Mutations in SPINT2 encoding the epithelial serine protease inhibitor hepatocyte growth factor activator inhibitor-2 (HAI-2) are associated with congenital tufting enteropathy. However, the functions of HAI-2 in vivo are poorly understood. Here we used tamoxifen-induced Cre-LoxP recombination in mice to ablate Spint2. Mice lacking Spint2 died within 6 days after initiating tamoxifen treatment and showed severe epithelial damage in the whole intestinal tracts, and, to a lesser extent, the extrahepatic bile duct. The intestinal epithelium showed enhanced exfoliation, villous atrophy, enterocyte tufts and elongated crypts. Organoid crypt culture indicated that Spint2 ablation induced Epcam cleavage with decreased claudin-7 levels and resulted in organoid rupture. These organoid changes could be rescued by addition of serine protease inhibitors aprotinin, camostat mesilate and matriptase-selective α-ketobenzothiazole as well as by co-deletion of Prss8, encoding the serine protease prostasin. These results indicate that HAI-2 is an essential cellular inhibitor for maintaining intestinal epithelium architecture.
**SPINT2** encodes the type 1 transmembrane protein hepatocyte growth factor activator inhibitor (HAI)-2 that has two extracellular Kunitz-type serine protease inhibitor domains, namely KD1 (N-terminal side Kunitz domain) and KD2 (C-terminal side Kunitz domain), a single path transmembrane domain and a short intracytoplasmic domain. The protease inhibitor domain of HAI-2 is homologous to that of HAI-1. HAI-2 and HAI-1 also have a similar anti-protease spectrum and are frequently co-expressed by epithelial cells. They regulate the activities of serum hepatocyte growth factor activator (HGFA), cellular type 2 transmembrane serine proteases (TTSP), particularly matriptase, and glycosylphosphatidylinositol-anchored serine protease, Prss8 (also known as prostatin). However, in a human cell line expressing both HAI-1 and HAI-2, HAI-2 could not compensate for loss of HAI-1 and vice versa, and silencing of **SPINT1** or **SPINT2** produces specific and differing phenotypes, suggesting that these two protease inhibitors have distinct roles in epithelial cells. Indeed, HAI-1 has a distinct cell surface localization, whereas HAI-2 localizes to the cytoplasm. In mice, deletion of either **Spint2** or **Prss8** is present. Therefore, a conditional knockout mouse system based on the Cre recombinase and the Cre recombinase expression results in the deletion of most of the **Spint2** gene coding region, including exons encoding KD1 (exon 2) and KD2 (exon 5) (Fig. 1a and Supplementary Fig. 1a). The floxed mice were then crossed with ROSA26-CreERT2 mice to generate Spint2LoxP/LoxP-CreERT2 mice. We then analyzed the effects of Spint2 deletion following intraperitoneal administration of tamoxifen to Spint2LoxP/LoxP-CreERT2 mice (homozygous Spint2 deletion) at 6 weeks of age to activate the Cre recombinase and compared the induced phenotypes with those of vehicle-treated controls (wild-type Spint2) and also with tamoxifen-treated Spint2LoxP/+CreERT2 mice (heterozygous Spint2 deletion). Within three days, tamoxifen-treated Spint2LoxP/LoxP-CreERT2 mice were lethargic and had significant weight loss (Fig. 1b). Three days after starting tamoxifen treatment, HAI-2 mRNA was hardly detectable in major HAI-2–expressing organs such as the intestinal tract (Fig. 1c). Genomic polymerase chain reaction (PCR) confirmed the DNA rearrangement in all tissues except for the brain (Supplementary Fig. 1b). Notably, the Spint2LoxP/LoxP-CreERT2 mice survived no more than 6 days after beginning tamoxifen treatment, indicating that the induced loss of HAI-2 protein had a lethal effect (Fig. 1d). Similar results were seen for another Spint2LoxP/LoxP-CreERT2 mouse derived from an independently targeted embryonic stem (ES) clone (ES89; Supplementary Table 1). 

### Results

**Lethal effect of spontaneous Hai-2 loss in mice.** To circumvent the embryonic lethality associated with HAI-2 deficiency, we engineered mice homozygous for Spint2 floxed alleles in which Cre recombinase expression results in the deletion of most of the Spint2 gene coding region, including exons encoding KD1 (exon 2) and KD2 (exon 5) (Fig. 1a and Supplementary Fig. 1a). The floxed mice were then crossed with ROSA26-CreERT2 mice to generate Spint2LoxP/LoxP-CreERT2 mice. We then analyzed the effects of Spint2 deletion following intraperitoneal administration of tamoxifen to Spint2LoxP/LoxP-CreERT2 mice (homozygous Spint2 deletion) at 6 weeks of age to activate the Cre recombinase and compared the induced phenotypes with those of vehicle-treated controls (wild-type Spint2) and also with tamoxifen-treated Spint2LoxP/+CreERT2 mice (heterozygous Spint2 deletion). Within three days, tamoxifen-treated Spint2LoxP/LoxP-CreERT2 mice were lethargic and had significant weight loss (Fig. 1b). Three days after starting tamoxifen treatment, HAI-2 mRNA was hardly detectable in major HAI-2–expressing organs such as the intestinal tract (Fig. 1c). Genomic polymerase chain reaction (PCR) confirmed the DNA rearrangement in all tissues except for the brain (Supplementary Fig. 1b). Notably, the Spint2LoxP/LoxP-CreERT2 mice survived no more than 6 days after beginning tamoxifen treatment, indicating that the induced loss of HAI-2 protein had a lethal effect (Fig. 1d). Similar results were seen for another Spint2LoxP/LoxP-CreERT2 mouse derived from an independently targeted embryonic stem (ES) clone (ES89; Supplementary Table 1).

**Hai-2 loss induced severe epithelial damage of the intestine.** To clarify the cause of lethality in Spint2LoxP/+LoxPCreERT2 mice, we sacrificed the mice 24, 36, 48, and 72 h after starting tamoxifen treatment. At 72 h, macroscopic phenotypes were observed in the gastrointestinal tracts of tamoxifen-treated Spint2LoxP/LoxP-CreERT2 mice, but not in other organs. In the treated mice, the length of the intestine was shorter compared to the control mice and the tissue showed diffuse mucosal damage. The stomach was expanded (Fig. 2a). Subsequent histopathological evaluation revealed successful ablation of the HAI-2 protein and diffuse epithelial damage with mucosal disorganization throughout the intestinal tracts of Spint2LoxP/LoxP-CreERT2 mice. In the small intestine, 72 h after starting the tamoxifen treatment Spint2 inactivation dramatically altered the mucosal architecture as evidenced by severe villus atrophy and significantly elongated crypts that showed proliferative activity (Fig. 2b). The ratio of villous length relative to the corresponding crypt depth was significantly decreased in tamoxifen-treated mice relative to vehicle-treated control mice (p = 0.0005) (Supplementary Fig. 2a). In the villous epithelium, stacking of enterocyte nuclei produced a tufted appearance compatible with descriptions of intestinal specimens from CTE patients (Fig. 2b). The time-course observation also indicated that the number of enterocytes positive for cleaved caspase-3 increased in the upper portion of the crypts and villus base 36 h after tamoxifen treatment (Supplementary Fig. 2b). After 48 h, increased exfoliation of single epithelial cells was present, with peeling of enterocyte sheets from the villous tip (Fig. 2c). These peeling cells were positive for staining with antibodies against cleaved caspase-3 and single-strand DNA (ssDNA), suggestive of apoptotic changes (Fig. 2c). Meanwhile, the number of Paneth cells decreased in the crypt base (Fig. 2d) in...
a statistically significant level ($p = 0.0028$, Student $t$-test, Supplementary Fig. 2c).

Diffuse epithelial damage was also evident in the large intestine. Both the cecum and colon showed obvious, diffuse epithelial damage after 3 days, with increased numbers of apoptotic bodies and exfoliating cells (Fig. 3a). The number of crypts was decreased by 48 h, followed by an active regeneration phase when elongated crypts became apparent. After 72 h, mucosal disorganization was evident, showing marked mucosal thickening with elongated crypts, reactive epithelial cell atypia, and small tufted structures consisting of stacked enterocytes on the surface (Fig. 3a, b). The elongated crypts were composed mostly of Ki67-positive proliferating cells, and many detached cells having pyknotic nuclei were also present (Fig. 3b). The number of goblet cells showed a time-dependent decrease after tamoxifen treatment (Fig. 3c). Collectively, the absence of Hai-2 proteins impaired the terminal differentiation capacity needed to establish a normal epithelial structure. However, no increase in the frequency of $\beta$-catenin nuclear translocation was observed during the observation period, indicating that abnormal Wnt/$\beta$-catenin signaling did not contribute to the Hai-2 loss-induced enteropathy (Supplementary Fig. 2d).

Although the glandular epithelium of the stomach also showed slight increased amounts of apoptosis and focal erosion, these changes were not as pronounced as those seen in the intestine (Supplementary Fig. 3). Thus, the marked stomach distention was likely secondary to intestinal dysfunction. Taken together, the marked structural damages and dysfunction of the intestinal epithelium likely underpin the lethality caused by spontaneous loss of Hai-2 in mice.
Extrahepatic biliary tracts were also affected by Hai-2 loss. As HAI-2/Hai-2 is widely expressed in epithelial tissues in humans and mice, we also searched for additional histopathological changes in other epithelial tissues. Significant alterations were observed in the epithelium of the gallbladder and extrahepatic bile duct of Spint2LoxP/LoxP CreERT2 mice. The gallbladder epithelial cells tended to have a hobnail appearance, occasionally forming a slight tufted structure and increased exfoliation (Fig. 4a). However, apoptotic bodies were not evident compared with that seen in the intestinal epithelium. Although the epithelium of the extrahepatic biliary tract showed similar changes, the intrahepatic small bile ducts and pancreatic duct were not notably affected by Hai-2 ablation (Fig. 4b and Supplementary Fig. 3). In accordance with this observation, Hai-2 expression levels were lower in the intrahepatic small bile ducts and pancreatic ducts compared with the epithelia of gallbladder and extrahepatic bile duct (Fig. 4). The skin, bronchopulmonary epithelial cells, esophageal epithelium, and renal tubules showed no obvious
**Fig. 3** Effects of Hai-2 ablation on large intestine.  
**a** Histology of the cecum from Spint2^loxP/loxP^CreERT2 mice after tamoxifen or vehicle (corn oil) treatment. Representative photos of HE from mice 24, 36, and 72 h after tamoxifen treatment are shown. Successful Hai-2 ablation was confirmed by immunohistochemistry.  
**b** Histology of the colon 72 h after tamoxifen treatment. Crypts were elongated with many exfoliating, degenerated cells present in the lumen. Arrows indicate a tuft formed by enterocytes. Higher magnification images are shown in the insets. Representative photos of Ki67 immunohistochemistry are also presented (right panel).  
**c** Decreased numbers of goblet cell in Spint2-deleted colon mucosa. Alcian-blue staining. The 25th and 75th percentile (boxes) and the median (bold line within the boxes) are plotted. Circle represents the value of each case. Goblet cells per crypt of Spint2-deleted mice (n = 5 and 11 for 48 h and 72 h after starting treatment, respectively) in the proximal and distal colon were counted and compared to that for vehicle-treated control mice (n = 8). p-value; Mann-Whitney U-test. Bars, 50 μm (**a-c**).
histopathological changes during the observation period (Supplementary Fig. 3).

Hai-2 loss led to destruction of intestinal organoids. Epithelial damage and disorganization of the intestinal architecture in Spint2-deleted mice could be a direct effect of Hai-2 loss in epithelial cells or arise from secondary stromal inflammation caused by leaky epithelia and intestinal microbial colonization. To distinguish between these possibilities, we performed in vitro three-dimensional (3-D) crypt organoid culture of Spint2LoxP/LoxPCreERT2 small intestine cells. Similar to in vivo observations, deterioration of organoid integrity began 24–28 h after treatment with 4-hydroxytamoxifen (4-OHT) (Fig. 5a and Supplementary Movies 1 and 2). At 36 h (Fig. 5b), destruction or rupture of crypt structure was apparent (Supplementary Movies 1 and 2), whereas the vehicle-treated organoids were intact (Supplementary Movie 3).

Disruption of Epcam/claudin-7 complex by Hai-2 loss. CSD patients, including those with syndromic-form CSD caused by SPINT2 mutation, show intestinal histology compatible with that observed in CTE caused by EPCAM mutations. Therefore, we performed immunohistochemical analysis of the expression and localization of Epcam and one of its essential partners in the intestinal epithelium, claudin-721,22. Hai-2-deficient epithelium showed decreased cell surface Epcam immunoreactivity and mislocalization within the cytoplasm of disorganized epithelial cells, particularly in cells forming tufts, as well as accompanying decreases in claudin-7 immunoreactivity (Fig. 6a). The disruption and mislocalization of Epcam peaked 48 h after tamoxifen treatment, as did increases in mucosal permeability (Fig. 6b, c). Loss of cell surface Epcam and claudin-7 immunoreactivity was also observed in Hai-2-deficient extrahepatic biliary epithelium (Fig. 6d).

We then analyzed Epcam and claudin-7 expression in 3-D intestinal organoid cultures from Spint2LoxP/LoxPCreERT2 mice. Immunoblot analysis showed enhanced levels of a 35-kDa cleavage product of Epcam in the absence of Hai-2 as well as markedly decreased claudin-7 levels (Fig. 7a). Immunofluorescence analysis of these proteins also showed significantly decreased cell surface Epcam and claudin-7 in organoids after 4-OHT treatment (Fig. 7a). On the other hand, Epcam and claudin-7 mRNA levels were not affected (Supplementary Fig. 4).
indicating that the decrease in cell surface Epcam and claudin-7 in response to Spint2-deletion occurred at a post-translational level. Importantly, addition of the protease inhibitor aprotinin to the culture media significantly suppressed Epcam cleavage and concomitant decreases in claudin-7 levels (Fig. 7a). Therefore, an excess of trypsin-like serine protease activity was likely responsible for Epcam cleavage. Notably, aprotinin rescued Hai-2 ablation-induced destruction in organoids (Fig. 7b). Similar in vitro results were also obtained following the addition of the synthetic serine protease inhibitor camostat mesilate (Fig. 7c). However, in vivo administration of camostat mesilate (oral or intraperitoneal) could not rescue the lethal phenotype of Spint2LoxP/LoxPCRcET2 mice after tamoxifen treatment (Supplementary Fig. 5). The ineffectiveness of camostat mesilate in vivo...
may be due to insufficient concentration of the inhibitor in the intestinal microenvironment in vivo, which will require further studies.

There were no significant changes in the expression of other junctional proteins, including E-cadherin, claudin-2 and ZO-1 in response to Hai-2 ablation (Fig. 7d). Taken together, these results indicate that Hai-2 is an essential cellular protease inhibitor needed to stabilize the Epcam/claudin-7 complex in the intestine.

**Prss8 was required for Epcam cleavage in Spint2−/− organoids.** In mouse studies, embryonic lethality of Spint2−/− mice could be prevented by reducing Prss8 (prostasin) activity. In the human intestinal epithelial cell line Caco2, HAI-2 regulates PRSS8, which is required for normal cell surface localization and function of matriptase. Moreover, in these cells, HAI-2 insufficiency leads to enhanced EpCAM cleavage by matriptase. These lines of evidence suggest that phenotypes induced by spontaneous ablation of Hai-2 may be secondary to dysregulation of the Prss8-matriptase axis. To explore this possibility, we crossed Spint2<sup>LoxP/LoxPCreERT2</sup> mice with Prss8<sup>LoxP/+</sup> mice to generate either Spint2<sup>LoxP/LoxPCreERT2</sup> or Spint2<sup>LoxP/LoxP<sub>Prss8</sub>CreERT2</sup> mice, which were treated with tamoxifen at 6 weeks of age. The mean body weight of Spint2<sup>LoxP/+ Prss8<sub>LoxP/LoxPCreERT2</sub></sup> mice did not decrease after tamoxifen treatment (Fig. 8a), suggesting that Prss8 is not essential for maintenance of the intestinal epithelium as reported previously. Deletion of Prss8 gene did not rescue the lethal phenotype caused by postnatal deletion of Spint2. After tamoxifen treatment, the body weight loss of Spint2<sup>LoxP/LoxP<sub>Prss8</sub>CreERT2</sup> mice was comparable to that of Spint2<sup>LoxP/LoxP</sup> CreERT2 littermate mice.
(Fig. 8a) and the intestinal tissues showed similar macroscopic changes (Fig. 8b). We next sought to determine whether Prss8 is required for Epcam cleavage in the presence of Hai-2 ablation in organoids prepared from the small intestines of Spint2^LoxP/LoxP^Prss8^LoxP/LoxP^CreERT2 mice. In contrast to the observation in vivo, co-deletion of Prss8 suppressed Epcam cleavage, stabilized claudin-7 levels (Fig. 8c, d) and rescued destruction of crypt organoids caused by Hai-2 ablation (Fig. 8e).

Matriptase cleaved Epcam in Spint2^−/−^ organoids. It has been reported that Prss8 cannot directly cleave Epcam whereas matriptase can17. Thus, we asked whether matriptase was responsible for the enhanced cleavage of Epcam in Spint2-deleted intestinal organoids due to dysregulated Prss8-matriptase axis. Transfection of matriptase short hairpin RNA (shRNA) lentiviral vector reduced the matriptase mRNA level by 56%, which partly alleviated the Epcam cleavage in Spint2-deleted organoids (Fig. 9a, b). In accordance with this observation, matriptase-selective synthetic inhibitor ZFH7185-8 suppressed the Hai-2 loss-induced Epcam cleavage in a dose-dependent manner, accompanying stabilization of claudin-7 (Fig. 9c). In contrast, hepsin-selective inhibitor ZFH7185-3 did not show notable effects (Fig. 9c).

Discussion
In this study, we demonstrated that acute ablation of the Hai-2 protein had a lethal effect on mice due to epithelial changes and intestinal dysfunction. Although increased exfoliation of epithelial
cells and in turn active cellular proliferation occurred in the intestinal crypts, epithelial regeneration was abnormal such that the intestinal mucosa eventually showed severely disorganized architecture, atrophic villi, continuous exfoliation and tufted formations composed of enterocytes, as well as markedly elongated crypts. Mice carrying the Spint2 deletion all died within 6 days of initiating tamoxifen treatment.

Congenital SPINT2 mutations induce a syndromic form of CSD, and the intestinal epithelium of patients suffering from this rare autosomal-recessive disorder shows histological changes similar to those seen in CTE\textsuperscript{15,16}. Conventional CTE is caused by a mutation in the EPCAM gene wherein abnormal EpCAM function destabilizes EpCAM-associated claudin-7, which eventually results in abnormally enhanced epithelial permeability\textsuperscript{15,21,22,25}. Our organoid culture study revealed that Hai-2 loss accelerates Epcam protein cleavage, which was suppressed by the addition of protease inhibitors targeting trypsin-like serine proteases. Therefore, excess cleavage of Epcam by serine proteases could occur in the absence of Hai-2. Evidence indicates that HAI-2/Hai-2 regulates PRSS8/Prss8 in epithelial cells such as enterocytes, which influences
matriptase localization and activation in the cells. Given that EpCAM is a matriptase substrate in human enterocytes, abnormal activity of the Prss8-matriptase axis could underpin the enhanced EpCAM cleavage that occurred in the absence of Hai-2. Indeed, co-deletion of Prss8 and Spint2 in organoids relieved the EpCAM cleavage in the absence of Hai-2, and consequently maintained the claudin-7 levels and rescued organoids from subsequent rupture. Moreover, silencing of matriptase in organoids or treatment of the organoids with matriptase-selective synthetic inhibitor alleviated the Hai-2 loss-induced EpCAM cleavage. Therefore, Hai-2 tightly regulates Prss8-matriptase axis in the intestinal epithelial cells and dysregulation of this axis results in excess EpCAM cleavage by matriptase and destabilization of the EpCAM-claudin-7 complex. Whereas co-deletion of Prss8 and Spint2 suppressed EpCAM cleavage caused by Spint2 deletion in organoids, Spint2/Prss8 co-deletion could not rescue the lethal phenotype associated with Spint2 deletion in mice. This discrepancy may be consistent with recent observations in another CTE model: Spint2−/−/Prss8R44Q/R44Q double mutant mice. In the double mutant model, homozygous R44Q mutation of Prss8 gene resulted in the impaired activation of prostasinzymogen (i.e.,zymogen-locked Prss8) and rescued embryonic lethality caused by the Spint2 deletion, and the intestinal development was normal in Spint2−/−/Prss8R44Q/R44Q fetus at E18.5. However, after birth, Spint2−/−/Prss8R44Q/R44Q mice showed severe growth retardation and died within a week with severe intestinal abnormalities reminiscent to CTE. Thus, homozygous R44Q mutation in Prss8 rescued Spint2 deletion-induced CTE only during the fetal period. Environmental factors, such as intestinal microbial colonization and food intake, may underpin the conflicting observations between mice in vivo after birth and organoids ex vivo that might be equivalent to the fetal intestine. Nonetheless, these lines of evidence raise the question of whether EpCAM cleavage is sufficient to induce the severe intestinal phenotype in vivo. In fact, the histological abnormalities observed in the colon mucosa of the Spint2-deletion mice in this study were more severe than those reported for EpCam mutant mice. Moreover, our Spint2 knockout mice showed epithelial abnormalities in the gallbladder and extrahepatic bile duct, which were not reported in the Epcam knockout studies. Therefore, the role of Hai-2 in epithelial cells may not be limited to stabilization of the Epcam/claudin-7 complex in the intestine, and Hai-2 may have additional, vital functions for epithelial cells in vivo. Hai-2/Prss8 2 immunoreactivity has been observed primarily in the cytoplasm, and it likely localizes to the endoplasmic reticulum (ER). Hai-2/Hai-2 may therefore have an important role in regulation of protein export in the ER and intracellular secretory pathways, which might be required to maintain epithelial integrity in vivo. Further studies to identify proteins interacting with Hai-2/Hai-2 will be required. Whether Hai-2 loss affects the activity of epithelial sodium channel (ENaC) also remains unclear. Prss8 and matriptase, which are both Hai-2 sensitive, are likely involved in ENaC activation, and patients carrying SPINT2 gene mutations often experience sodium diarrhea. Although the severe effect of spontaneous Hai-2 ablation precluded analysis of ENaC function in our mouse model in vivo, ex vivo intestinal organoids from these mutant mice could provide a tool for future studies to explore the roles of Hai-2 and Prss8 in intestinal ENaC activation. Finally, the effect of Hai-2 loss on intestinal stem cells also awaits analysis. Given that the number of Lgr5-positive intestinal stem cells remained unchanged in Epcam-deleted mice, intestinal stem cells may not be a primary target in the Spint2 deletion-induced phenotype. However, Paneth cells maintain the stem cell niche in intestinal crypts, and the number of Paneth cells was decreased by Spint2 deletion. Therefore, the maintenance of stem cells may indeed be disturbed in the Spint2-deleted intestine. The conditional Spint2 knockout mouse described in this report is the second mouse model of Hai-2 deficiency-induced CTE next to Spint2−/−/Prss8R44Q/R44Q double mutant mouse recently reported by Szabo and Bugge. Although both models show similar CTE morphology, there are a couple of differences in regard to histological findings. First, mucosal damage, inflammation and apoptotic epithelial cells were more evident in our model compared to the double mutant model. Second, Ki67-positive cells in the intestinal crypts increased significantly in our model, but not in the double mutant model. In addition, abnormalities observed in the extrahepatic biliary epithelium of our mutant mice were not reported in the double mutant mice. These differences may be due to the method of Spint2 ablation: acute Spint2 ablation in postnatal period in our model versus genetically Spint2 null condition in the double mutant model. The strength of the Spint2-Prss8 double mutant model may be an early-onset intestinal failure after birth, which likely mimics the patients of congenital CTE. However, Prss8 mutation is not reported in the CTE patients. The current conditional Spint2 knockout mouse model has wild-type Prss8 and has an advantage in this regard. Another important advantage of the current model is the availability of organoid culture. The intestinal organoids from Spint2Lox/Lox/Prss8Lox/LoxCreERT2 mice reproduces molecular changes induced by Hai-2 deficiency in vitro, providing an indispensable research tool for the study of CTE. In conclusion, Hai-2 is a vital epithelial protease inhibitor that is needed to maintain the integrity of the intestine and extrahepatic biliary epithelia as well as to stabilize the Epcam/claudin-7 complex. Our Spint2-mutant mouse model together with the intestinal organoid culture from these mutant mice would provide a useful tool for detailed studies of Hai-2 function in epithelial cells and the screening of therapeutic compounds for patients carrying SPINT2 mutations. Successful inhibition of
Methods

involving animals. The required portions of the mouse Committee, in accordance with international guidelines for biomedical research performed under the approval of the University of Miyazaki Animal Research

β-actin mouse mAb (Sigma) and anti-β-actin antibody (Dako, Carpinteria, CA).

Spint2

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

Generation of Spint2 conditional knockout mice. All of the animal work was performed under the approval of the University of Miyazaki Animal Research Committee, in accordance with international guidelines for biomedical research involving animals. The required portions of the mouse Spint2 gene were amplified by PCR from a mouse (C57BL/6) genomic bacterial artificial chromosome library and subcloned into a pBluescript II SK + phagemid vector (Agilent Technologies, Palo Alto, CA), which was used to construct a targeting vector. LoxP sites were inserted into exons 2 and 6 to delete all transcript variants of the Spint2 gene followed activation of Cre recombinase. A neomycin resistance gene (neo) cassette for positive selection was inserted into intron 6 and was flanked by short flippase recognition target (FRT) sites. The targeting vector also contained a diphtheria toxin expression cassette for negative selection and had an MfeI site for screening by Southern blotting. The targeting vector was linearized with SceI and transfected into C57BL/6 strain ES cells by electroporation. Correctly targeted ES clones were selected using G418 (Sigma). Two independently targeted ES clones (ES16 and ES89) were obtained and microinjected into morulae of ICR mice. The resulting chimeras were mated with C57BL/6 mice. Germline transmission of the targeted allele was detected by progeny coat color and PCR. FLPeR mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and the mutant mice were cross-mated for more than nine generations with C57BL/6 mice (Charles River Laboratories, Portage, MI). Then, Spint2loxPfloxP/+neo+ mice were crossed to the B6-background FLPeR mice to remove the neo cassette. Heterozygous offspring (Spint2loxPfloxP/) were crossed to produce homozygous mutant offspring (Spint2loxPflaxP). Spint2loxPflaxP mice were further crossed with ROSA26-CreERT2 mice (The Jackson Laboratory) to generate ROSA26-CreERT2 mice with the floxed Spint2 gene (hereafter Spint2flaxPflaxPCreERT2). To activate the CreERT2 recombinase, 6-

Fig. 9 Matriptase is responsible for Epcam cleavage. a Knockdown efficacy of matriptase (encoded by St14 gene) shRNA in organoids from Spint2loxPflaxPCreERT2 mice. RNA was extracted from mock-transfected control (Cont) and matriptase shRNA lentivirus vector-transfected (KD) organoids and matriptase mRNA levels were verified by quantitative RT-PCR. The 25th and 75th percentile (boxes) and the median (bold line within the boxes) are plotted. Circle represents the value of each measurement. *p = 0.029 (Student t-test; n, 4). b Control and matriptase-knockdown organoids were treated with 4-OHT (1 µM) or vehicle only (ethanol) for 28 h. Immunoblot analysis of Epcam and claudin-7 (left panel) and representative morphology of organoids (right panel) are shown. c Dose-dependent effects of matriptase-selective inhibitor (ZFHT185-8) on Epcam cleavage and claudin-7 in Spint2loxPflaxPCreERT2 organoids with or without 4-OHT treatment (28 h). Hepsin-selective inhibitor (ZFHT185-3) was also used as a control (lower panel). Representative photos of organoids are also shown

Hai-2 loss-induced Epcam cleavage by an exogenous serine protease inhibitor in an ex vivo organoid culture model could be important for developing treatments for intestinal failure induced by Spint2 mutations.

Methods

Antibodies. The following anti-mouse antibodies were used: anti-Hai-2 goat polyclonal antibody (pAb) (R & D Systems, Minneapolis, MN); anti-Epcam (ProteinTech., Rosemont, IL); anti-β-catenin (Sigma, St. Louis, MO); anti-cleaved caspase-3 (Cell Signaling, Boston, MA); anti-β-actin mouse mAb (Sigma) and anti-β-actin antibody (Dako, Carpinteria, CA).

Spint2

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)

MAL

β-actin

Claudin-7

Epcam

4-OHT

Cont

KD

4-OHT

Cont

KD

0 1 5 10 0 1 5 10

(kM)

Epcam

Claudin-7

β-actin

ZFHT185-8

ZFHT185-3

0 1 5 10 0 1 5 10

(kM)
week-old male mice were treated by intraperitoneal injection of 50 mg (135 μmol)/kg tamoxifen (Sigma) dissolved in corn oil for 3 consecutive days. Successful DNA rearrangement was validated by genomic PCR and reverse-transcription (RT)-PCR using DNA and total RNA, respectively, extracted from the tissues. The primers used for the validation described above are shown in Supplementary Fig. 1 and Supplementary Table 2. The site of each primer for genomic PCR is indicated in Fig. 1a. Unless otherwise indicated, data from the 16 μg/mouse line are presented.

To generate Sprt2 and Prss8 double conditional knockout mice, Sprt2+/-;CreERT2 mice were crossed with Prss8loxP/loxP mice. Primer sequences for genotyping of the Prss8 mutant mice are shown in Supplementary Table 2.

**Histological analysis.** For histological analysis, mice tissues were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. To evaluate crypt morphology of ileum, adjacent sections were stained with alcian-blue, counterstained with hematoxylin and eosin (HE). Villus length and crypt depth were measured using Olympus cellSens imaging software (v1.15). To count the number of Paneth cells, ten randomly selected areas were photographed at 200x magnification from each mouse small intestine. Then, two independent investigators counted the number of Paneth cells per crypt. To count the number of goblet cells, large intestinal tissues were stained with alcian-blue, and revealed with nickel, cobalt-3,3'-diaminobenzidine (Thermo Fisher Scientific) treatment for 30 min at room temperature (RT). For anti-Hai-2 pAb (1:250 dilution), Ventana Discovery system (Ventana Medical System, Tucson, AZ) was used according to the manufacturer’s instructions. For immunostaining of sdDNA, deparaffinized tissue sections were incubated with PBS containing 0.2 mg/mL saponin (Nacalai Tesque, Kyoto, Japan) and 20 μg/mL Proteinase K (Nacalai Tesque) at RT for 20 min. After washing the slides in distilled water, sections were heated in preheated 50% formamide in distilled water at 56 °C for 20 min, followed by treatment with 3% H2O2 in PBS for 10 min. After blocking in 3% bovine serum albumin in PBS, the sections were incubated with primary antibodies for 16 h at 4 °C: anti-Ki-67 (1:100 dilution), anti-cleaved caspase-3 (1:300 dilution), anti-Lysozyme (1:50 dilution), anti-Epcam (1:250 dilution), anti-claudin-7 (1:100 dilution), and anti-β-catenin (1:2000 dilution). After washing with PBS, the sections were incubated with Envision® labeled polymer reagents (DAKO) for 30 min at room temperature (RT). For anti-Hai-2 pAb (1:250 dilution), Ventana Discovery system (Ventana Medical System, Tucson, AZ) was used according to the manufacturer’s instructions. For immunostaining of sdDNA, deparaffinized tissue sections were incubated with PBS containing 0.2 mg/mL saponin (Nacalai Tesque, Kyoto, Japan) and 20 μg/mL Proteinase K (Nacalai Tesque) at RT for 20 min. After washing the slides in distilled water, sections were heated in preheated 50% formamide in distilled water at 56 °C for 20 min, followed by treatment with 3% hydrogen peroxide for 10 min and washing in PBS. After blocking in 3% non-fat dry milk for 20 min at 37 °C, the sections were incubated with anti-SDNA (1:700 dilution) in 1% non-fat dry milk at RT for 30 min. The sections were then washed in PBS and incubated with Envision-labeled polymer reagent (DAKO) for 30 min at RT. The reaction was revealed with nickel, cobalt-3,3'-diaminobenzidine (Thermo Fisher Scientific) and counterstained with hematoxylin.

**Intestinal permeability assay.** To assess intestinal barrier function, an intestinal permeability assay was performed as previously described31,32 except for Hai-2 and ssDNA. Brieﬂy, intestinal permeability assay was performed as previously described31. Briefly, mice were autopsied and tissues were collected at the indicated time point, fixed in 4% formaldehyde in PBS, and embedded in paraffin. Intestinal permeability was assessed by using fluorescein isothiocyanate (FITC)-dextran (4 kDa, Sigma) administered to the mice by gavage 4 h after starting tamoxifen treatment. Three hours after administration of FITC-dextran, blood samples were obtained from the right ventricle, and EDTA-containing plasma samples were collected by centrifugation at 960 g for 20 min at 4 °C. Plasma (75 μL) was diluted in 250 μL PBS, and the fluorescence was quantified using a fluorometer (DXT800 Multimode Detector; Beckman Coulter, Fullerton, CA) with excitation and emission wavelengths of 485 and 535 nm, respectively. FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran.

**Camostat mesilate administration.** Camostat mesilate (Ono Pharmaceutical, Osaka, Japan) was administered to the mice to addition to the diet or by gavage, or intraperitoneal injection. For dietary administration, mice were given a diet containing 0.1% camostat mesilate three days before starting tamoxifen treatment until termination. For gavage or intraperitoneal injection, mice were treated with camostat mesilate solution (30 μg/μL water; 200 mg/kg) every 12 h beginning at the same time as tamoxifen treatment was initiated.

**Matriptase-selective synthetic inhibitor.** General procedure for the preparation of a ketobenzothiazole (kbt) serine protease inhibitors for HGFA, matriptase or another TTSP namely hepsin has been reported previously33 and described in Supplementary Methods. Fluorescent kinetic enzyme inhibitor assays were performed to analyze the specificity of the kbt inhibitors using either recombinant catalytic domains of HGFA, matriptase (Prss8, Prss9, and R&D Systems) or (Prss10, R&D Systems) in black 384 plates (Corning #3575, Corning, NY) (Supplementary Methods). Two kbt inhibitors, ZFFH1785-8 and -3, were prepared (Supplemental Methods).

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Author contributions
Conceived and designed the experiments: H.K.; Performed the experiments and data analysis: M.K., K.Y., N.T., T.F., F.Y., J.W.J., H.K.; Contributed reagents/materials/analysis tools: K.S., K.K., Y.H., J.W.J.; Wrote the paper: M.K., K.Y., H.K.

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