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RESEARCH ARTICLE

Delineation of proteolytic and non-proteolytic functions of the membrane-anchored serine protease prostasin

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ABSTRACT

The membrane-anchored serine proteases prostasin (PRSS8) and matriptase (ST14) initiate a cell surface proteolytic pathway essential for epithelial function. Mice expressing only catalytically inactive prostasin are viable, unlike prostasin null mice, indicating that at least some prostasin functions are non-proteolytic. Here we used knock-in mice expressing catalytically inactive prostasin (*Prss8*$^{Ki/Ki}$) to show that the physiological and pathological functions of prostasin vary in their dependence on its catalytic activity. Whereas prostasin null mice exhibited partial embryonic and complete perinatal lethality, *Prss8*$^{Ki/Ki}$ mice displayed normal prenatal and postnatal survival. Unexpectedly, catalytically inactive prostasin caused embryonic lethality in mice lacking its cognate inhibitors HAI-1 (SPINT1) or HAI-2 (SPINT2). Proteolytically inactive prostasin, unlike the wild-type protease, was unable to activate matriptase during placentation. Surprisingly, all essential functions of prostasin in embryonic and postnatal development were compensated for by loss of HAI-1, indicating that prostasin is only required for mouse development and overall viability in the presence of this inhibitor. This study expands our knowledge of non-proteolytic functions of membrane-anchored serine proteases and provides unexpected new data on the mechanistic interactions between matriptase and prostasin in the context of epithelial development.

KEY WORDS: Cell surface proteolysis, Serine protease, Epithelial development, Placental labyrinth

INTRODUCTION

Trypsin-like serine proteases are a large family of proteolytic enzymes that reside within the extracellular and pericellular space of all vertebrate species. They are emerging as major regulators of a variety of biological processes in the context of tissue development and homeostasis owing to their involvement in the activation of hormones, growth factors and cytokines, the activation and ectodomain shedding of signaling receptors and adhesion molecules, regulation of ion fluxes and extracellular matrix remodeling (Afonina et al., 2015; Chin et al., 2008; Naldini et al., 1992; Picard et al., 2008; Scudamore et al., 1998; Shimomura et al., 1993; Vallet et al., 1997; Wang et al., 1994; Yan et al., 2000).

Prostasin [or protease, serine 8 (PRSS8); also known as CAP1] and matriptase [also known as suppression of tumorigenicity 14 (ST14), MT-SP1, PRSS14] are trypsin-like membrane-anchored serine proteases expressed in epithelial compartments of mouse and human tissues (Antalis et al., 2011; Bergum et al., 2012; List et al., 2007; Szabo and Bugge, 2011). Studies using genetically modified mouse strains suggest that the two proteases are part of a single proteolytic cascade that plays a central role in the development and homeostasis of a variety of epithelia. Mice with a systemic loss of either prostasin or matriptase display strain-dependent complete or partial embryonic lethality, and all of the matriptase or prostasin null mice that survive until term die shortly after birth due to fatal dehydration (Hummler et al., 2013; Leyvraz et al., 2005; List et al., 2002; Peters et al., 2014). Detailed phenotypic analysis revealed that the matriptase/prostasin pathway is essential for terminal epidermal differentiation as well as tight junction formation and, as a result, the loss of matriptase or prostasin function leads to a severe defect in epidermal barrier function, and an abnormal hair follicle maturation (Leyvraz et al., 2005; List et al., 2009, 2003). Subsequent studies using tissue-specific knockout mice to bypass the postnatal lethality revealed that matriptase and/or prostasin play crucial roles in epithelial development and function in a wide variety of mouse tissues including placenta, skin, salivary gland, intestines, lungs and thymus (Frateschi et al., 2012; List et al., 2009; Malsure et al., 2014; Planes et al., 2010; Szabo et al., 2014).

Activity of the matriptase/prostasin pathway during development is controlled by two transmembrane serine protease inhibitors: hepatocyte growth factor activator inhibitor 1 (HAI-1) and HAI-2 (also known as SPINT1 and SPINT2, respectively). HAI-1 is essential for placental differentiation and overall embryonic and postnatal survival (Nagaike et al., 2008; Szabo et al., 2007; Tanaka et al., 2005). Loss of HAI-2 is associated with an embryonic lethality around embryonic day (E) 8.5-9.5, and a high frequency of defects in neural tube closure and a mid-gestational embryonic lethality due to a placental failure in matriptase heterozygous mice (Mitchell et al., 2001; Szabo et al., 2009a, 2012). Importantly, all developmental defects in HAI-1- and HAI-2-deficient mice are rescued in whole or in part by simultaneous inactivation of either matriptase or prostasin, thus demonstrating a crucial contribution of the matriptase/prostasin proteolytic pathway to the lethality observed in these mice (Szabo et al., 2009a, 2012).

We recently generated knock-in mice that carry a point mutation resulting in the substitution of the catalytic serine 238 in prostasin with alanine and which therefore express only a catalytically inactive variant of the protease (Peters et al., 2014; Szabo et al., 2012). Unexpectedly, these mice did not exhibit profound defects in epidermal development and barrier function, nor the perinatal lethality observed in prostasin null mice (Peters et al., 2014). Loss of prostasin enzymatic activity did result in abnormal hair growth and delayed skin wound healing, indicating that at least some of the physiological functions of prostasin are proteolytic.

In this study, we performed a systematic analysis of physiological, pathophysiological and biochemical processes previously reported to require prostasin to determine the specific

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contribution of the proteolytic and non-proteolytic functions of the protease to these processes. We found that prostasin proteolytic function is essential for matriptase zymogen activation during establishment of the feto-maternal barrier in the placenta. Unexpectedly, although devoid of catalytic activity and the capacity to activate placental matriptase, prostasin caused defects in placental differentiation and prenatal lethality in mice lacking HAI-1 or HAI-2. Equally unexpectedly, the absence of HAI-1 completely compensated for the loss of all proteolytic and non-proteolytic functions of prostasin, as mice with combined deficiency in HAI-1 and prostasin displayed normal development and postnatal survival. Taken together, these findings provide important new insights into prostasin function and its mechanistic relationship with other components of the matriptase/prostasin proteolytic pathway.

RESULTS

Prostasin is a crucial contributor to mouse mid-gestational embryonic development

The essential role of prostasin in mouse postnatal survival is well established (Leyvraz et al., 2005; Peters et al., 2014; Szabo et al., 2012). Reports of its role in embryonic development, however, show far less concordance. A study by Hummler et al. (2013) reported that loss of prostasin in an inbred C57 strain leads to defects in placental differentiation and complete embryonic lethality. By contrast, we have recently reported 50-100% viability of either matriptase or prostasin null embryos in outbred strains (Peters et al., 2014; Szabo et al., 2009a, 2014). The importance of matriptase and prostasin during embryogenesis thus appears to be strongly dependent on the genetic background. To enable a direct comparison of the phenotypes resulting from the specific loss of prostasin proteolytic function in mice expressing catalytically inactive prostasin (prostasin knock-in, $Prss8^{Ki/Ki}$) and those resulting from a complete loss of all prostasin functions (prostasin null, $Prss8^{-/-}$), we established genetically background-matched colonies of $Prss8^{Ki/+}$ and $Prss8^{+/-}$ breeder mice by first interbreeding mice carrying $Prss8^{Ki}$ and $Prss8^{-}$ alleles. The resulting $Prss8^{-/-}$ and $Prss8^{Ki/Ki}$ offspring of these $Prss8^{+/-}\times Prss8^{+/-}$ and $Prss8^{Ki/+}\times Prss8^{Ki/+}$ breeding pairs were then used throughout this study.

Analysis of the prenatal survival of offspring from $Prss8^{+/-}\times Prss8^{+/-}$ breeders showed a partial but highly significant decrease in prenatal viability in mice lacking prostasin, as fewer than half of the expected number of $Prss8^{-/-}$ mice were observed at birth (Fig. 1A; $P<0.003$, χ^2). Allele distributions in embryos extracted on or before E12.5 revealed the expected numbers of $Prss8^{-/-}$ mice (Fig. 1B,C). All of the $Prss8^{-/-}$ embryos extracted before E12.5 appeared normal, indicating that the loss of prostasin did not affect pre-implantation and early post-implantation development (Fig. 1D). At E13.5, however, the survival of the $Prss8^{-/-}$ mice decreased sharply, with only 51% of the expected number of prostasin-deficient embryos extracted between E13.5 and E14.5 being found alive (Fig. 1B,C; $P<0.04$, χ^2). The survival rate then remained virtually unchanged for the rest of the prenatal period, with the number of living $Prss8^{-/-}$ embryos reaching 49% between E15.5 and E16.5 ($P<0.04$, χ^2) and 46% at birth, suggesting that prostasin is specifically required for the relatively narrow interval of mid-gestational development (Fig. 1A-C).

Macroscopic inspection of living and non-living $Prss8^{-/-}$ embryos at E13.5 and E14.5 did not reveal any obvious growth retardation and/or developmental abnormality that would explain the sudden loss of viability (Fig. 1D′). This would be consistent with a recent report showing that defects in placental rather than embryonic development result in loss of mid-gestational viability in prostasin-deficient C57 mice (Hummler et al., 2013). Importantly, however, in contrast to Hummler et al. (2013) microscopy analysis of placental tissues from living E13.5 $Prss8^{-/-}$ embryos did not reveal any obvious differences in the overall histological structure or differentiation of the prostasin- and HAI-1-expressing labyrinthine epithelium, when compared with the prostasin-expressing wild-type littermate control tissues (Fig. 1E-G′). Indeed, histomorphometric quantification of the basic structural characteristics of the placental labyrinth of $Prss8^{-/-}$ E13.5 placentas, including the thickness and cross-sectional area of the labyrinth, as well as the density of fetal vessels within the labyrinth, all showed values comparable to those of wild-type littermates (Fig. 2A-C).

To determine whether the crucial contribution of prostasin to development after mid-gestation in our genetic background is mediated by prostasin proteolytic activity, or by its non-proteolytic functions, we next analyzed prenatal survival of $Prss8^{Ki/Ki}$ mice. Contrary to the prostasin full knockouts, no decrease in survival was observed in $Prss8^{Ki/Ki}$ mice at any stage of embryonic development, and these mice were born in the expected Mendelian ratio (Fig. 2D-F). To further validate this finding, mice carrying $Prss8^{Ki}$ and $Prss8^{-}$ alleles were also directly interbred to generate $Prss8^{Ki/-}$ breeding pairs capable of producing $Prss8^{Ki/Ki}$ and $Prss8^{-/-}$ offspring within the same litter. Consistent with the findings presented above, analysis of the newborn offspring of the $Prss8^{Ki/-}$ breeders revealed a highly significant decrease in the embryonic survival of $Prss8^{-/-}$ mice compared with their $Prss8^{Ki/Ki}$ littermates (Fig. 2G). At birth, $Prss8^{Ki/Ki}$ mice vastly outnumbered $Prss8^{-/-}$ newborns (Fig. 2G; observed ratio 67:23, expected ratio 1:1, $P<0.0001$, χ^2). Survival of $Prss8^{Ki/-}$ mice also appeared to be affected, with an observed ratio between $Prss8^{Ki/Ki}$ and $Prss8^{Ki/-}$ newborns of 67:88 (Fig. 2G; expected 1:2, $P<0.01$, χ^2). These data show that prostasin supports embryonic survival after a mid-gestational checkpoint and that this is independent of its proteolytic activity.

Prostasin proteolytic activity is required for placental matriptase zymogen activation

We have previously reported that the expression level of matriptase protein and its tissue localization are unaltered in $Prss8^{-/-}$ embryos, but these embryos display a complete absence of the active two-chain form of matriptase in the placenta (Szabo et al., 2012). We have also recently reported that catalytically inactive prostasin stimulates matriptase autoactivation in a reconstituted cell-based system (Friis et al., 2013), suggesting that placental matriptase activation could be either proteolysis dependent or independent. To investigate this, placental tissues from $Prss8^{-/-}$, $Prss8^{Ki/Ki}$ and their respective wild-type littermate controls were analyzed for the presence of the active two-chain form of matriptase. Expression analysis of placental tissues from $Prss8^{Ki/Ki}$ embryos at the time of active labyrinth differentiation (E12.5) by immunohistochemistry and western blot did not reveal any obvious effect of the loss of prostasin enzymatic activity on the levels of total prostasin and matriptase proteins (Fig. 3A,B), or their respective patterns of expression in placental epithelium (Fig. 3C-F). Direct detection of active matriptase in placental tissues by western blot is not feasible owing to very low signal intensity (Szabo et al., 2012). Therefore, we determined the amount of active matriptase that formed inhibitor complexes with its cognate inhibitor HAI-1, which selectively binds the active two-chain form of matriptase (Benaud et al., 2001; Lee et al., 2005).

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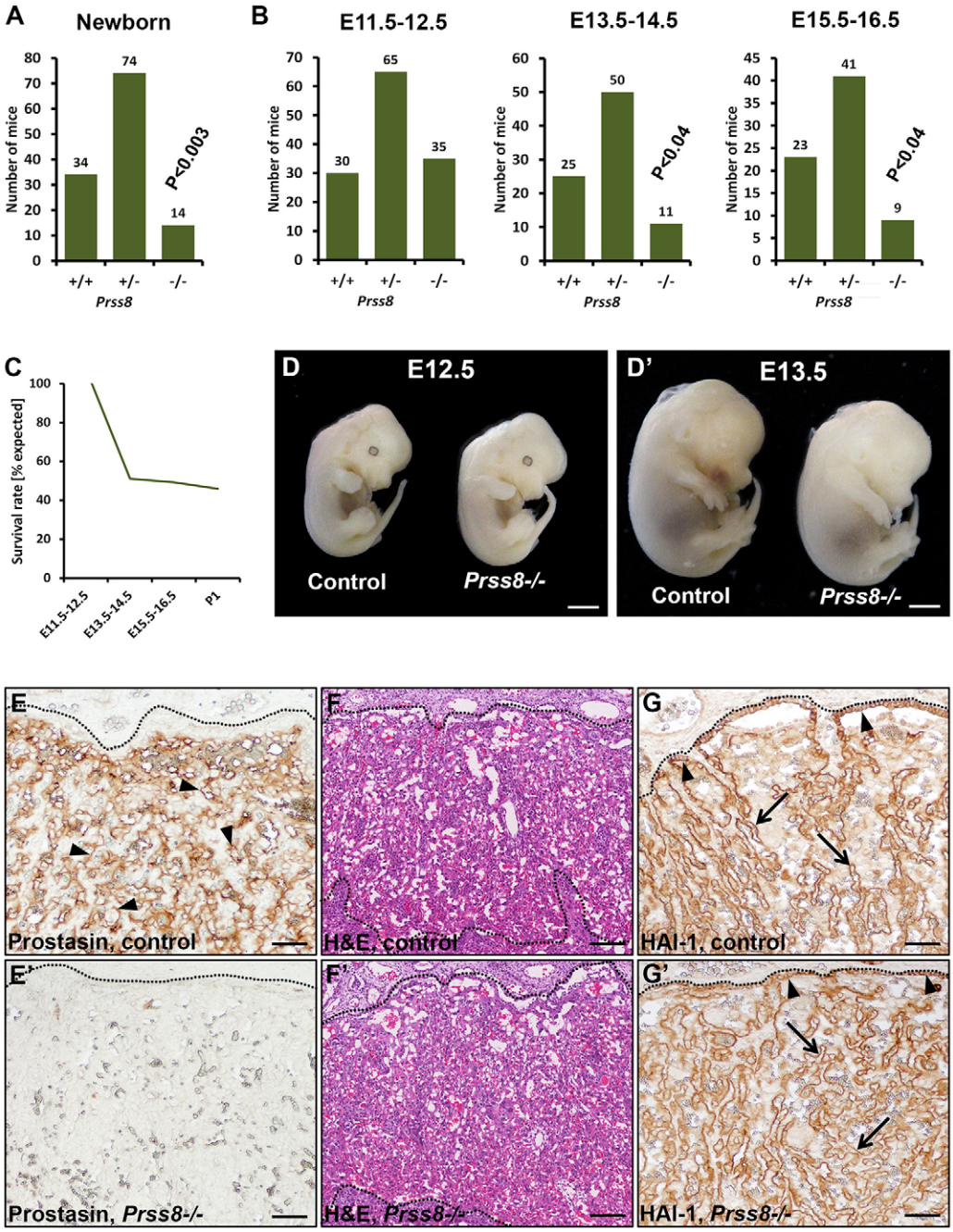

Fig. 1. Loss of prostasin function leads to partial embryonic lethality. (A,B) Distribution of *Prss8* genotypes in (A) newborn offspring and in (B) embryos of the indicated stages from $Prss8^{+/-}$×$Prss8^{+/-}$ breeding pairs. Loss of prostasin function led to a significant decrease in viability after E12.5. (C) Relative survival of $Prss8^{-/-}$ mice throughout embryonic development (observed versus expected number of mice based on normal Mendelian distribution) based on data presented in A and B. A decline in survival of 49%, 51% and 55% was observed in prostasin-deficient mice for E13.5-E14.5, E15.5-E16.5 and at birth [postnatal day (P) 1], respectively. (D,D′) Macroscopic appearance of control and $Prss8^{-/-}$ embryos at E12.5 (D) and E13.5 (D′). Prostasin-deficient mice show no growth retardation or any obvious developmental abnormality. (E,E′) Expression of prostasin in placental tissues of control (E) and $Prss8^{-/-}$ (E′) embryos at E13.5. Prostasin was detected only within the labyrinth layer in control tissues (arrowheads) and was completely absent in the placentas from *Prss8*-deficient mice. (F-G′) General histological appearance (F,F′, H&E staining) and the branching of the labyrinthine epithelium (G,G′, anti-HAI-1 immunostaining) in control (F,G) and *Prss8*-deficient (F′,G′) placental tissues. The extent of the labyrinth layer is outlined (dotted lines). Loss of prostasin did not affect the overall structure of the labyrinth or the appearance of HAI-1-positive chorionic (arrowheads) and labyrinthine (arrows) epithelium. Each image is representative of three independent stainings, each carried out on at least two tissue samples of the same genotype. Scale bars: 1 mm in D,D′; 50 µm in E,E′; 100 µm in F,F′; 75 µm in G,G′.

Placental lysates from control mice revealed detectable amounts of isolated matriptase serine protease domain (SPD), which is formed by activation site cleavage (Fig. 3G, lanes 2 and 3). By contrast, only minimal amounts of active matriptase were detected in placentas expressing catalytically inactive prostasin, with no obvious difference in the levels of detectable matriptase SPD between $Prss8^{Ki/Ki}$ and $Prss8^{-/-}$ mice (Fig. 3G, compare lanes 4 and 5 with 6 and 7), suggesting that activation of matriptase in the developing placental labyrinth is dependent on prostasin proteolytic activity.

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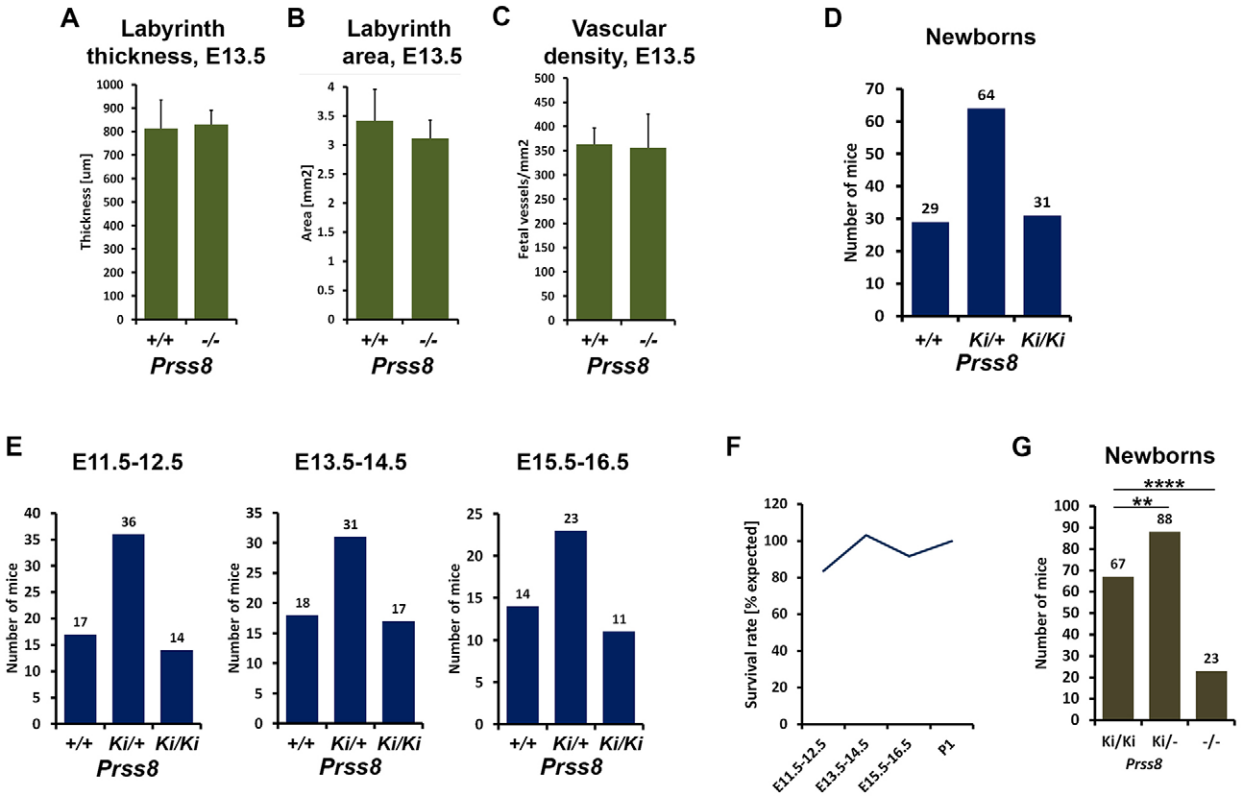

Fig. 2. Prenatal survival is not affected by loss of prostasin proteolytic activity. (A-C) Morphometric analysis of the thickness (A), cross-sectional area (B) and the density of fetal vasculature (C) of the placental labyrinth of control (*Prss8*$^{+/+}$, *n*=8) and prostasin-deficient (*Prss8*$^{-/-}$, *n*=5) mice at E13.5. Absence of prostasin did not visibly affect the structure of the labyrinth layer. Error bars indicate s.d. (D,E) Distribution of *Prss8* genotypes in (D) newborn offspring and (E) embryos of the indicated stages from *Prss8*$^{Ki/+}$×*Prss8*$^{Ki/+}$ breeding pairs. Loss of prostasin proteolytic function did not reduce prenatal viability. (F) Relative survival of *Prss8*$^{Ki/Ki}$ mice between E11.5 and birth (P1) (observed versus expected number of mice based on normal Mendelian distribution) based on data presented in D and E. (G) Distribution of *Prss8* genotypes in newborn offspring from *Prss8*$^{Ki/-}$×*Prss8*$^{Ki/-}$ breeding pairs. Numbers of *Prss8*$^{-/-}$ and *Prss8*$^{Ki/-}$ mice were significantly reduced compared with *Prss8*$^{Ki/Ki}$ littermate controls (χ^2, observed versus expected based on normal survival of *Prss8*$^{Ki/Ki}$ mice). ***P*<0.01, *****P*<0.0001.

Loss of prostasin proteolytic activity does not restore embryonic survival in HAI-1- or HAI-2-deficient mice

Loss of the transmembrane serine protease inhibitors HAI-1 and HAI-2 in mice results in complete embryonic lethality (Fan et al., 2007; Mitchell et al., 2001; Szabo et al., 2009a; Tanaka et al., 2005). Prenatal survival of both HAI-1- and HAI-2-deficient mice can, however, be fully restored by genetic inactivation of either matriptase or prostasin, indicating a crucial contribution of the prostasin/matriptase pathway to the demise of mice lacking either of the two HAI proteins (Szabo et al., 2009a,b, 2007, 2012). To establish whether the prostasin-dependent loss of viability of HAI-deficient mice requires prostasin proteolytic activity, we interbred mice carrying HAI-1 or HAI-2 null alleles (*Spint1*$^{+/-}$ or *Spint2*$^{+/-}$, respectively) with mice carrying both prostasin null and catalytically inactive knock-in alleles and analyzed their offspring at birth.

Consistent with our previous reports, expression of prostasin wild-type protein led to a complete loss of prenatal viability in mice lacking HAI-1. Thus, no *Spint1*$^{-/-}$ mice carrying two or one wild-type allele of prostasin (*Spint1*$^{-/-}$;*Prss8*$^{+/+}$ or *Spint1*$^{-/-}$;*Prss8*$^{+/-}$) were observed at birth (Fig. 4A; both $P<0.0001$, χ^2). As anticipated, complete loss of prostasin was able to restore embryonic survival in *Spint1*$^{-/-}$ mice (Fig. 4A; *Spint1*$^{-/-}$;*Prss8*$^{-/-}$, $P=0.51$, χ^2). Surprisingly, however, breeding of *Spint1*$^{+/-}$;*Prss8*$^{Ki/+}$ mice did not produce any viable *Spint1*$^{-/-}$;*Prss8*$^{Ki/Ki}$ mice at birth, indicating that the elimination of prostasin proteolytic function is not sufficient for the completion of prenatal development in the absence of the HAI-1 protein (Fig. 4B; $P<0.0005$, χ^2).

Collection of embryos from *Spint1*$^{+/-}$;*Prss8*$^{+/-}$ or *Spint1*$^{+/-}$;*Prss8*$^{Ki/+}$ breeding pairs between E12.5 and E15.5 showed a complete absence of living *Spint1*$^{-/-}$;*Prss8*$^{+/+}$ mice (Fig. 4C,D, $P<0.02$ and $P<0.05$, respectively, χ^2). As expected from the analysis of newborn mice (Fig. 4A), the viability of HAI-1-deficient mice was completely restored at both embryonic stages in a prostasin null background (Fig. 4C; *Spint1*$^{-/-}$;*Prss8*$^{-/-}$). Importantly, viability was also improved by the selective loss of prostasin proteolytic activity, with about half of the expected *Spint1*$^{-/-}$;*Prss8*$^{Ki/Ki}$ embryos still alive at E12.5-E13.5 (Fig. 4D, left; 52%, $P=0.41$, χ^2). However, this effect was transient, and no living *Spint1*$^{-/-}$;*Prss8*$^{Ki/Ki}$ embryos were detected after E14.5 (Fig. 4D, right; $P<0.02$, χ^2).

To investigate whether the extended lifespan of HAI-1-deficient mice in the prostasin knock-in background was caused by improved placental function, we next performed a histological analysis of placental tissues extracted from *Spint1*$^{-/-}$ embryos that were *Prss8*$^{+/+}$, *Prss8*$^{Ki/Ki}$ or *Prss8*$^{-/-}$ at E12.5. Control tissues exhibited well-formed placental labyrinth with a highly branched fetal vasculature (Fig. 4E), whereas placentas from *Spint1*$^{-/-}$;*Prss8*$^{+/+}$ mice showed a complete absence of the labyrinth structure and no invading fetal vessels (Fig. 4F,I,J). Prostasin full deficiency was able to restore placental differentiation, as evidenced by the thickness and area of the labyrinth layer in the midline cross-section of the tissue as

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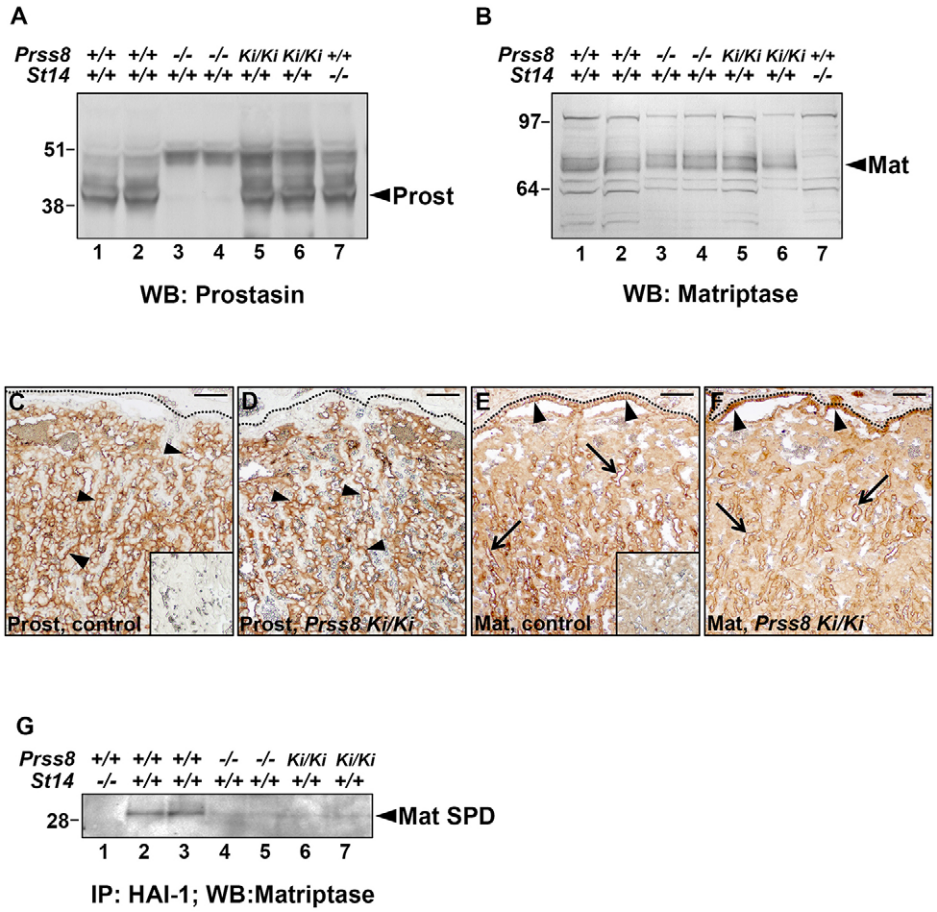

Fig. 3. Prostasin-mediated activation of matriptase during embryogenesis requires prostasin proteolytic activity. (A,B) Western blot (WB) detection of prostasin (A) and single-chain matriptase (B) in the fetal part of E12.5 control (lanes 1 and 2), prostasin-deficient (lanes 3 and 4), prostasin knock-in (lanes 5 and 6) and matriptase-deficient (lane 7) placentas. Loss of prostasin proteolytic activity did not affect total expression of prostasin (Prost) or matriptase (Mat) proteins. Positions of molecular weight markers (kDa) are shown on the left. (C-F) Representative images showing immunohistochemical staining for prostasin (C,D) and matriptase (E,F) in placental tissues of control (C,E) and *Prss8*$^{Ki/Ki}$ (D,F) embryos at E12.5. The pattern of expression of prostasin within the labyrinth layer (C,D, arrowheads) and matriptase in chorionic and labyrinthine epithelium (E,F, arrowheads and arrows, respectively) was not affected in *Prss8*$^{Ki/Ki}$ placentas. Insets show corresponding staining in age-matched prostasin-deficient (C) and matriptase-deficient (E) tissues. (G) Western blot detection of an active two-chain form of matriptase after anti-HAI-1 immunoprecipitation (IP) in the E12.5 matriptase-deficient (*Prss8*$^{+/+}$;*St14*$^{-/-}$, lane 1), control (*Prss8*$^{+/+}$;*St14*$^{+/+}$, lanes 2 and 3), prostasin-deficient (*Prss8*$^{-/-}$;*St14*$^{+/+}$, lanes 4 and 5) and prostasin knock-in (*Prss8*$^{Ki/Ki}$;*St14*$^{+/+}$, lanes 6 and 7) placenta. The expected position of the matriptase serine protease domain (Mat SPD) is indicated. Loss of prostasin proteolytic function prevents conversion of matriptase zymogen into the active two-chain form. Blots (A,B,G) are representative of three independent experiments. Each image (C-F) is representative of three independent stainings, each carried out on at least two tissue samples of the same genotype. Scale bars: 50 µm.

well as the density of the fetal vasculature within the labyrinth layer of *Spint1*$^{-/-}$;*Prss8*$^{-/-}$ mice, which were all comparable to those of wild-type controls (Fig. 4G,I,J). Even though the loss of prostasin proteolytic activity also partially restored labyrinth differentiation in *Spint1*$^{-/-}$;*Prss8*$^{Ki/Ki}$ mice, the structure of the labyrinth layer remained abnormal with strongly reduced cross-sectional area and branching of fetal vasculature (Fig. 4H-J).

Loss of HAI-2 leads to an early embryonic lethality with no surviving *Spint2*$^{-/-}$ mice identified at or after E10.5 (Mitchell et al., 2001; Szabo et al., 2012) (Fig. 5C). The complete loss of prostasin protein compensates for the loss of HAI-2 during embryogenesis, as evidenced by the presence of living *Spint2*$^{-/-}$;*Prss8*$^{-/-}$ mice at birth (Mitchell et al., 2001; Szabo et al., 2012) (Fig. 5A). By contrast, a similar analysis did not reveal any surviving *Spint2*$^{-/-}$;*Prss8*$^{Ki/Ki}$ embryos, suggesting that the selective loss of prostasin proteolytic activity is not sufficient to prevent embryonic lethality in HAI-2-deficient mice (Fig. 5B). Furthermore, the loss of prostasin proteolytic activity was not sufficient to extend the life of *Spint2*$^{-/-}$ mice when compared with mice expressing wild-type alleles of prostasin, as suggested by a complete lack of living *Spint2*$^{-/-}$;*Prss8*$^{Ki/Ki}$ mice among the embryos extracted at E10.5 and E11.5 (Fig. 5C; $P<0.01$, χ^2). As expected from the analysis of the newborn mice (Fig. 5A), *Spint2*$^{-/-}$;*Prss8*$^{-/-}$ embryos were found alive and in the expected Mendelian ratio (Fig. 5C). These data indicate that the prostasin-dependent embryonic lethality observed in mice lacking either of the two HAI proteins is mediated by non-proteolytic functions of prostasin, although its proteolytic activity does contribute to placental defects in mice lacking HAI-1.

Inactivation of HAI-1, but not HAI-2, results in prostasin-independent postnatal survival

Loss of prostasin function leads to a catastrophic loss of the epidermal barrier, the absence of whiskers at birth, and a complete perinatal lethality (Leyvraz et al., 2005; Peters et al., 2014; this study). Unexpectedly, analysis of postnatal survival in the offspring from *Spint1*$^{+/-}$;*Prss8*$^{+/-}$ breeding pairs showed that, whereas all of the HAI-1-expressing *Prss8*$^{-/-}$ mice (*Spint1*$^{+/+}$;*Prss8*$^{-/-}$ or *Spint1*$^{+/-}$;*Prss8*$^{-/-}$) died within the first few days after birth, mice

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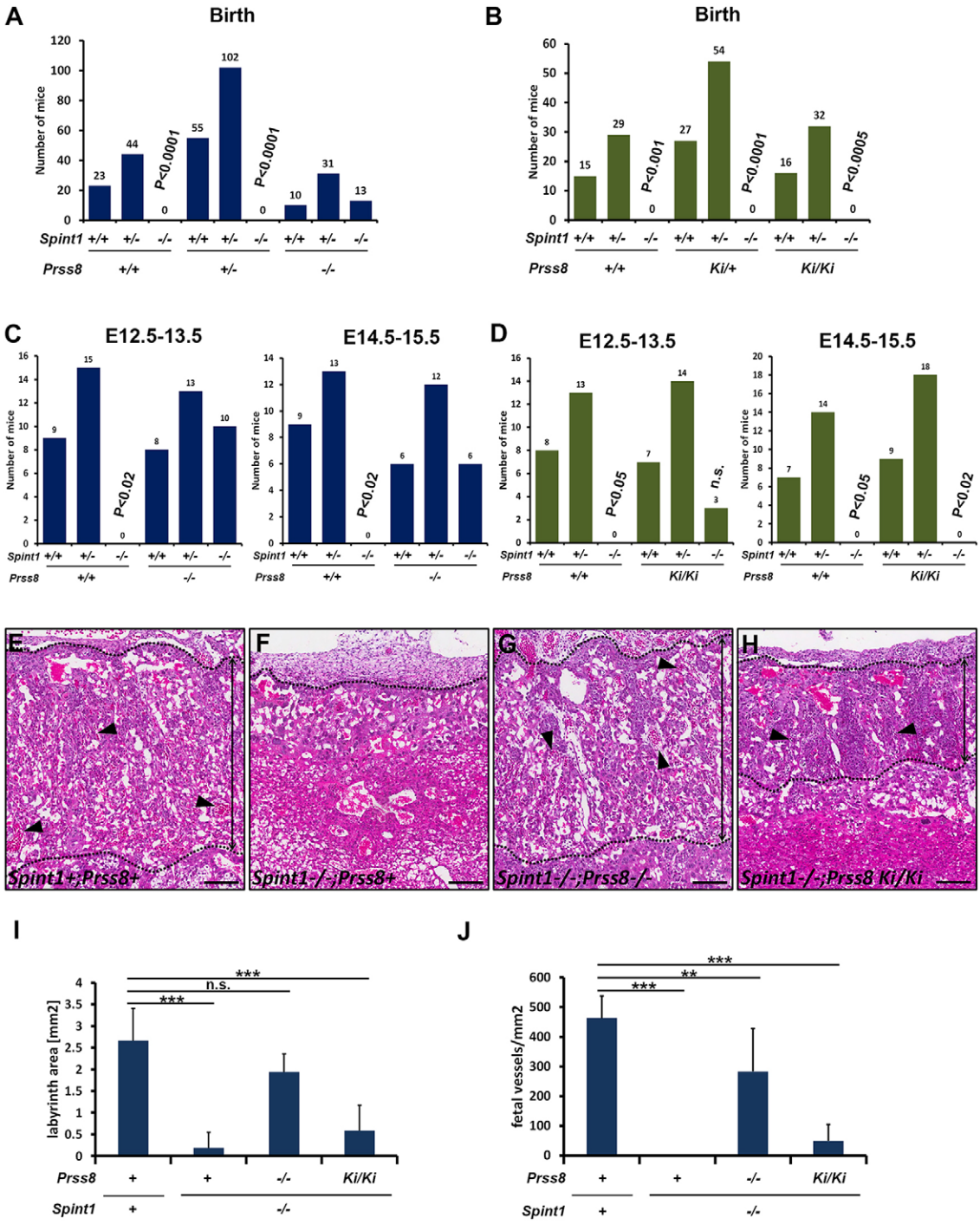

Fig. 4. Embryonic lethality in HAI-1-deficient embryos is not rescued by loss of prostasin proteolytic activity. (A,B) Distribution of *Spint1* (HAI-1) genotypes in newborn offspring from *Spint1*$^{+/-}$;*Prss8*$^{+/-}$×*Spint1*$^{+/-}$;*Prss8*$^{+/-}$ (A) and *Spint1*$^{+/-}$;*Prss8*$^{Ki/+}$×*Spint1*$^{+/-}$;*Prss8*$^{Ki/+}$ (B) breeding pairs. No *Spint1*$^{-/-}$ mice were observed among prostasin-sufficient (*Prss8*$^{+/+}$ or *Prss8*$^{+/-}$) mice. Embryonic survival of HAI-1-deficient mice was completely restored in prostasin null mice (*Prss8*$^{-/-}$) but not in mice lacking prostasin catalytic activity (*Prss8*$^{Ki/Ki}$). (C,D) Distribution of *Spint1* genotypes in the offspring from *Spint1*$^{+/-}$;*Prss8*$^{+/-}$×*Spint1*$^{+/-}$;*Prss8*$^{+/-}$ (C) or *Spint1*$^{+/-}$;*Prss8*$^{Ki/+}$×*Spint1*$^{+/-}$;*Prss8*$^{Ki/+}$ (D) breeding pairs at E12.5-E13.5 and E14.5-E15.5. *Spint1*$^{-/-}$ embryos are detected in the expected numbers in the *Prss8*$^{-/-}$ background. Elimination of prostasin catalytic activity transiently improves the survival of *Spint1*$^{-/-}$ mice. (E-H) Representative images showing H&E-stained placental tissues of control (E, *Spint1*$^{+}$;*Prss8*$^{+}$), HAI-1-deficient (F, *Spint1*$^{-/-}$;*Prss8*$^{+}$), HAI-1/prostasin double-deficient (G, *Spint1*$^{-/-}$;*Prss8*$^{-/-}$), and HAI-1-deficient prostasin knock-in (H, *Spint1*$^{-/-}$;*Prss8*$^{Ki/Ki}$) mice. The thickness of the placental labyrinth is indicated by the double-headed arrows between the dotted lines (E,G,H). No labyrinth structure was detected in HAI-1-deficient placenta (F). Fetal vessels (E,G,H, arrowheads) within the labyrinth were restored to near control levels in HAI-1/prostasin double-deficient mice (G) but strongly reduced in placentas from the HAI-1-deficient prostasin knock-in (H). Each image in is representative of three independent stainings, each carried out on at least two tissue samples of the same genotype. (I,J) Quantification of the labyrinth cross-sectional area (I) and the density of fetal vessels in the placental labyrinth (J) of control (*Spint1*$^{+}$;*Prss8*$^{+}$, *n*=8) and HAI-1-deficient (*Spint1*$^{-/-}$) embryos expressing wild-type (*Prss8*$^{+}$, *n*=4), null (*Prss8*$^{-/-}$, *n*=6) and catalytically inactive knock-in (*Prss8*$^{Ki/Ki}$, *n*=6) alleles of prostasin. Complete loss of prostasin expression restored labyrinth development to near-normal levels, whereas only a modest improvement was seen in the absence of prostasin catalytic activity compared with HAI-1-deficient (*Spint1*$^{-/-}$) mice. ***P*<0.01, ****P*<0.001, Student's *t*-test, two-tailed. Error bars indicate s.d. Scale bars: 100 µm.

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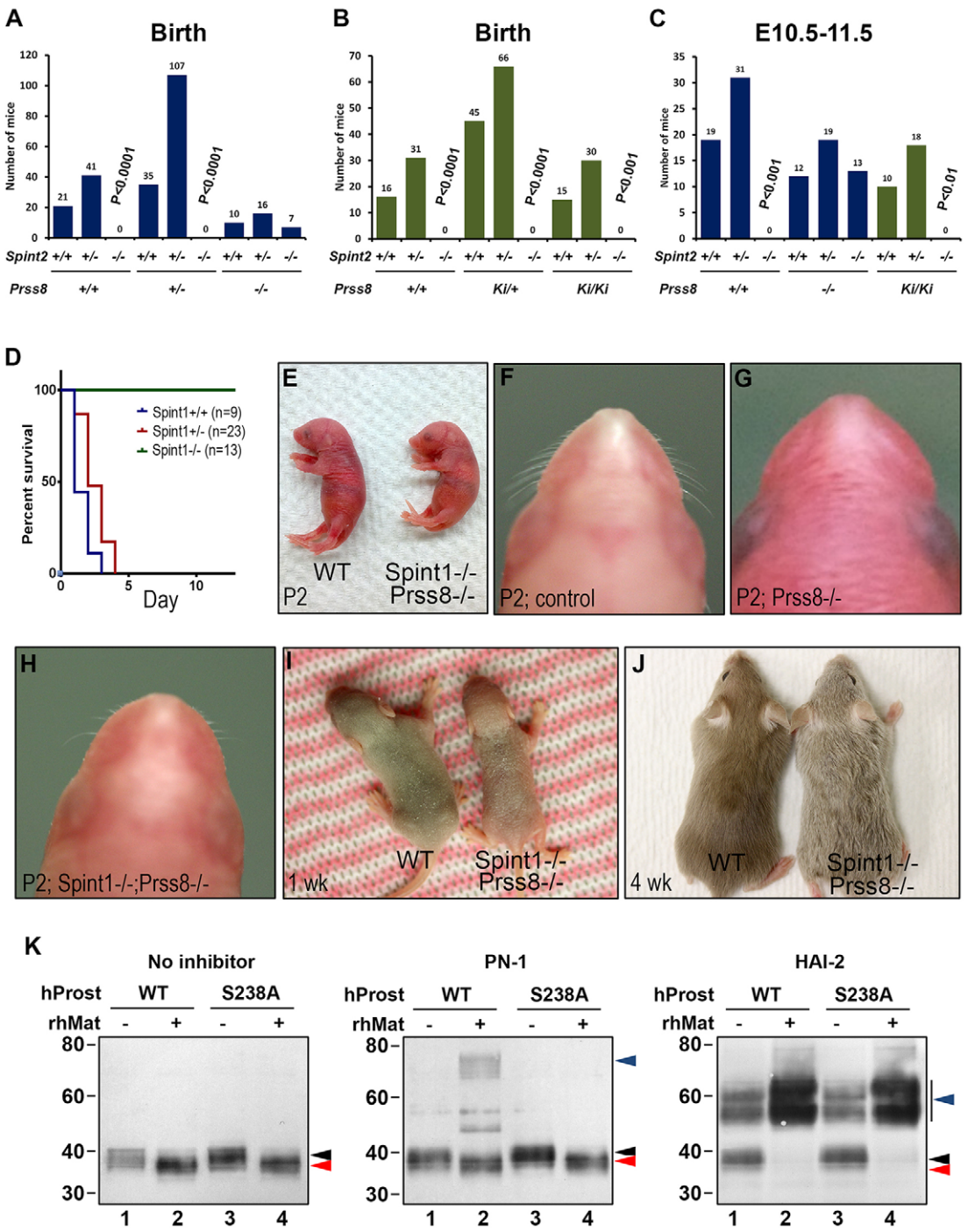

Fig. 5. Catalytically inactive prostasin does not restore viability in HAI-2-deficient embryos. (A,B) Distribution of *Spint2* (HAI-2) genotypes in newborn offspring from *Spint2*$^{+/-}$;*Prss8*$^{+/-}$×*Spint2*$^{+/-}$;*Prss8*$^{+/-}$ (A) and *Spint2*$^{+/-}$;*Prss8*$^{Ki/+}$×*Spint2*$^{+/-}$;*Prss8*$^{Ki/+}$ (B) breeding pairs. Whereas *Spint2*$^{-/-}$ mice were born in the expected ratio in the *Prss8*$^{-/-}$ background, there were none in the prostasin-sufficient (*Prss8*$^{+/+}$ or *Prss8*$^{+/-}$) or *Prss8*$^{Ki/Ki}$ backgrounds. (C) Distribution of *Spint2* genotypes in the offspring from *Spint2*$^{+/-}$;*Prss8*$^{+/-}$×*Spint2*$^{+/-}$;*Prss8*$^{+/-}$ (blue bars) and *Spint2*$^{+/-}$;*Prss8*$^{Ki/+}$×*Spint2*$^{+/-}$;*Prss8*$^{Ki/+}$ (green bars) breeding pairs at E10.5-E11.5. *Spint2*$^{-/-}$ embryos are detected in expected numbers in the *Prss8*$^{-/-}$ but not in the *Prss8*$^{Ki/Ki}$ background. (D-J) Inactivation of HAI-1 eliminates the need for prostasin during development. (D) Postnatal survival of born *Prss8*$^{-/-}$ mice that are wild-type (*Spint1*$^{+/+}$), *Spint1*$^{+/-}$ or *Spint1*$^{-/-}$. Whereas all prostasin-deficient mice expressing HAI-1 protein die within the first few days after birth, prostasin/HAI-1 double-deficient mice show no decrease in viability [P<0.0001, log-rank (Mantel-Cox) test]. (E) Macroscopic appearance of control (WT) and *Spint1*$^{-/-}$;*Prss8*$^{-/-}$ mice at P2. *Spint1*$^{-/-}$;*Prss8*$^{-/-}$ exhibit notably smaller body size. (F-H) Representative image of whiskers in control (F), *Prss8*$^{-/-}$ (G) and *Spint1*$^{-/-}$;*Prss8*$^{-/-}$ (H) mice at P2. Unlike prostasin single knockouts, double-deficient mice present with visible, although shorter whiskers. (I,J) Macroscopic appearance of control (WT) and *Spint1*$^{-/-}$;*Prss8*$^{-/-}$ mice at 1 (I) and 4 (J) weeks of age. Double-deficient mice initially present with delayed body hair growth and ichthyotic skin. Both defects subside within the first month of life. Each image (E-J) is representative of at least five animals of the same genotype. (K) Western blot detection of prostasin after incubation of no inhibitors (left), PN-1 (middle) or HAI-2 (right) with wild-type (WT, lanes 1 and 2) or catalytically inactive (S238A, lanes 3 and 4) pro-prostasin zymogen (black arrowheads, lanes 1 and 3) and matriptase-activated double-chain prostasin (red arrowheads, lanes 2 and 4). PN-1 only formed SDS-stable inhibitory complexes (blue arrowheads) with a double-chain form of wild-type prostasin, whereas HAI-2-prostasin complexes were detected with both the wild-type and S238A double-chain and, to a lesser extent, the one-chain zymogen forms of prostasin. Positions of molecular weight markers (kDa) are shown on the left.

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deficient in HAI-1 and prostasin (*Spint1*$^{-/-}$;*Prss8*$^{-/-}$) did not exhibit any postnatal lethality and were all found alive at weaning and when followed for up to 4 months (Fig. 5D). Thus, the essential role of prostasin in postnatal survival is completely circumvented in mice lacking HAI-1. At birth, mice lacking both prostasin and HAI-1 did not display any gross developmental abnormalities (Fig. 5E). In contrast to *Prss8*$^{-/-}$ mice, the *Spint1*$^{-/-}$;*Prss8*$^{-/-}$ newborn mice did present with visible whiskers, although these were shorter than those of wild-type littermate controls (Fig. 5F-H). The initial eruption of body hair was also markedly delayed in *Spint1*$^{-/-}$;*Prss8*$^{-/-}$ mice (Fig. 5I). The body size, whisker and body hair growth all normalized between 2 and 3 weeks of age, and the adult double-deficient mice did not exhibit any obvious defects compared with the HAI-1- and prostasin-sufficient littermate controls (Fig. 5J). Similar analysis of the offspring from *Spint2*$^{+/-}$;*Prss8*$^{+/-}$ breeding pairs did not reveal any effect of the loss of HAI-2 on the survival of prostasin-deficient mice, as all of the 33 born *Prss8*$^{-/-}$ mice (Fig. 5A) died within 48 h after birth, irrespective of their *Spint2* genotype. These findings indicate that the absence of HAI-1, but not of HAI-2, can completely compensate for the loss of essential functions of prostasin during postnatal development.

The above findings could be explained by HAI-2, unlike HAI-1, directly inhibiting prostasin rather than matriptase activity, so that only the loss of HAI-1, but not of HAI-2, can compensate for the loss of prostasin by removing inhibition of matriptase and increasing its activity to a level required to sustain development and survival. However, since HAI-2 is also essential for survival in *Prss8*$^{Ki/Ki}$ mice (Fig. 5B), in this scenario the inhibitor would also need to regulate non-proteolytic functions of prostasin, predicting an effective binding of HAI-2 to the catalytically inactive protease. To test this hypothesis, we incubated zymogen or matriptase-activated double-chain forms of wild-type and S238A prostasin with recombinant HAI-2 or PN-1, which is a serpin-type (SERPINE2) cognate inhibitor of prostasin. As expected, PN-1, which requires proteolytic cleavage of its reactive center loop to form a stable complex with a protease, only bound the active double-chain form of wild-type prostasin (Fig. 5K). By contrast, double-chain forms of both wild-type and catalytically inactive prostasin were very efficiently sequestered by HAI-2 (Fig. 5K). HAI-2 also formed complexes with the zymogen forms of wild-type and S238A prostasin, although at reduced levels. These findings are consistent with the ability of HAI-2 to regulate both proteolytic and non-proteolytic functions of prostasin.

DISCUSSION

An overwhelming body of evidence suggests that prostasin, matriptase, HAI-1 and HAI-2 constitute a system of proteases and protease inhibitors that acts on the surface of epithelial cells to regulate multiple aspects of development, postnatal homeostasis and tissue remodeling. First, loss-of-function studies in matriptase- and prostasin-deficient mice reveal virtually identical effects of the elimination of the two proteases on overall survival, epidermal differentiation and barrier formation, hair growth as well as lethality of HAI-1- and HAI-2-deficient embryos (Leyvraz et al., 2005; List et al., 2002, 2003; Szabo et al., 2012). Second, several recent reports document the ability of prostasin to stimulate matriptase activity in cell culture as well as in tissues, suggesting that prostasin is likely to act upstream of matriptase (Buzza et al., 2013; Camerer et al., 2010; Friis et al., 2013; Szabo et al., 2012). Third, prostasin and matriptase form protein complexes when co-expressed in epithelial cells (Friis et al., 2013). Finally, defects in epidermal development and barrier formation in prostasin null mice that express near-normal levels of the activated two-chain form of matriptase in the epidermis show that, even in its active form, matriptase is unable to perform its essential skin functions in the absence of prostasin (Friis et al., 2016; Szabo et al., 2012).

Surprisingly, our recent work indicates that at least some of the reported physiological functions of prostasin during development are preserved in mice that express a catalytically inactive variant of the protease (Peters et al., 2014). Here, we performed a comprehensive analysis of previously reported functions of prostasin during embryonic and postnatal development, and show that specific prostasin-mediated processes vary in their degree of dependence on its proteolytic activity (Table 1). Thus, catalytically inactive prostasin fully supports embryonic development, postnatal survival and prostasin-dependent aspects of terminal epidermal differentiation and epidermal barrier formation, but is unable to mediate prostasin-dependent zymogen activation of matriptase during placental differentiation. Surprisingly, this inability to promote activation of placental matriptase only transiently extends the survival of mice lacking the cognate matriptase/prostasin inhibitor HAI-1 and shows no effect on the embryonic lethality observed in HAI-2-deficient mice. These paradoxical findings could most easily be explained by an ability of prostasin to allosterically stimulate the activity of the one-chain zymogen, in addition to the two-chain 'active' form of matriptase (Fig. 6). This is consistent with our recent report that catalytically inactive prostasin is found in complex with a zymogen-locked matriptase in cultured epithelial cells (Friis et al., 2016).

An explanation for the varying degree of dependence of prostasin biological functions on its proteolytic activity remains to be established. It is clear that, at least in one tissue, prostasin mediates the proteolytic activation of matriptase zymogen, as evidenced by a loss of the two-chain form of the matriptase in prostasin null placentas. However, the presence of active matriptase in the epidermis of prostasin-deficient mice suggests that additional proteases are likely to contribute to matriptase zymogen activation in other tissues and/or biological processes (Friis et al., 2016; Szabo et al., 2012). As a result, matriptase zymogen activation and the full activity of the matriptase/prostasin pathway may, depending on circumstances, exhibit complete, partial or no dependence on prostasin proteolytic function. Also, various biological processes might require different levels of matriptase activity for successful completion. It is therefore plausible that although non-proteolytic functions of prostasin are sufficient to stimulate the enzymatic activity of single- or two-chain matriptase to levels that support some processes, such as epidermal development, its full activation, dependent also on prostasin proteolytic functions, is required during

Table 1. Prostasin-mediated functions during development and their dependence on prostasin proteolytic activity

Prostasin-mediated function/process	Dependence on proteolytic activity
Embryonic survival	No
Postnatal survival	No
Epidermal differentiation	No
Embryonic lethality in HAI-2-deficient mice	No
Epidermal barrier function	Partial
Whisker and hair growth	Partial
Embryonic lethality in HAI-1-deficient mice	Partial
Cutaneous wound healing	Partial or complete*
Matriptase activation in placenta	Complete

*Unable to determine the effect of complete loss of prostasin proteolytic and non-proteolytic functions.

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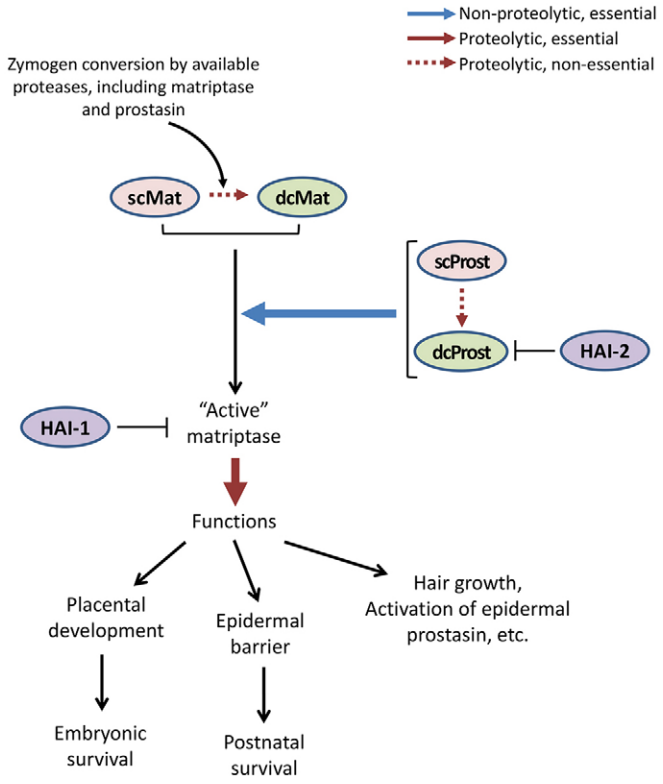

Fig. 6. Proposed mechanism of the physiological functions of the matriptase/prostasin proteolytic pathway during development. Prostasin acts as a co-factor to enhance matriptase proteolytic activity during prenatal and perinatal development by an as yet unidentified mechanism that might include interaction with the substrate or protection from inhibition. This function does not require the proteolytic activity of prostasin, but is essential to raise the enzymatic activity of matriptase towards its target substrates to the levels required for normal embryonic and postnatal survival. However, the requirement for prostasin in development and survival can be negated by removal of pathway inhibition by HAI-1, which is expected to increase overall matriptase activity, and suggests that prostasin does not directly execute the developmental functions of the matriptase/prostasin pathway, and that HAI-1 is a direct inhibitor of matriptase rather than of prostasin activity. However, HAI-2 appears to primarily target prostasin, rather than matriptase, function. Notably, while conversion of matriptase and prostasin zymogens to their respective two-chain forms is expected to increase the activity of the matriptase/prostasin pathway, it is not required for the completion of embryonic development or the acquisition of epidermal barrier (Friis et al., 2016; S. Friis and T.H.B., unpublished). sc, single chain; dc, double chain.

other processes, such as hair growth and wound closure. Alternatively, prostasin could have some proteolytic and/or non-proteolytic functions that are independent of matriptase activity, including acting as a non-enzymatic co-factor to other trypsin-like serine proteases or directly cleaving as yet unidentified downstream targets. Although we cannot formally exclude this possibility, the fact that the loss of prostasin function leads to phenotypes that are virtually identical to those observed in the absence of matriptase would suggest that the two proteases regulate developmental functions as part of a single proteolytic system.

Genetic background appears to have a considerable effect on the phenotypes associated with the loss of prostasin or matriptase, in particular during embryonic development, as documented by complete embryonic lethality in an inbred C57BL/6J background but partial or complete prenatal survival in several prostasin- or matriptase-deficient strains of mixed background (Hummler et al., 2013; Leyvraz et al., 2005; List et al., 2002; Peters et al., 2014; Szabo et al., 2009a, 2014; R.S. and T.H.B., unpublished). This could also explain our inability to observe previously described structural defects in the development of the placental labyrinth as the cause of death in C57BL/6J *Prss8*$^{-/-}$ embryos (Hummler et al., 2013). However, it should be noted that our current findings do not eliminate defects in placental function as the most likely reason for the lethality in mice lacking prostasin.

Probably the most surprising finding of this study is the restoration of prostasin-dependent functions and the full viability of prostasin-deficient mice when they also lack HAI-1. This strongly suggests that the regulation of processes such as epidermal barrier formation, hair growth and overall survival by prostasin is mediated by modulation of the activity of heterologous proteases, and is consistent with the proposed role of prostasin as a co-factor in the stimulation of matriptase activity (Fig. 6). Restored epidermal barrier and survival of prostasin/HAI-1 double-deficient mice can then be explained by the compensation of a decreased activity of matriptase in the absence of prostasin-mediated 'activation' by a sufficient increase in HAI-1-free, and presumably more active, matriptase. This is supported by the observation that loss of essential functions of the matriptase/prostasin pathway in skin development, when caused by loss of matriptase rather than loss of prostasin, cannot be restored by a removal of pathway inhibition through the elimination of HAI-1 (Szabo et al., 2007), identifying matriptase as the protease that executes the function of the pathway (Fig. 6).

Loss of HAI-2 did not have a visible impact on phenotypes associated with prostasin deficiency. It is therefore likely that although both HAI-1 and HAI-2 are required for the regulation of matriptase/prostasin activity during development, their mechanisms of action differ. Thus, while it is widely accepted that HAI-1 forms inhibitory complexes with matriptase *in vivo* and is likely to directly regulate matriptase activity at the cell surface, HAI-2 might exert its function by primarily regulating prostasin activity (Fig. 6). This would explain why the loss of HAI-2, unlike that of HAI-1, cannot compensate for the loss of prostasin by removing inhibition of matriptase. Indeed, we have previously documented that, in a cell culture-based system, prostasin efficiently co-immunoprecipitates with HAI-2, and the effect of HAI-2 on matriptase activation and shedding in this system is largely prostasin dependent (Friis et al., 2014). Furthermore, the isolation of prostasin complexes with HAI-2 from human milk was recently reported, showing a direct interaction between the two proteins *in vivo* (Lai et al., 2016). Interaction of HAI-2 with catalytically inactive prostasin, as shown in our current study, suggests that HAI-2 can inhibit both proteolytic and non-proteolytic functions of prostasin and can explain why HAI-2 remains essential for development and survival in the *Prss8*$^{Ki/Ki}$ background. Although we cannot formally exclude the possibility that, in addition to its effect on prostasin, HAI-2 can also contribute to the inhibition of other serine proteases, including matriptase, this does not appear to be crucial for mouse embryonic survival.

It is important to underscore that the current study focuses on functions of the matriptase/prostasin pathway in placental differentiation and skin development and its function in embryonic and postnatal survival. Additional studies will be needed to determine the extent to which our model applies to the reported functions of matriptase and prostasin in other tissues.

MATERIALS AND METHODS

Mouse strains

All experiments were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium

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following standard operating procedures and were approved by the NIDCR Institutional Animal Care and Use Committee. Matriptase-deficient (*St14*$^{-/-}$), prostasin-deficient (*Prss8*$^{-/-}$), HAI-1-deficient (*Spint1*$^{-/-}$) and HAI-2-deficient (*Spint2*$^{-/-}$), as well as prostasin knock-in mice expressing proteolytically inactive protein (*Prss8*$^{Ki/Ki}$) have been described previously (List et al., 2002; Peters et al., 2014; Szabo et al., 2009a, 2007). All studies used mice of mixed 129S6/Sv;NIH BlackSwiss;FVB/NJ;C57Bl/6J genetic background and were littermate controlled. Ear or tail clips of newborn or 2-week-old mice were subjected to genomic DNA extraction and genotyped by PCR (see Table S1 for primer sequences).

Analysis of embryonic and placental tissues

Females were euthanized at designated time points by CO_2 asphyxiation and embryos were extracted by Caesarian section. Tail clips or yolk sacs of individual embryos were washed in PBS, subjected to genomic DNA extraction and genotyped by PCR (Table S1). To analyze development of the placental labyrinth, placental tissues were fixed for 24 h in 4% paraformaldehyde (PFA), processed into paraffin, sectioned, and stained with Hematoxylin and Eosin (H&E), or used for immunohistochemistry as described below. Overall thickness of the placental labyrinth was measured as the distance between the undifferentiated chorionic epithelium and the labyrinth-supporting spongiotrophoblast layer on a single midline cross-section. To evaluate branching of the fetal vasculature in the E12.5 and E13.5 placentas, a midline cross-section of each placenta was immunostained with anti-CD31 (PECAM1) antibody (see below), followed by the manual counting of individual profiles of CD31-stained vessels within the placental labyrinth. All tests were performed using tissues from at least five mice of each genotype. Only living embryos with detectable heartbeat were used for the morphometric analysis. The observed values were statistically analyzed using a two-sample, two-tailed Student's *t*-test.

Immunohistochemistry

Antigens from 5 µm paraffin sections were retrieved by incubation for 10 min at 100°C with 1 mM EDTA pH 8.0 for CD31 staining, or by incubation for 20 min at 100°C in 0.01 M sodium citrate buffer pH 6.0 for all other antigens. The sections were blocked with 2.5% bovine serum albumin (fraction V, MP Biomedicals) in PBS, and incubated with primary antibody overnight at 4°C (for detailed information on antibodies, see Table S2). Bound antibodies were visualized using biotin-conjugated secondary antibodies (Table S2) and a Vectastain ABC Kit (Vector Laboratories) using 3,3′-diaminobenzidine as the substrate (Sigma-Aldrich). All microscopy images were acquired on an Olympus BX40 microscope using an Olympus DP70 digital camera system.

Western blotting

Fresh tissues were homogenized and lysed in buffer containing 1% Triton X-100 and 0.5% sodium deoxycholate in PBS. The homogenates were cleared by centrifugation at 12,000 ***g*** for 10 min at 4°C and the protein concentration determined by BCA assay (Pierce). 80 µg total protein was loaded on 4-12% SDS-PAGE under reducing conditions and analyzed by western blotting, with incubation with primary antibody overnight at 4°C followed by secondary antibody conjugated to alkaline phosphatase for 1 h at room temperature (Table S2). Alkaline phosphatase activity was visualized using nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate substrates.

Detection of active matriptase in mouse embryonic tissues

Lysates from three placentae of the same genotype were combined and pre-incubated with 100 µl GammaBind G Sepharose beads (GE Healthcare Life Sciences) for 30 min at 4°C with gentle agitation. The samples were spun at 1000 ***g*** for 1 min to remove the beads, and the supernatant then incubated with 3 µg goat anti-mouse HAI-1 antibody (R&D Systems, Table S2) and 80 µl GammaBind G Sepharose beads for 3 h at 4°C. The samples were spun at 1000 ***g*** for 1 min, the supernatant removed, and the beads washed three times with 1 ml ice-cold PBS containing 1% Triton X-100 and 0.5% sodium deoxycholate. The beads were then mixed with 30 µl SDS loading buffer (Invitrogen) containing 0.25 M β-mercaptoethanol, incubated for 5 min at 99°C, and cooled on ice for 2 min. The samples were spun at 1000 ***g*** for 1 min and the released proteins then resolved by 4-12% polyacrylamide SDS-PAGE and analyzed by western blot using sheep anti-human matriptase (R&D Systems) primary antibody and peroxidase-conjugated donkey anti-sheep (Sigma-Aldrich) secondary antibodies (Table S2). The signal was visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Formation of prostasin-HAI inhibitory complexes

The preparation of wild-type and S238A variants of human prostasin has been described (Netzel-Arnett et al., 2006; Szabo et al., 2012). To detect prostasin-HAI inhibitory complexes, PI-PLC-released pro-prostasin variants were left untreated or first activated by incubation with 10 nM human recombinant matriptase serine protease domain (R&D Systems) for 20 min at 37°C. 100 ng prostasin zymogen or matriptase-activated prostasin in 50 mM Tris-HCl pH 8.0, 100 mM NaCl buffer was then incubated with 300 ng human recombinant PN-1 or HAI-2 (both R&D Systems) for 30 min at room temperature. Reduced but non-boiled samples were analyzed by western blotting as described above (for antibodies see Table S2).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.S. and T.H.B. designed the study; R.S., T.L. and D.E.P. performed experiments and analyzed data; R.S. and T.H.B. wrote the manuscript.

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Supplementary information

Supplementary information available online at
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