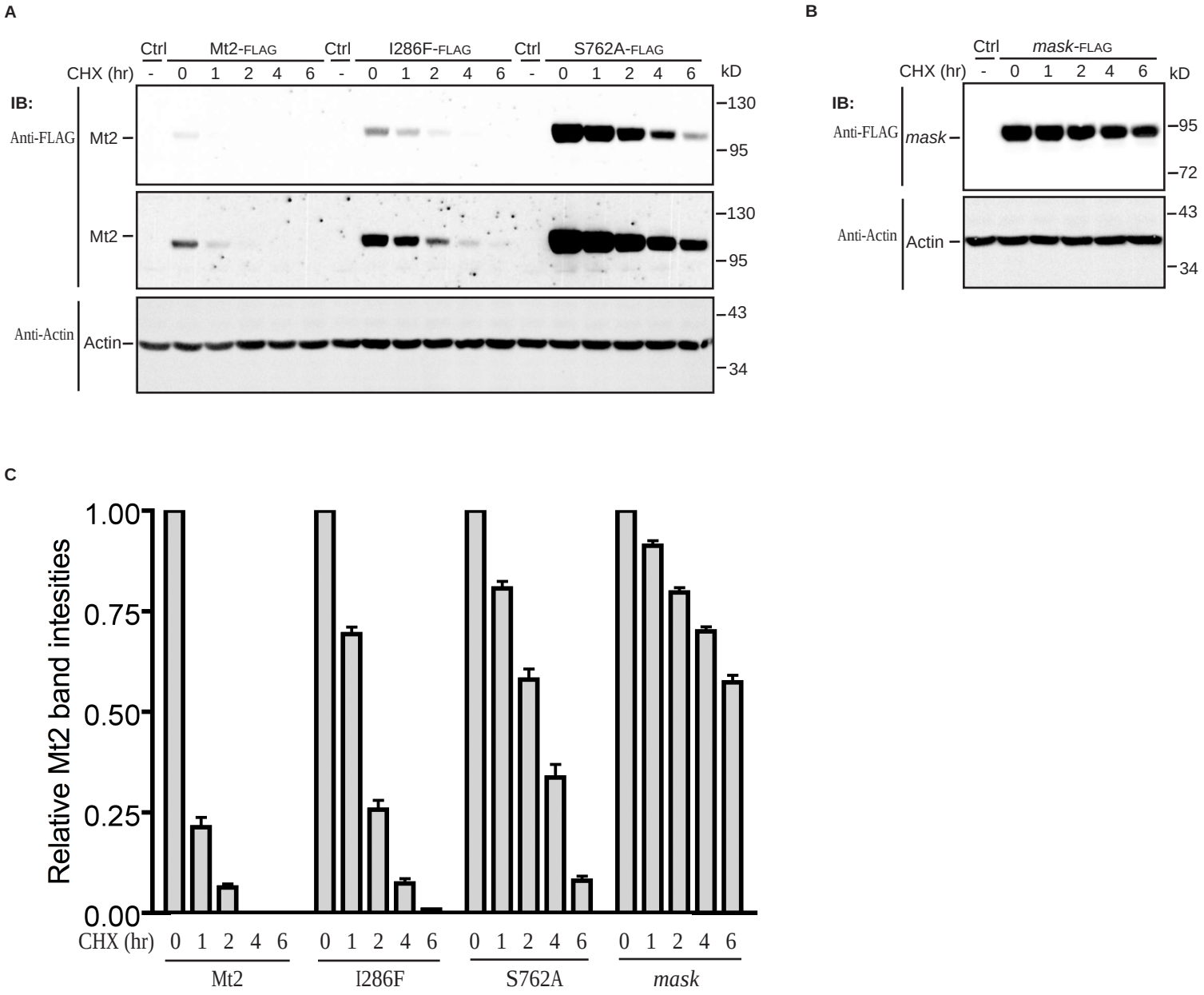

Figure S4. *Analysis of the degradation rates of cellular Mt2, Mt2^{I286F}, Mt2^{S762A}, and Mt2^{mask} in Hep3B cells.* Hep3B cells in 12-well plates were transfected with an equal amount of pCMV9-Mt2, Mt2^{I286F}, Mt2^{S762A}, or Mt2^{mask} plasmid DNA. At about 2 day post-transfection, fresh medium with 100 μg/mL of cycloheximide (CHX) was changed to block protein biosynthesis. After 0, 1, 2, 4, and 6 hr of incubation, cell lysate was collected for immunodetection of Mt2 and mutants using an anti-FLAG antibody (**A/B**). All experiments were repeated three times with consistent results. **C)** Quantification of Mt2 and mutant bands in **A & B**.

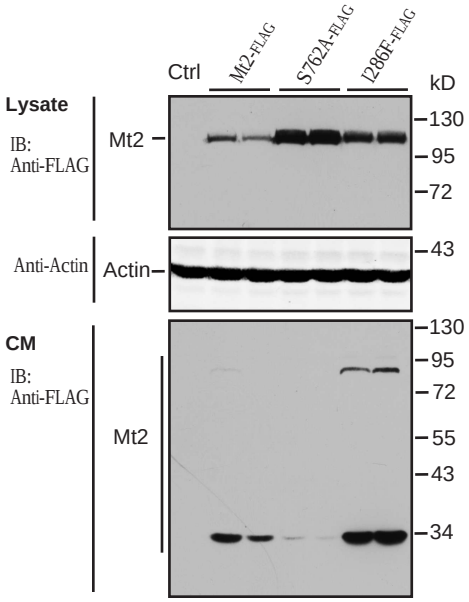

Figure S5. *I286F mutation in Mt2 does not affect the catalytic domain shedding.* HEK293 cells were transfected with equal amount of pCMV9-Mt2, $Mt2^{S762A}$, or $Mt2^{I286F}$ plasmid DNA. At about 30 hr post-transfection, medium was changed to Opti-MEM/1% FCS. Analysis was performed after another 18 hr of incubation. About 150 µg of cell lysate proteins and TCA-precipitated proteins from the conditioned medium (CM) were subjected to SDS-PAGE and immunodetection by using anti-FLAG HRP and anti-β actin. Experiments were repeated at least three times with consistent results.

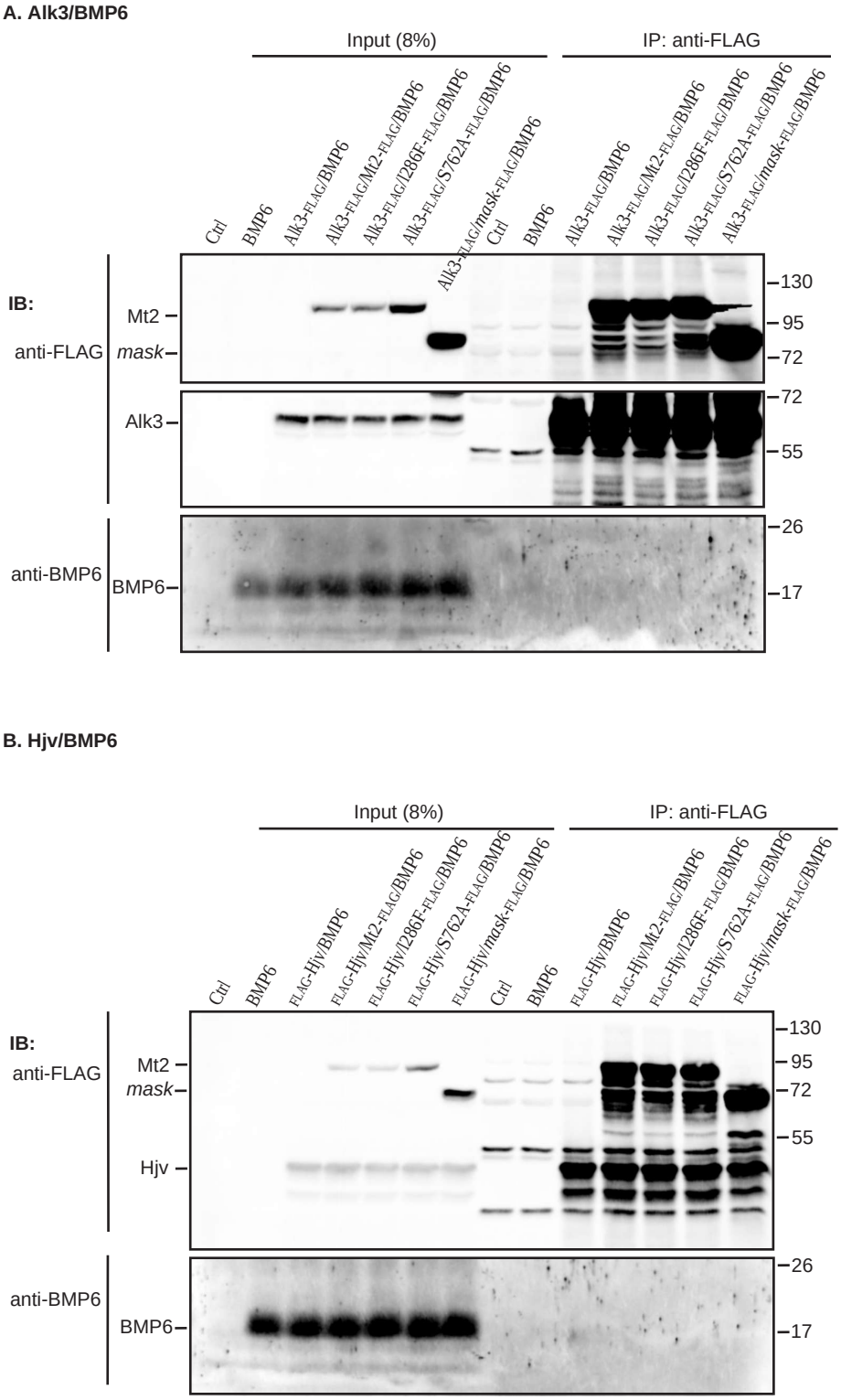

Figure S6. *Co-immunoprecipitation analysis of Alk3* (**A**) *and Hjv* (**B**) *with BMP6 in the absence or presence of Mt2 or mutants*. HEK293 cells were transfected individually with pCMV6-Mt2, $Mt2^{I286F}$, $Mt2^{S762A}$, $Mt2^{mask}$, Alk3, or pCMV9-Hjv. Un-transfected cells were used as control (Ctrl). Alk3 or Hjv lysates (~400 µl) were mixed sequentially with BMP6 (300 ng; R&D #507-BP) and control or Mt2 or mutant lysates (~400 µl). After overnight incubation at 4˚C, Alk3, Hjv, and Mt2 was pulled down by using anti-FLAG affinity gel (Sigma #A2220), and eluted by using 3xFLAG Peptide at ~200 µg/ml (Sigma F4799). About 8% of input and the entire eluates were subjected to SDS-PAGE, followed by immunodetection of Alk3/Hjv/Mt2 and BMP6 using mouse anti-FLAG and goat anti-BMP6 antibody (sc-7406; Santa Cruz Biotechnology), respectively. The experiments were repeated three times with consistent results.

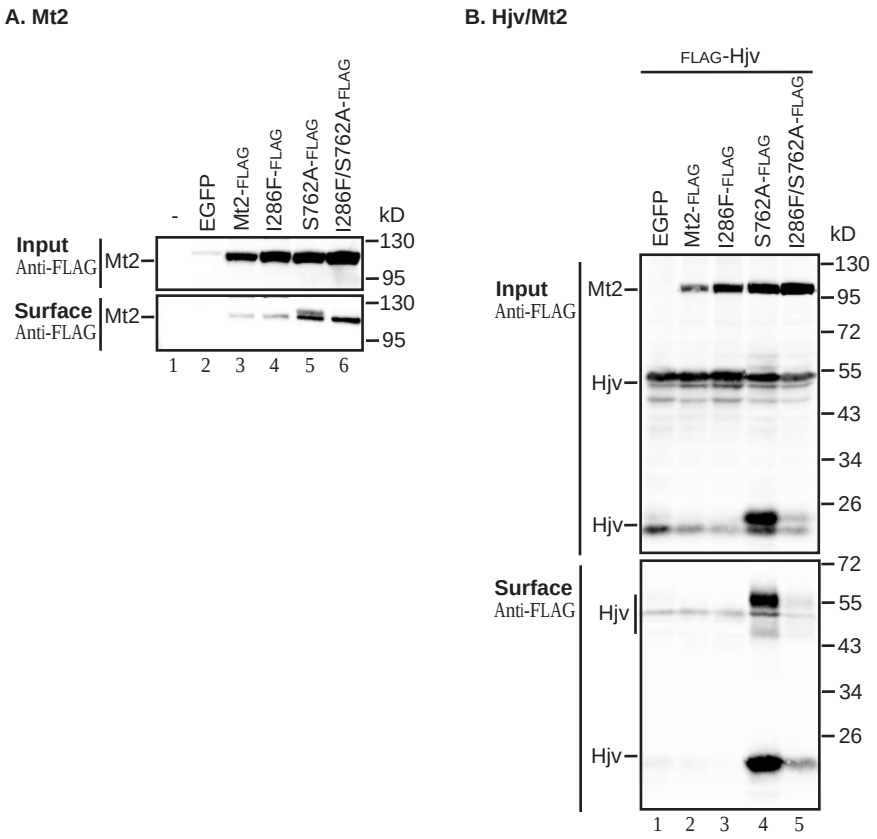

Figure S7. *Cell surface expression of $Mt2^{I286F/S762A}$ and the effects of $Mt2^{I286F/S762A}$ on cell surface Hjv in HEK293 cells.* **A**) Cell surface expression of $Mt2^{I286F/S762A}$. HEK293 cells were transfected with equal amount of pEGFP-N1 (EGFP), pCMV9-Mt2, $Mt2^{I286F}$, $Mt2^{S762A}$, or $Mt2^{I286F/S762A}$ plasmid DNA. At about 2 days post-transfection, cell surface proteins were biotinylated at 4˚C, followed by pull-down 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. **B**) Effects of $Mt2^{I286F/S762A}$ on cell surface Hjv. pCMV9-Hjv was co-transfected with an equal amount of pEGFP-N1 (EGFP), pCMV9-Mt2, $Mt2^{I286F}$, $Mt2^{S762A}$, or $Mt2^{I286F/S762A}$ plasmid DNA into HEK293 cells. At about 2 days post-transfection, cell surface proteins were biotinylated at 4˚C, followed by pull-down 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 for Mt2, $Mt2^{I286F}$, $Mt2^{S762A}$, $Mt2^{mask}$, and Hjv. All experiments were repeated at least three times with consistent results.

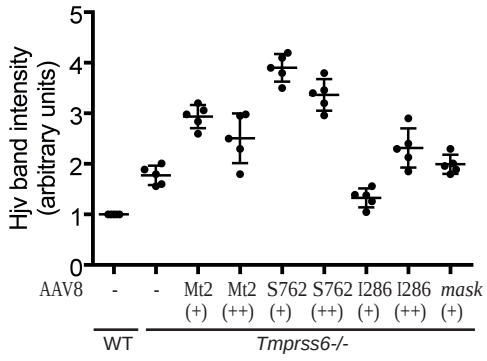

Figure S8. *Quantification of Hjv bands from* ***Figure 6A/B***.

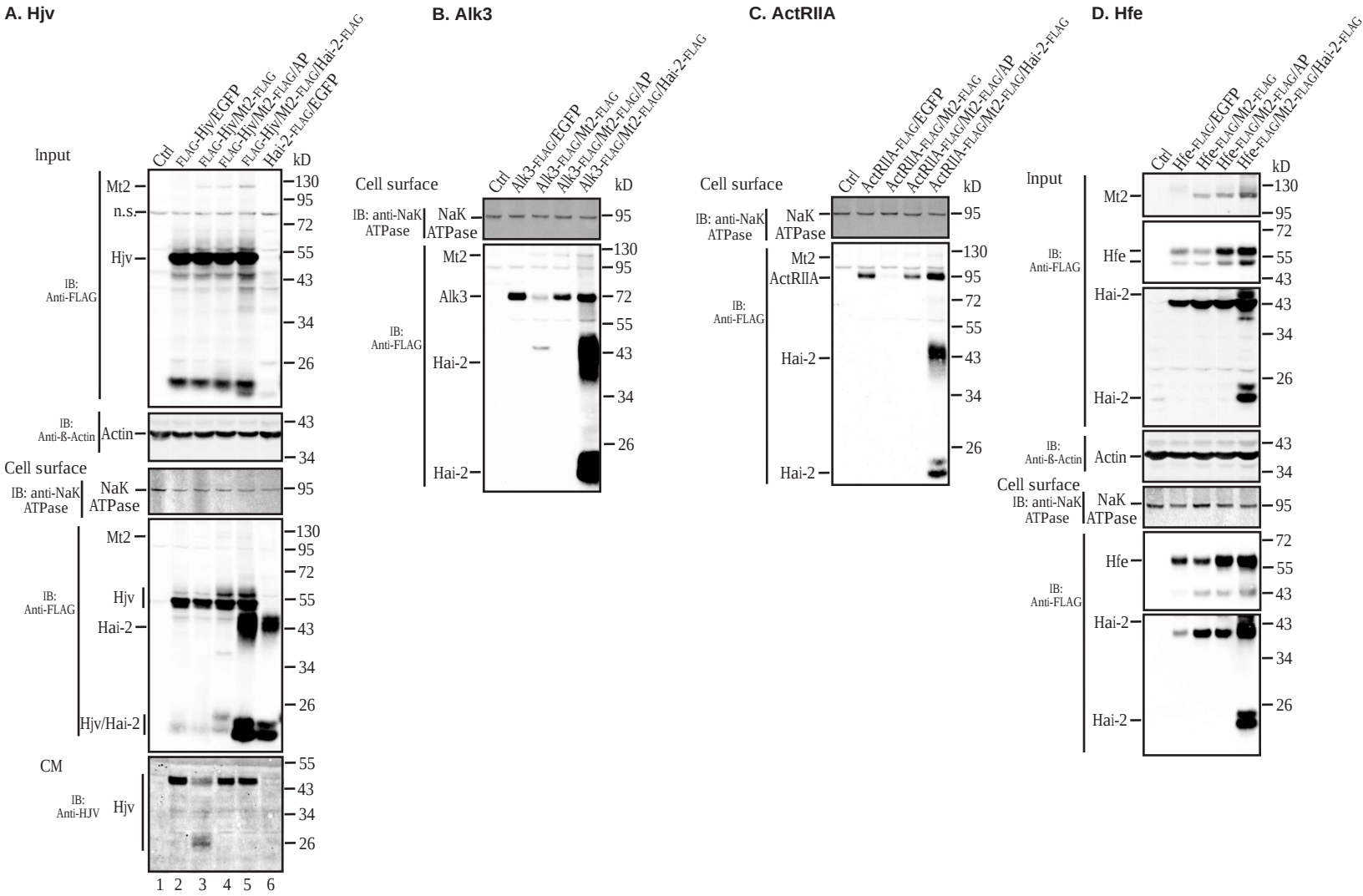

Figure S9. *Hai-2 inhibits Mt2 cleavage of Hjv*, *Alk3, ActRIIA, and Hfe in Hep3B cells.* **A-C)** Hai-2 inhibition of Mt2 cleavage of Hjv (**A**), Alk3 (**B**), and ActRIIA (**C**). pCMV9-Hjv, pCMV6-Alk3, or pCMV6-ActRIIA was co-transfected with pCMV6-Mt2 and pCMV6-Hai-2 or pEGFP-N1 at 1:1:1 ratios of plasmid DNA into Hep3B cells. At about 2 days post-transfection, cell surface proteins were biotinylated, followed by pull-down of biotinylated proteins and immunodetection as described in the legends to **Figure 2A-C**. **D)** Hai-2 inhibition of Mt2 cleavage of Hfe. Hep3B cells were co-transfected with pCMV6-Hfe, pJB-1-B2M, pCMV6-Mt2, and Hai-2 or pEGFP-N1 at 1:1:1:1 ratios of plasmid DNA. Biotinylation were the same as described above for Hjv, Alk3, and Hfe. Cellular and cell surface Mt2, Hfe, and Hai-2 were immunodetected by using an anti-FLAG antibody. All experiments were repeated at least three times with consistent results.