Short Communication

Loss of Matriptase Suppression Underlies *Spint1* Mutation-Associated Ichthyosis and Postnatal Lethality

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Hepatocyte growth factor activator inhibitor-1 (HAI)-1 is an epithelial Kunitz-type transmembrane serine protease inhibitor that is encoded by the *SPINT1* gene. HAI-1 displays potent inhibitory activity toward a large number of trypsin-like serine proteases. HAI-1 was recently shown to play an essential role in postnatal epithelial homeostasis. Thus, *Spint1*-deficient mice were found to display severe growth retardation and are unable to survive beyond postnatal day 16. The mice present histologically with overt hyperkeratosis of the forestomach, hyperkeratosis and acanthosis of the epidermis, and hypotrichosis associated with abnormal cuticle development. In this study, we show that loss of inhibition of a proteolytic pathway that is dependent on the type II transmembrane serine protease, matriptase, underlies the detrimental effects of postnatal *Spint1* deficiency. Matriptase and HAI-1 precisely co-localize in all tissues that are affected by the *Spint1* disruption. *Spint1*-deficient mice that have low matriptase levels, caused by a hypomorphic mutation in the *St14* gene that encodes matriptase, not only survived the neonatal period but were healthy and displayed normal long-term survival. Furthermore, a detailed histological analysis of neonatal, young adult, as well as aged mice did not reveal any abnormalities in *Spint1*-deficient mice that have low matriptase levels. This study identifies matriptase suppression as an essential function of HAI-1 in postnatal tissue homeostasis. (Am J Pathol 2009, 174:2015–2022; DOI: 10.2353/ajpath.2009.090053)

operate in the pericellular environment to facilitate embryonic development tissue homeostasis, tissue remodeling, and tissue repair. These enzymes are synthesized as inactive precursors, or zymogens, that are converted to their catalytically active form by endoproteolytic cleavage within a highly conserved activation site. This activation step is irreversible, and proteolysis is terminated by specific macromolecular protease inhibitors that bind directly to the active site of their cognate proteases.^{1–3} Inhibition of trypsin-like serine proteases is mediated by a large number of serine protease inhibitors belonging to one of three structurally and functionally distinct families: serpin-type inhibitors, Kazal-type inhibitors, and Kunitz-type inhibitors.^{1–3} Hepatocyte growth factor activator-1 (HAI-1) is a recently discovered membrane-associated Kunitz-type serine protease inhibitor. This peculiar serine protease inhibitor, which is structurally distinct from other previously described Kunitz-type inhibitors, is a type I transmembrane glycoprotein that consists of an N-terminal MANEC-domain followed by two extracellular Kunitz-type inhibitory domains that are separated by a single low-density lipoprotein receptor type a repeat, and a C-terminal transmembrane/cytoplasmic tail domain.^{4–8} HAI-1 is widely expressed in embryonic and extra-embryonic epithelia of the developing embryo, as well as in simple, stratified and pseudo-stratified epithelia of the adult organs of humans and mice.^{9–13} HAI-1 was originally identified as the cognate inhibitor of the trypsin-like serine protease, hepatocyte growth factor activator. However, the isolated Kunitz-type inhibitor domains of HAI-1 were subsequently shown to display potent inhibitory activity toward a large number of other trypsin-like serine proteases when analyzed *in vitro*, suggesting that HAI-1

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Trypsin-like serine proteases constitute a large family of secreted and membrane-bound proteolytic enzymes that

could have multiple inhibitory targets and be a key player in many physiological processes.^{14–20}

The widespread epithelial expression of HAI-1 in embryonic and adult epithelial tissues would also predict an essential function of the transmembrane serine protease inhibitor in mammalian development and adult tissue homeostasis. Indeed, inactivation of the *Spint1* gene encoding HAI-1 in mice resulted in embryonic lethality at mid-gestation due to placental failure caused by agenesis of the placental labyrinth.^{13,21,22} This uniform lethality of *Spint1* null embryos at mid-gestation has complicated the analysis of the function of HAI-1 in adult tissue homeostasis. However, a recent elegant study by Nagaike and co-workers bypassed the embryonic lethality imposed by the placental requirement for HAI-1 by injection of *Spint1* null embryonic stem cells into wild-type blastocysts, leading to the generation of chimeric mice with high-grade deletion of *Spint1* in the embryo proper, but sufficient placental *Spint1* to support development to term.²³ These high-grade *Spint1*^{−/−} chimeric mice were unremarkable at birth. However, they uniformly suffered from severe growth retardation and were incapable of surviving beyond postnatal day 16. Histopathological examination revealed that *Spint1*^{−/−} mice displayed a range of abnormalities of keratinized epithelium, including hyperplasia of the forestomach, severe ichthyosis of the skin with inflammatory cell infiltration, and abnormal hair development. However, the molecular basis for these dramatic manifestations of HAI-1 ablation was not determined, and several possible explanations were proposed by the authors of the study.²³

Matriptase (also known as MT-SP1, epithin, and TADG15) is a modular trypsin-like serine protease encoded by the *ST14* gene that belongs to the recently established type II transmembrane serine protease family.^{24–30} Like HAI-1, matriptase is widely expressed in both embryonic and adult epithelia of mice and humans.^{9–11,13,22,31} Homozygosity for mutations in the *ST14* gene was recently shown to be the etiological origin of two human syndromes termed congenital ichthyosis, follicular atrophoderma, hypotrichosis, and hypohidrosis and autosomal recessive ichthyosis with hypotrichosis.^{32–36} Mice with wholesale deletion of the *St14* gene complete embryonic development, but they die shortly after birth as a consequence of compromised epidermal barrier function.^{37,38} However, an *St14* hypomorphic mouse strain, generated by insertion of a retroviral targeting vector into intron 1 of the *St14* gene, which displays 1% residual matriptase mRNA levels in the epidermis and up to 18% residual mRNA levels in other tissues, has normal postnatal and long-term survival, revealing that low matriptase suffices to maintain epithelial homeostasis.³⁹

In the present study, we hypothesized that the detrimental effects of postnatal loss of HAI-1 were caused by excess matriptase activity. Indeed, using a genetic approach, we demonstrate in this study that the requirement of HAI-1 for postnatal mouse development and epithelial homeostasis is mechanistically linked to the inhibition of matriptase. Thus, whereas *Spint1*^{−/−} mice with one or two wild-type *St14* alleles died *in utero*, *Spint1*^{−/−} mice on the

St14 hypomorphic mouse background not only completed embryonic development, but were viable, healthy, and displayed normal long-term survival. This study identifies matriptase as a critical inhibitory target for HAI-1 in keratinized squamous epithelium, shows that HAI-1 inhibition of protease targets besides matriptase is not essential for mouse health and long-term survival, and reveals that a delicate balance between matriptase and HAI-1 maintains homeostasis of adult mammalian tissues.

Materials and Methods

Mice and Tissue Acquisition

All procedures involving live animals were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium, following Institutional Guidelines and standard operating procedures. The generation and genotyping of the *Spint1* null mice and β -galactosidase-tagged *St14* hypomorphic mice have been described.^{13,39,40} Histological analysis, immunohistochemistry and whole mount X-gal staining was performed exactly as described.^{10,13,31,38,40}

Blood Chemistry Analysis

To isolate serum, blood was collected from the periportal vein of freshly euthanized animals, clotted for 2 hours at room temperature, and centrifuged at 800 × *g* for 10 minutes at room temperature. The clear upper phase was transferred into a new tube and immediately used for the analysis or stored at −80°C. The biochemical analysis of mouse sera from at least three animals of each genotype and sex was performed by the Department of Laboratory Medicine at the National Institutes of Health Clinical Center (Bethesda, MD).

Intestinal Epithelial Permeability

Ten μ l per gram of body weight of 22 mg/ml fluorescein isothiocyanate (FITC)-labeled dextran (average 4,000 g/mol, Sigma, St. Louis, MO) in PBS was injected directly into the stomach of 6 month-old animals using a 1.5-inch long, bulb-tipped gastric gavage needle (Roboz, Gaithersburg, MD) attached to a 1-ml syringe. After four h, the animals were euthanized by CO₂ inhalation, blood was extracted, and serum was prepared as described above. Fifty μ l of serum was then diluted 1:3 in 1× PBS (Gibco-Invitrogen, Carlsbad, CA) and the concentration of FITC-dextran was determined by reading the fluorescence at 535 nm after excitation at 485 nm using a Victor3V spectrophotometer (PerkinElmer, Waltham, MA).

Detection of Matriptase Protein in Mouse Skin

Whole skins from newborn mice were homogenized in glass homogenizers in ice-cold RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.1% SDS)

with a protease inhibitor cocktail (Sigma). The homogenate was cleared by centrifugation for 10 minutes at $16,000 \times g$ at 4°C . The protein content was determined with the BCA protein assay kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Seventy-five μg of total protein and 15 ng of recombinant mouse matriptase serine protease domain (R&D Systems, Minneapolis, MN) was separated on 4% to 12% NuPAGE Bis/Tris gel (Invitrogen, Carlsbad, CA) under reducing conditions and transferred onto polyvinylidene difluoride membrane (Invitrogen). Matriptase was detected using 1 $\mu\text{g}/\text{ml}$ sheep anti-human matriptase primary antibody (R&D Systems) and donkey anti-sheep secondary antibody conjugated to alkaline phosphatase (Sigma), diluted 1:10,000.

Body Fat Content Determination

Mice were anesthetized by isoflurane inhalation and the body area excluding the head and the tail was subjected to a dual energy X-ray absorptiometry whole body scan using a Lunar PIXImus small animal scanner (GE Lunar Corp., Madison, WI) according to the manufacturer's instructions. The amount of body fat (g) and body fat content (%) were then determined using PIXImus software. At least four animals of each genotype and sex were analyzed.

Results

Low Matriptase Negates HAI-1 Requirement during Early Postnatal Development

Matriptase co-localizes with HAI-1 in the epidermis (Figure 1A, 1B) and can be detected in its proteolytically activated form (Figure 1C). Mice with a combined whole-sale deletion of the *Spint1* and *St14* genes complete embryonic development, but die shortly after birth as a consequence of matriptase deficiency, thus precluding the analysis of the importance of matriptase regulation by HAI-1 in adult tissue homeostasis.¹³ On the other hand, greatly lowering matriptase levels in mice has no major adverse effect on postnatal health and survival.³⁹ Therefore, to query the potential role of excess matriptase proteolytic activity in the postnatal lethality of mice devoid of HAI-1,²³ we reduced the level of matriptase in *Spint1*^{-/-} null mice, rather than eliminate the protease altogether. *Spint1*^{+/-} mice were first bred with mice carrying one *St14* hypomorphic allele and one *St14* null allele (*St14*^{-/hypo}) mice. Breeding of the ensuing *St14*^{+/-hypo}; *Spint1*^{+/-} offspring to *St14*^{+/-}; *Spint1*^{+/-} offspring subsequently produced the following strains: *Spint1*^{+/+} and *Spint1*^{+/-} mice on an *St14*^{+/+}, *St14*^{+/-hypo}, or *St14*^{+/-} background (hereafter termed *St14*^{+/-}; *Spint1*^{+/-} mice), *Spint1*^{-/-} mice on an *St14*^{+/+}, *St14*^{+/-hypo} or *St14*^{+/-} background (hereafter termed *St14*^{+/-}; *Spint1*^{-/-} mice), *Spint1*^{+/+} and *Spint1*^{+/-} mice on an *St14*^{-/hypo} background (hereafter termed *St14*^{-/hypo}; *Spint1*^{+/-} mice), and *Spint1*^{-/-} mice on an *St14*^{-/hypo} background (hereafter termed *St14*^{-/hypo}; *Spint1*^{-/-} mice). In agreement with

previous studies,^{13,21,22} no *St14*^{+/-}; *Spint1*^{-/-} mice were identified among 119 offspring genotyped at weaning (Figure 1D). Interestingly, however, *St14*^{-/hypo}; *Spint1*^{-/-} mice were found with the expected frequency in weaned offspring, revealing that reduced levels of matriptase negate not only the requirement for placental HAI-1 expression during development, but also the requirement of HAI-1 for survival within the postnatal period. The reported demise of high-grade *Spint1*^{-/-} chimeric mice is preceded by severe growth retardation (30% at postnatal day 7 and 60% at day 13) when compared with their low-grade chimeric littermates.²³ To determine the possible contribution of excess matriptase activity to the failure of HAI-1-deficient pups to gain weight during the pre-weaning period, we determined the weight of *St14*^{-/hypo}; *Spint1*^{-/-} pups and their *St14*^{-/hypo}; *Spint1*⁺; and *St14*⁺; *Spint1*⁺ littermates every 24 hours between postnatal day 1 and 19 (Figure 1E). Interestingly, *St14*^{-/hypo}; *Spint1*^{-/-} pups displayed only a minimal residual growth retardation, when compared with either *St14*^{-/hypo}; *Spint1*⁺ or *St14*⁺; *Spint1*⁺ littermates. Like high-grade *Spint1*^{-/-} chimeric pups,²³ *St14*^{-/hypo}; *Spint1*^{-/-} pups were unremarkable at birth (data not shown). Unlike high-grade *Spint1*^{-/-} chimeric pups, however, *St14*^{-/hypo}; *Spint1*^{-/-} pups remained outwardly normal and did not develop pronounced hyperkeratosis, or grossly abnormal pelage hair and whiskers, displaying only the minimal phenotypes observed in their *St14*^{-/hypo}; *Spint1*⁺ littermates³⁹ (Figure 1F and 2A). In agreement with the outward healthy appearance, a careful histological examination of keratinized tissues (Figure 1G, H, I, and J, and data not shown) as well as non-keratinized tissues (data not shown) of pre-weaning *St14*^{-/hypo}; *Spint1*^{-/-} pups revealed no apparent abnormalities. Notably, the pervasive epidermal acanthosis with neutrophilic infiltration reported in high-grade *Spint1*^{-/-} chimeric pups²³ was entirely absent. The hair follicle morphology was also unremarkable, as was the appearance of the forestomach. Taken together, these data show that lowering the level of matriptase in mice negates the strict requirement of HAI-1 for growth, survival, and tissue homeostasis and restores normal early postnatal development.

Normal Long-Term Survival, but Reduced Body Fat Accumulation in HAI-1-Deficient Mice with Low Matriptase

Prospective cohorts of male and female *St14*^{-/hypo}; *Spint1*^{-/-} mice and their *St14*^{-/hypo}; *Spint1*⁺ or *St14*⁺; *Spint1*⁺ littermates were next established to determine the long-term consequences of complete HAI-1 deficiency in mice with low matriptase. The mice were monitored by bi-weekly weight determination and visual inspection for 1 year. At the termination of the experiment, the mice were subjected to body-fat mass and blood chemistry analysis, as well as detailed histopathological examination. No increase in postweaning mortality was observed in neither male nor female *St14*^{-/hypo}; *Spint1*^{-/-} mice (Figure 2B). Likewise, the appearance of skin, fur,

Figure 1. Decrease in matriptase activity rescues postnatal lethality, ichthyosis and hair follicle defects in HAI-1-deficient mice. **A–B:** Matriptase (**A**, blue staining) and HAI-1 (**B**, brown staining) co-localize in hair follicle matrix (**A**, **B** arrowheads) and in suprabasal layers of interfollicular epidermis (**A**, **B** arrows). **C:** Reduced SDS-polyacrylamide gel electrophoresis/Western blot of skin extracts from two pairs of newborn wild-type $St14^{+/+}$ mice (lanes 1 and 3), littermate $St14^{-/-}$ mice (lane 2 and 4), and 15 ng purified mouse recombinant active matriptase serine protease domain (rMatSP, lane 5). The presence of a ~30 kDa immunoreactive protein species in $St14^{+/+}$ (indicated by arrow at right), but not in $St14^{-/-}$ extracts, which co-migrates with purified recombinant matriptase serine protease domain shows the presence of activated matriptase in the epidermis. Bands observed in both $St14^{+/+}$ and $St14^{-/-}$ extracts represent cross reactivity. The positions of molecular weight markers in kDa are indicated at left. **D:** Distribution of genotypes of newborn mice from $St14^{+/+}; Spint1^{+/+} \times St14^{-/-}; Spint1^{+/+}$ breeding pairs. No $Spint1^{-/-}$ pups were detected that carried one or two wild-type $St14$ alleles ($St14^{+}$), while $Spint1^{-/-}$ pups on an $St14^{-/-}$ background were present with the expected Mendelian frequency. **E:** Average pre-weaning weight gain of $St14^{+/+}; Spint1^{+/+}$ ($N = 54$), $St14^{-/-}; Spint1^{+/+}$ ($N = 7$), and $St14^{-/-}; Spint1^{-/-}$ ($N = 14$) pups. **F:** Representative example of the outward appearance of $St14^{-/-}; Spint1^{-/-}$, $St14^{-/-}; Spint1^{+/+}$, and $St14^{+/+}; Spint1^{+/+}$ mice at day 17. **G–J:** H&E staining of histological sections of skin (**G**, **H**) and forestomach (**I**, **J**) of 17 day-old $St14^{-/-}; Spint1^{-/-}$ mice (**G**, **I**) and their $St14^{+/+}; Spint1^{+/+}$ littermates (**H**, **J**). No hyperkeratosis, acanthosis, or neutrophil infiltration has been observed in HAI-1-deficient tissues with low matriptase. Scale bars = 25 μ m (**G**, **H**); 50 μ m (**I**, **J**). Scale bars for **A** and **B** = 50 μ m.

whiskers, teeth, eyes, and external genitalia of these mice was indistinguishable from $St14^{-/-}; Spint1^{+/+}$ or $St14^{+/+}; Spint1^{+/+}$ littermates (Figure 2C and data not shown). The microscopic appearance of the interfollicular and follicular epidermis (Figure 2, D–G), esophagus (Figure 2, H–K), and forestomach (Figure 2, L–O), as well as all other organs examined (data not shown) was unremarkable. These findings demonstrate that the complete loss of HAI-1 has no major adverse effect on the long-term health of mice with low matriptase in the absence of intrinsic or external challenges. However, both female

and male mice were markedly leaner than $St14^{-/-}; Spint1^{+/+}$ or $St14^{+/+}; Spint1^{+/+}$ littermates (Figure 3A and B). Peripheral instantaneous X-ray imager analysis confirmed that body fat accumulation in $St14^{-/-}; Spint1^{-/-}$ female mice was significantly reduced (71% and 65% relative to $St14^{-/-}; Spint1^{+/+}$ or $St14^{+/+}; Spint1^{+/+}$ mice, respectively), whereas the reduction in body fat content in $St14^{-/-}; Spint1^{-/-}$ male mice did not reach significance. However, measurement of body length revealed that both male and female $St14^{-/-}; Spint1^{-/-}$ mice were slightly shorter in stature (females: 90% and 89%

Figure 2. HAI-1 is dispensable for postnatal development and long-term survival in the presence of low matriptase activity. **A:** Macroscopic image of the head region of a 17 day-old *St14*^{-/-}*hypo*; *Spint1*^{-/-} mouse showing normal development of whiskers and lack of scaling on ears and around eyes. **B:** Long term survival of female *St14*⁺; *Spint1*⁺ (*N* = 29), *St14*^{-/-}*hypo*; *Spint1*⁺ (*N* = 25), and *St14*^{-/-}*hypo*; *Spint1*^{-/-} (11), and male *St14*⁺; *Spint1*⁺ (*N* = 26), *St14*^{-/-}*hypo*; *Spint1*⁺ (*N* = 20), and *St14*^{-/-}*hypo*; *Spint1*^{-/-} (*N* = 8) mice. No effect of the loss of HAI-1 on overall survival of mice with low matriptase activity was observed. **C:** Representative example of the appearance of 9 months-old control *St14*⁺; *Spint1*⁺, *St14*^{-/-}*hypo*; *Spint1*^{-/-}, and *St14*^{-/-}*hypo*; *Spint1*⁺ mice. HAI-1-deficient mice with low matriptase are outwardly normal (**D–O**). H&E staining (**D–N**) and HAI-1 immunostaining (**E–O**) of histological sections of skin (**D–G**), esophagus (**H–K**), and forestomach (**L–O**) of 12 month-old *St14*⁺; *Spint1*⁺ (**D, E; H, I; L, M**) or *St14*^{-/-}*hypo*; *Spint1*^{-/-} (**F, G; J, K; N, O**) mice. No apparent abnormalities were detected in any of the keratinized tissues of HAI-1-deficient mice with low matriptase. **Arrows** indicate the expression of HAI-1 protein. Scale bars = 25 μ m (**D–O**).

relative to *St14*^{-/-}*hypo*; *Spint1*⁺ or *St14*⁺; *Spint1*⁺ mice, respectively, *P* < 0.01; males: 96% and 94% relative to *St14*^{-/-}*hypo*; *Spint1*⁺ or *St14*⁺; *Spint1*⁺ mice, respectively, did not reach significance). Both HAI-1 and matriptase are highly expressed throughout the mouse gastrointestinal tract.^{10,31,40} However, the intestinal barrier perme-

ability in *St14*^{-/-}*hypo*; *Spint1*^{-/-} mice, as measured by FITC-dextran gavage, was similar to that of control *St14*⁺; *Spint1*⁺ mice (Figure 3C). Likewise, blood chemistry analysis was uninformative as to the cause of the reduced body fat accumulation and shorter stature (Figure 3, D and E, and data not shown).

Figure 3. Decreased body weight but normal intestinal barrier function and biochemical parameters in HAI-1-deficient mice with low matriptase. **A, B:** Body weight of 6 to 52 week-old female (**A**) *ST14⁺;Spint1⁺* (*N* = 29), *ST14^{-hypo};Spint1⁺* (*N* = 25), and *ST14^{-hypo};Spint1^{-/-}* (*N* = 11), and male (**B**) *ST14⁺;Spint1⁺* (*N* = 26), *ST14^{-hypo};Spint1⁺* (*N* = 20), and *ST14^{-hypo};Spint1^{-/-}* (*N* = 8) mice. **C:** Concentration of FITC-labeled dextran in the blood of 12 month-old *ST14⁺;Spint1⁺* (females *N* = 6, males *N* = 12) and *ST14^{-hypo};Spint1^{-/-}* (females *N* = 4, males *N* = 5) mice 4 hours after instillation of FITC-labeled dextran into the stomach by oral gavage. No significant increase in intestinal permeability was observed in HAI-1-deficient animals with low matriptase. Error bars indicate SD (**D, E**). Biochemical analysis of the blood of 12 month *ST14⁺;Spint1⁺* (females *N* = 10 (**D**), males *N* = 15 (**E**), *ST14^{-hypo};Spint1⁺* (females *N* = 10, males *N* = 14), and *ST14^{-hypo};Spint1^{-/-}* (females *N* = 3, males *N* = 5) mice. No significant differences between HAI-1-expressing and HAI-1-deficient animals were identified. Error bars indicate SD. Abbreviations: ALT/GPT, alanine aminotransferase/glutamate-pyruvate transaminase; AST/GOT, aspartate aminotransferase/glutamate-oxaloacetate transaminase; BUN, blood urea nitrogen; CK, creatine kinase; LD, lactate dehydrogenase.

Discussion

The current study provides the mechanistic explanation for the requirement of HAI-1 for maintenance of epithelial integrity within the postnatal period.²³ Indeed, we identified the suppression of proteolysis dependent on the type II transmembrane serine protease matriptase as a critical *in vivo* function for HAI-1. Thus, whereas HAI-1-deficient mice displayed severe growth retardation and uniform lethality within the early postnatal period and presented with profound defects in keratinized epithelium, HAI-1-deficient mice with a low level of matriptase gained weight at near-normal rates in the postnatal period, had unaltered long-term survival, were outwardly healthy, and histologically unremarkable. Matriptase is an autoactivating protease that is capable of activating other serine protease zymogens, including the glycosylphosphatidylinositol-anchored membrane serine protease, prostatic, and receptor-bound urokinase plasminogen activator.^{41–44} Therefore, it is formally possible that HAI-1 does not exert its essential inhibitory functions through a direct interaction with matriptase, but rather acts on target proteases whose activation is matriptase-dependent. How-

ever, HAI-1 and matriptase co-localize in tissues affected by the absence of HAI-1 and a proteolytically shed form of HAI-1 has been found in a complex with shed matriptase in conditioned medium from immortalized epithelial cells and even in human milk.⁴⁵ Taken together, this suggests that HAI-1 most likely maintains tissue homeostasis through direct inhibition of matriptase.

This study identifies HAI-1 as one critical postnatal inhibitor of matriptase. However, it does not exclude that other potential matriptase inhibitors, such as HAI-2,¹⁰ antithrombin III, α 1-antitrypsin, and α 2-antiplasmin⁴⁶ have equally important roles in the regulation of matriptase activity in the epidermis or in other adult tissues.

Despite the widespread expression of HAI-1 and its potent inhibitory activity against multiple trypsin-like proteases, we nevertheless found that reduction of matriptase-dependent proteolysis in mice devoid of HAI-1 sufficed to ensure normal postnatal development, health, and long-term survival. Thus, the direct inhibition of hepatocyte growth factor activator, trypsin, hepsin, prostatic, or other trypsin-like serine proteases was not

critical to maintain tissue homeostasis. Functional redundancy in the inhibition of these other potential targets is one plausible explanation. In this regard it should be noted that HAI-1 and the closely related inhibitor HAI-2 (placental bikunin) display highly overlapping protease specificity *in vitro* and co-localize in most adult epithelia.^{10,14–20} Alternatively, HAI-1 inhibition of these other candidate protease targets may not be physiologically relevant or may only become critical in the context of external environmental or intrinsic challenges, such as tissue injury, infection or metabolic restrictions.

Reduced accumulation of body fat was the only notable feature that distinguished HAI-1-deficient mice with low matriptase levels from mice with low matriptase. No obvious reason for the leanness of these mice was pinpointed by histological, intestinal barrier function, or blood chemistry analysis. Two molecular explanations for this are likely: inadequate inhibition of a target protease different from matriptase, or excessive or spatially undesired activity of the residual matriptase. In support of the latter, we have found that supraphysiological levels of matriptase can cause the rapid demise in multiple epithelia (Karin List, Peter Kosa, Roman Szabo, and Thomas H. Bugge, unpublished data). In summary, the current study has revealed that inhibition of the type II transmembrane serine protease, matriptase, is an essential function of HAI-1 that maintains adult tissue homeostasis.

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References

- Puente XS, Sanchez LM, Overall CM, Lopez-Otin C: Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* 2003, 4:544–558
- Rawlings ND, Barrett AJ: Families of serine peptidases. *Methods Enzymol* 1994, 244:19–61
- Rau JC, Beaulieu LM, Huntington JA, Church FC: Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost* 2007, 5 Suppl 1:102–115
- Marlor CW, Delaria KA, Davis G, Muller DK, Greve JM, Tamburini PP: Identification and cloning of human placental bikunin, a novel serine protease inhibitor containing two Kunitz domains. *J Biol Chem* 1997, 272:12202–12208
- Itoh H, Kataoka H, Hamasuna R, Kitamura N, Kono M: Hepatocyte growth factor activator inhibitor type 2 lacking the first Kunitz-type serine proteinase inhibitor domain is a predominant product in mouse but not in human. *Biochem Biophys Res Commun* 1999, 255:740–748
- Muller-Pillasch F, Wallrapp C, Bartels K, Varga G, Friess H, Buchler M, Adler G, Gress TM: Cloning of a new Kunitz-type protease inhibitor with a putative transmembrane domain overexpressed in pancreatic cancer. *Biochim Biophys Acta* 1998, 1395:88–95
- Kawaguchi T, Qin L, Shimomura T, Kondo J, Matsumoto K, Denda K, Kitamura N: Purification and cloning of hepatocyte growth factor activator inhibitor type 2, a Kunitz-type serine protease inhibitor. *J Biol Chem* 1997, 272:27558–27564
- Shimomura T, Denda K, Kitamura A, Kawaguchi T, Kito M, Kondo J, Kagaya S, Qin L, Takata H, Miyazawa K, Kitamura N: Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. *J Biol Chem* 1997, 272:6370–6376
- Oberst MD, Johnson MD, Dickson RB, Lin CY, Singh B, Stewart M, Williams A, al-Nafussi A, Smyth JF, Gabra H, Sellar GC: Expression of the serine protease matriptase and its inhibitor HAI-1 in epithelial ovarian cancer: correlation with clinical outcome and tumor clinicopathological parameters. *Clin Cancer Res* 2002, 8:1101–1107
- Szabo R, Hobson JP, List K, Molinolo A, Lin CY, Bugge TH: Potent inhibition and global co-localization implicate the transmembrane kunitz-type serine protease inhibitor hai-2 in the regulation of epithelial matriptase activity. *J Biol Chem* 2008, 283:29495–29504
- Oberst M, Anders J, Xie B, Singh B, Ossandon M, Johnson M, Dickson RB, Lin CY: Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo. *Am J Pathol* 2001, 158:1301–1311
- Kataoka H, Suganuma T, Shimomura T, Itoh H, Kitamura N, Nabeshima K, Kono M: Distribution of hepatocyte growth factor activator inhibitor type 1 (HAI-1) in human tissues. Cellular surface localization of HAI-1 in simple columnar epithelium and its modulated expression in injured and regenerative tissues. *J Histochem Cytochem* 1999, 47:673–682
- Szabo R, Molinolo A, List K, Bugge TH: Matriptase inhibition by hepatocyte growth factor activator inhibitor-1 is essential for placental development. *Oncogene* 2007, 26:1546–1556
- Denda K, Shimomura T, Kawaguchi T, Miyazawa K, Kitamura N: Functional characterization of Kunitz domains in hepatocyte growth factor activator inhibitor type 1. *J Biol Chem* 2002, 277:14053–14059
- Kirchhofer D, Peek M, Lipari MT, Billici K, Fan B, Moran P: Hepsin activates pro-hepatocyte growth factor and is inhibited by hepatocyte growth factor activator inhibitor-1B (HAI-1B) and HAI-2. *FEBS Lett* 2005, 579:1945–1950
- Kirchhofer D, Peek M, Li W, Stamos J, Eigenbrot C, Kadkhodayan S, Elliott JM, Corpuz RT, Lazarus RA, Moran P: Tissue expression, protease specificity, and Kunitz domain functions of hepatocyte growth factor activator inhibitor-1B (HAI-1B), a new splice variant of HAI-1. *J Biol Chem* 2003, 278:36341–36349
- Herter S, Piper DE, Aaron W, Gabriele T, Cutler G, Cao P, Bhatt AS, Choe Y, Craik CS, Walker N, Meininger D, Hoey T, Austin RJ: Hepatocyte growth factor is a preferred *in vitro* substrate for human hepsin, a membrane-anchored serine protease implicated in prostate and ovarian cancers. *Biochem J* 2005, 390:125–136
- Delaria KA, Muller DK, Marlor CW, Brown JE, Das RC, Rocznik SO, Tamburini PP: Characterization of placental bikunin, a novel human serine protease inhibitor. *J Biol Chem* 1997, 272:12209–12214
- Fan B, Wu TD, Li W, Kirchhofer D: Identification of hepatocyte growth factor activator inhibitor-1B as a potential physiological inhibitor of prostasin. *J Biol Chem* 2005, 280:34513–34520
- Qin L, Denda K, Shimomura T, Kawaguchi T, Kitamura N: Functional characterization of Kunitz domains in hepatocyte growth factor activator inhibitor type 2. *FEBS Lett* 1998, 436:111–114
- Tanaka H, Nagaike K, Takeda N, Itoh H, Kohama K, Fukushima T, Miyata S, Uchiyama S, Uchinokura S, Shimomura T, Miyazawa K, Kitamura N, Yamada G, Kataoka H: Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is required for branching morphogenesis in the chorioallantoic placenta. *Mol Cell Biol* 2005, 25:5687–5698
- Fan B, Brennan J, Grant D, Peale F, Rangell L, Kirchhofer D: Hepatocyte growth factor activator inhibitor-1 (HAI-1) is essential for the integrity of basement membranes in the developing placental labyrinth. *Dev Biol* 2007, 303:222–230
- Nagaike K, Kawaguchi M, Takeda N, Fukushima T, Sawaguchi A, Kohama K, Setoyama M, Kataoka H: Defect of hepatocyte growth factor activator inhibitor type 1/serine protease inhibitor. Kunitz type 1 (Hai-1/Spint1) leads to ichthyosis-like condition and abnormal hair development in mice. *Am J Pathol* 2008, 173:1–12
- Kim DR, Sharmin S, Inoue M, Kido H: Cloning and expression of novel mosaic serine proteases with and without a transmembrane domain from human lung. *Biochim Biophys Acta* 2001, 1518:204–209
- Lin CY, Anders J, Johnson M, Sang QA, Dickson RB: Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity. *J Biol Chem* 1999, 274:18231–18236
- Takeuchi T, Shuman MA, Craik CS: Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci USA* 1999, 96:11054–11061
- Tanimoto H, Underwood LJ, Wang Y, Shigemasa K, Parmley TH, O'Brien TJ: Ovarian tumor cells express a transmembrane serine

- protease: a potential candidate for early diagnosis and therapeutic intervention. *Tumour Biol* 2001, 22:104–114
28. Szabo R, Bugge TH: Type II transmembrane serine proteases in development and disease. *Int J Biochem Cell Biol* 2008, 40:1297–1316
29. Hooper JD, Clements JA, Quigley JP, Antalis TM: Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. *J Biol Chem* 2001, 276:857–860
30. Szabo R, Wu Q, Dickson RB, Netzel-Arnett S, Antalis TM, Bugge TH: Type II transmembrane serine proteases. *Thromb Haemost* 2003, 90:185–193
31. List K, Hobson JP, Molinolo A, Bugge TH: Co-localization of the channel activating protease prostaticin/(CAP1/PRSS8) with its candidate activator, matriptase. *J Cell Physiol* 2007, 213:237–245
32. Tursen U, Kaya TI, Ikizoglu G, Aktekin M, Aras N: Genetic syndrome with ichthyosis: congenital ichthyosis, follicular atrophoderma, hypotrichosis, and woolly hair; second report. *Br J Dermatol* 2002, 147:604–606
33. Lestringant GG, Kuster W, Frossard PM, Happle R: Congenital ichthyosis, follicular atrophoderma, hypotrichosis, and hypohidrosis: a new genodermatosis? *Am J Med Genet* 1998, 75:186–189
34. Alef T, Torres S, Hausser I, Metze D, Tursen U, Lestringant GG, Hennies HC: Ichthyosis, follicular atrophoderma, and hypotrichosis caused by mutations in ST14 is associated with impaired profilaggrin processing. *J Invest Dermatol* 2008, 129:862–869
35. Avrahami L, Maas S, Pasmanik-Chor M, Rainshtein L, Magal N, Smitt J, van Marle J, Shohat M, Basel-Vanagaite L: Autosomal recessive ichthyosis with hypotrichosis syndrome: further delineation of the phenotype. *Clin Genet* 2008, 74:47–53
36. Basel-Vanagaite L, Attia R, Ishida-Yamamoto A, Rainshtein L, Ben Amitai D, Lurie R, Pasmanik-Chor M, Indelman M, Zvulunov A, Saban S, Magal N, Sprecher E, Shohat M: Autosomal recessive ichthyosis with hypotrichosis caused by a mutation in ST14. encoding type II transmembrane serine protease matriptase. *Am J Hum Genet* 2007, 80:467–477
37. List K, Haudenschild CC, Szabo R, Chen W, Wahl SM, Swaim W, Engelholm LH, Behrendt N, Bugge TH: Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. *Oncogene* 2002, 21:3765–3779
38. List K, Szabo R, Wertz PW, Segre J, Haudenschild CC, Kim SY, Bugge TH: Loss of proteolytically processed filaggrin caused by epidermal deletion of matriptase/MT-SP1. *J Cell Biol* 2003, 163:901–910
39. List K, Currie B, Scharschmidt TC, Szabo R, Shireman J, Molinolo A, Cravatt BF, Segre J, Bugge TH: Autosomal ichthyosis with hypotrichosis syndrome displays low matriptase proteolytic activity and is phenocopied in ST14 hypomorphic mice. *J Biol Chem* 2007, 282:36714–36723
40. List K, Szabo R, Molinolo A, Nielsen BS, Bugge TH: Delineation of matriptase protein expression by enzymatic gene trapping suggests diverging roles in barrier function, hair formation, and squamous cell carcinogenesis. *Am J Pathol* 2006, 168:1513–1525
41. Lee SL, Dickson RB, Lin CY: Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J Biol Chem* 2000, 275:36720–36725
42. Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS: Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem* 2000, 275:26333–26342
43. Netzel-Arnett S, Currie BM, Szabo R, Lin CY, Chen LM, Chai KX, Antalis TM, Bugge TH, List K: Evidence for a matriptase-prostaticin proteolytic cascade regulating terminal epidermal differentiation. *J Biol Chem* 2006, 281:32941–32945
44. Kilpatrick LM, Harris RL, Owen KA, Bass R, Ghorayeb C, Bar-Or A, Ellis V: Initiation of plasminogen activation on the surface of monocytes expressing the type II transmembrane serine protease matriptase. *Blood* 2006, 108:2616–2623
45. Lin CY, Anders J, Johnson M, Dickson RB: Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. *J Biol Chem* 1999, 274:18237–18242
46. Tseng IC, Chou FP, Su SF, Oberst M, Madayiputhiya N, Lee MS, Wang JK, Sloane DE, Johnson M, Lin CY: Purification from human milk of matriptase complexes with secreted serpins: mechanism for inhibition of matriptase other than HAI-1. *Am J Physiol Cell Physiol* 2008, 295:C423–C431