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Hepsin regulates TGFβ signaling via fibronectin proteolysis

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Abstract

Transforming growth factor-beta (TGFβ) is a multifunctional cytokine with a well-established role in mammary gland development and both oncogenic and tumor-suppressive functions. The extracellular matrix (ECM) indirectly regulates TGFβ activity by acting as a storage compartment of latent-TGFβ, but how TGFβ is released from the ECM via proteolytic mechanisms remains largely unknown. In this study, we demonstrate that hepsin, a type II transmembrane protease overexpressed in unknown. In this study, we demonstrate that hepsin, a type II transmembrane protease overexpressed in breast cancer; fibronectin; hepsin; TGFβ

Introduction

The human genome encodes 566 proteases, and of these, 273 have been found in extracellular compartments or the lumen of secretory compartments, 277 in intracellular compartments, and a small fraction at the plasma membrane (Overall & Blobel, 2007). Extracellular proteolysis plays a key role in regulating the physical properties of the extracellular matrix (ECM) through turnover, which, for example, affects branching morphogenesis in the mammary gland (Wise-man et al., 2003; Green & Lund, 2005; Lu et al., 2011). Another critical role of extracellular proteolysis lies in the processing of growth factor pro-forms (e.g., pro-HGF; Herter et al., 2005, pro-MSP Ganesan et al., 2011, and EGF Higashiyama et al., 2011). Therefore, extracellular proteolysis orchestrates the interaction between ECM remodeling and growth factor signaling during development and tissue regeneration (Mohammed & Khokha, 2005; Fukushima et al., 2018). Most studies on proteolytic regulation of the ECM have focused on the diverse class of matrix metalloproteinases (MMPs) (Chang & Werb, 2001). Interestingly, in addition to MMPs, serine proteases have been demonstrated to be involved in pericellular proteolysis of ECM components and plasma membrane proteins, allowing localized ECM remodeling and receptor signaling regulation (Del Rosso et al., 2002).

Hepsin is a protease belonging to the type II transmembrane serine protease (TTSP) family, a special group of membrane-anchored proteases whose enzymatic activity is confined to the pericellular space (Hooper et al., 2001), making TTSPs accessible for function-blocking antibodies and small molecule inhibitors (Antalis et al., 2010). Hepsin knockout mice display defects in inner ear development and have altered kidney function (Guipponi et al., 2007; Olingr et al., 2019). A recent study reported reduced liver size and browning of fat tissue in hepsin knockout mice, leading to a...
reduction of body fat in mice on a high-fat diet (Li et al., 2020). Apart from a role for hepsin-mediated cleavage of pro-HGF in the liver (Hsu et al., 2012) and laminin-332 during invasion (Klezovitch et al., 2004; Tripathi et al., 2008; Pant et al., 2018a), the molecular mechanisms underlying other physiological functions of hepsin remain poorly defined.

Hepsin is one of the most frequently overexpressed proteins in prostate cancer (Dhanasekaran et al., 2001; Luo et al., 2001; Magee et al., 2001; Stamey et al., 2001; Welsh et al., 2001; Ernst et al., 2002; Chen et al., 2003; Stephan et al., 2004). Hepsin is also very frequently overexpressed in breast cancer, in up to 70% of breast tumors (Tervonen et al., 2016). Hepsin overexpression is suggested to promote cancer progression and metastasis (Tanimoto et al., 1997; Klezovitch et al., 2004; Tervonen et al., 2016), and several hepsin-regulated oncogenic pathways have been described. For example, overactive hepsin damages epithelial integrity and promotes degradation of the basement membrane (Klezovitch et al., 2004; Tripathi et al., 2008; Partanen et al., 2012; Tervonen et al., 2016), and hepsin directly cleaves oncogenic growth factors pro-HGF and pro-MSP (Herter et al., 2005; Ganesan et al., 2011). However, given that most proteases have a broad substrate range, additional signaling pathways downstream of hepsin probably exist that contribute to its oncogenic properties.

Here, we demonstrate that hepsin controls TGFβ signaling via proteolytic regulation of fibronectin. The findings are corroborated in vivo by using a new hepsin CRISPR knockout mouse model, alongside an established model of hepsin overexpression in breast cancer.

Results

CRISPR/Cas9-generated Hpn knockout mice manifest diminished liver size and partial loss of hearing

We generated hepsin knockout mice with CRISPR/Cas9-mediated gene editing using a guide RNA targeting exon 4 of the Hpn gene. Genetic analysis revealed a 50 bp frameshift deletion in the coding region of the Hpn gene (Fig 1A), which resulted in the loss of the hepsin protein in mice homozygous for the frameshift allele (Figs 1A and EV1A). Hpn knockout mice did not display any obvious histopathological changes, gross morphological, or tissue architecture deviations, nor did they show any differences in external appearance, lean mass, free fluid, or body fat (Fig EV1B–D). Consistent with Hpn knockout mice created via traditional homologous recombination methods (Wu et al., 1998; Li et al., 2020), our CRISPR hepsin knockout (Hpn−/−) mice displayed diminished liver size and reduced hearing (Figs 1B and C, and EV1E).

Hpn−/− mice are deficient in TGFβ signaling in the mammary gland and show increased ductal branching

Hepsin is expressed in human and mouse epithelial tissues, where it typically localizes to desmosomal and hemidesmosomal junctions (Miao et al., 2008). Moreover, in the mammary gland, oncogenic deregulation of hepsin has been coupled to loss of epithelial integrity (Partanen et al., 2012; Tervonen et al., 2016). These findings prompted us to examine whether the loss of hepsin might affect signaling pathways relevant to cohesiveness or morphogenesis of the mammary epithelial tissue. Whole mammary glands were isolated from wild-type and Hpn−/− mice and analyzed in parallel by RNAseq and Reverse Phase Protein Array (RPRA). The RPRA analysis revealed Hpn knockout-specific changes in the levels of 12 proteins, including osteopontin, peristin, and endostatin (Fig EV2A and B). RNAseq analysis demonstrated that while the expression levels of mRNAs encoding 2 of these proteins were also altered, expression changes in other 10 proteins were not accompanied by corresponding changes in mRNA expression, suggesting that hepsin regulates proteomes via both transcription-associated and post-translational/proteolytic mechanisms (Fig EV2A–D).

Gene Set Enrichment Analysis (GSEA) was performed on the RNAseq data. Functional clustering of gene signatures affected by the absence of hepsin suggests the downregulation of TGFβ1 signaling in Hpn−/− mammary glands (Fig 1D and E, Table EV1). TGFβ signaling regulation occurs via two discrete activation steps: the processing of latent-TGFβ in the extracellular compartment and TGFβ receptor-induced activation of downstream signaling, which includes phosphorylation of receptor-associated Smads (R-Smads), such as Smad2 and Smad3 (Derynick & Budi, 2019). To confirm the downmodulation of TGFβ signaling in Hpn−/− mice, we determined...
the levels of phospho-Smad2/3 in tissue extracts from mammary, lung, kidney, liver, skin, and spleen. The level of phosphorylated Smad2/3 was significantly decreased in Hpn+/− mammary tissue (Fig 1F), indicating deficient canonical TGFβ signaling in the mammary gland upon loss of hepsin. Reduced phosphorylated Smad2/3 levels were not observed in the other tissues examined (Fig 1F), suggesting that this phenomenon is specific to the mammary gland.

TGFβ is a well-established suppressor of mammary duct branching and proliferation (Ewan et al., 2002; Ingman & Robertson, 2008; Moses & Barcellos-Hoff, 2011). Analysis of mammary gland whole mounts from 6-week-old wild-type and Hpn+/− littermates showed
that loss of hepsin increased duct branching into the fat pad (Fig 1G). This phenotype resembles the one observed in TGFβ1−/− mice (Ewan et al., 2002; Ingman & Robertson, 2008), thus showing that loss of hepsin affects mammary morphogenesis in a manner consistent with reduced TGFβ signaling.

**Hepsin overexpression activates TGFβ signaling in a Wap-Myc model of breast cancer**

The TGFβ pathway is commonly dysregulated in human cancer but its impact on tumorigenesis is highly contextual—while TGFβ has tumor-suppressive effects, it also exerts pro-tumorigenic effects by modulating processes such as cell invasion, production of ECM, and inflammatory immune responses (Yeung et al., 2013; Bellomo et al., 2016; Mariathasan et al., 2018; Tauriello et al., 2018). To test whether ectopic overexpression of hepsin induces TGFβ signaling in the context of tumorigenesis, we made use of a previously published tumor syngraft model of Myc-driven breast cancer (Partanen et al., 2012; Utz et al., 2020). Wap-Myc mammary tumor cells, which express high Myc levels, were isolated from donor mice, transduced with the pN2D1-HPN lentiviral construct that allows doxycycline (DOX)-induced hepsin overexpression (Tervonen et al., 2016), and subsequently transplanted into recipient mice (Fig 2A). Consistent with the notion that hepsin promotes TGFβ signaling, Western blot analysis revealed increased phospho-Smad2/3 and upregulation of the TGFβ signaling downstream target SNAIL in Wap-Myc tumors with DOX-induced hepsin overexpression compared with control (DOX−) tumors (Fig 2B). RNAsq analysis of these tumors provided additional evidence for TGFβ pathway upregulation by hepsin as the most significantly upregulated gene signatures corresponded to the TGFβ signaling pathway (Figs 2C, D, and E, and EV3A and B). The largest cluster of gene signatures affected by hepsin overexpression, however, was related to the ECM and integrins (Fig 2C).

**Hepsin regulates the extracellular activation of TGFβ signaling**

Pro-TGFβ protein, which consists of both the mature TGFβ and latency-associated peptide (LAP), is released from cells in a latent form. This so-called TGFβ small latent complex (SLC) consists of the receptor-binding TGFβ growth factor dimer (12 kDa Western blot band under reducing conditions) and a dimer of the latency-associated peptide (LAP) (40 kDa Western blot band under reducing conditions) (Miyazono et al., 1988), where LAP inhibits the TGFβ growth factor dimer from binding and activating TGFβ receptors. The latent-TGFβ SLC is stored in the ECM through a covalent bond between LAP and latent-TGFβ-binding protein (LTBP), forming the large latent complex (LLC), which binds to fibronectin and fibrillins (Fig 3A) schematically depicts the TGFβ-LAP-LTBP complex). TGFβ can be released from LAP-LTBP, and thus from ECM storage, and activated by multiple types of stimuli, such as reactive oxygen species, proteases, or an acidic environment (Lyons et al., 1988, 1990; Sato & Rifkin, 1989; Yu & Stamenkovic, 2000; Jenkins, 2008; Soumi et al., 2010; Liu & Desai, 2015). Active TGFβ then mediates the tetramerization of type II TGFβ receptor (TGFβR2) and type I TGFβ receptor (TGFβR1), leading to phosphorylation of downstream receptor-associated R-Smads. Subsequently, phosphorylated R-Smads form oligomeric complexes with other Smads and translocate to the nucleus to regulate the transcription of target genes (Derynck & Budi, 2019).

Interestingly, further comparison of protein lysates from Wt and Hpn−/− mammary glands exposed markedly increased levels of LAP in Hpn−/− mammary glands, indicating accumulation of latent-TGFβ (Fig 3B). TGFβ1 mRNA levels were not altered by the loss of hepsin (Fig EV3C), suggesting that these effects are due to post-transcriptional events. To find further evidence for accumulation of latent-TGFβ, we syngrafted Wt Wap-Myc tumor cells into the fat pads of either Wt or Hpn−/− syngeneic hosts (Fig 3C) and measured phospho-Smad2/3 levels in tumor lysates. In this experiment, we observed increased phospho-Smad2/3 levels in tumors grown in Hpn−/− mice (Fig 3D), consistent with a model where latent-TGFβ1 accumulates in ECM stores in mammary glands in the absence of hepsin and processed TGFβ1 is liberated by tumor cells, leading to activation of the TGFβ1 pathway. While these results leave room for several other interpretations, at this stage they prompted us to investigate two possible lines of inquiry: (i) Hepsin or one or more proteases regulated by hepsin could directly cleave LAP, which releases active TGFβ from SLC, or (ii) hepsin could be needed for the release of latent-TGFβ from ECM stores, after which hepsin-independent processes convert latent-TGFβ into its active form.

To address whether hepsin directly or indirectly contributes to proteolytic processing of latent-TGFβ1 LAP in cells, we performed several assays using the MCF10A-pN2D20-HPN cell line (Tervonen et al., 2016). We established that DOX-induced ectopic hepsin expression enhances Smad2/3 phosphorylation in this cell line (Fig 3E), thus demonstrating its suitability to model hepsin-mediated regulation of TGFβ1 signaling. Importantly, hepsin-induced phosphorylation of Smad2/3 was rescued by the TGFβ1 inhibitor galunisertib, confirming that the hepsin-induced changes in pSmad2/3 levels were dependent on TGFβ receptor signaling (Fig 3E). First, we tested whether hepsin overexpression increases proteolytic processing of the inhibitory LAP peptide by incubating MCF10A-pN2D20-HPN cells, with (DOX+) and without (DOX−) hepsin overexpression, with purified latent-TGFβ1 (LAP-TGFβ; SLC) for 24 h before harvesting conditioned medium and cell extracts for Western blotting (Fig 3F). No cleavage of the full-length LAP peptide was observed upon hepsin overexpression, indicating that hepsin does not affect proteolytic processing of the LAP peptide. Secondly, we examined whether adding exogenous latent-TGFβ1 further potentiates hepsin-induced PAI-1 induction in MCF10A-pN2D20-HPN cells (Fig EV3D) and E). While hepsin overexpression and treatment with exogenous latent-TGFβ each enhanced TGFβ receptor signaling (PAI-1 expression was used as a readout (Cichon et al., 2016)), the combination of hepsin overexpression and latent-TGFβ did not have an additive effect (Fig EV3D), further suggesting that hepsin does not stimulate extracellular cleavage of TGFβ1 LAP. Thirdly, using a TGFβ ELISA assay (Brown et al., 1990), we measured active and total TGFβ levels in conditioned medium from TGFβ1-overexpressing MCF10A-pN2D20-HPN cells with (DOX+) or without (DOX−) hepsin overexpression (Fig 3G). While this TGFβ ELISA assay only detects active TGFβ1 (Brown et al., 1990), we utilized heat treatment of the conditioned medium to activate all TGFβ1 and thus to effectively measure total TGFβ1 levels. If hepsin would directly or indirectly promote cleavage of the LAP peptide, we expect hepsin overexpression to increase active TGFβ1 levels in
the conditioned medium, i.e., released from the insoluble ECM. We did not observe such an increase in active TGFβ1 levels (Fig 3G; first 2 columns), however, providing additional evidence that hepsin does not affect cleavage of TGFβ1 LAP. We did, however, observe a modest but significant increase in total TGFβ (after heat activation of TGFβ) in the conditioned medium of hepsin overexpressing cells (Fig 3G; last 2 columns), which is consistent with the second hypothesis mentioned above, i.e., that hepsin could be needed for the release of latent-TGFβ from ECM stores. Lastly, we performed an in vitro protease assay, which showed that recombinant hepsin is unable to proteolytically process recombinant latent-TGF-β1 LAP (Fig 3H), suggesting that hepsin does not directly cleave LAP.

Together, the experiments in Figs 3B, E–H, and EV3D strongly suggest that hepsin causes accumulation of latent-TGFβ1 and promotes TGFβ signaling via mechanisms other than direct proteolytic cleavage of latent-TGFβ.

Hepsin downregulates the levels of ECM protein fibronectin

To identify hepsin-regulated ECM proteins, we examined both lysates and culture supernatants collected from MCF10A-pND20-HPN cells with or without hepsin overexpression. The lysates and supernatants were separated on SDS–PAGE under reducing or non-reducing conditions followed by Coomassie staining, which revealed eight protein bands visibly affected by hepsin overexpression (Fig 4A). These proteins were analyzed by mass spectrometry, identifying fibronectin (FN1) as one of the proteins showing reduced expression in culture supernatant upon hepsin overexpression (Fig 4A). These proteins were analyzed by mass spectrometry, identifying fibronectin (FN1) as one of the proteins showing reduced expression in culture supernatant upon hepsin overexpression (Fig 4A). Hepsin-dependent downmodulation of fibronectin was of great interest, given the well-established role for fibronectin in the storage of TGFβ family growth factors, and thus in TGFβ signaling (Dallas et al., 2005; Robertson et al., 2015). Immunoblotting of the cell culture supernatant from the same cells showed accumulation...
of lower MW fibronectin fragments upon hepsin overexpression, which could indicate that reduced fibronectin levels in the cell extracts are due to fibronectin degradation (Fig 4B). Immunoblot for fibronectin in lysates of MCF10A-pIND20-HPN cells with or without hepsin overexpression confirmed the reduction in full-length fibronectin levels upon hepsin overexpression (Fig 4C).

To determine whether fibronectin could be a target for direct proteolytic processing by hepsin, we performed in vitro cleavage assays with purified proteins. This experiment showed that hepsin can cleave full-length fibronectin in vitro (Fig 4D, upper panel). Using different N-terminal and C-terminal fragments, we mapped the hepsin cleavage site to the N-terminal 70 kDa part of fibronectin A B C D E F G H

Figure 3. Hepsin is a regulator of TGFβ storage.
A Schematic illustration of proteins involved in TGFβ storage in the extracellular matrix. The small latent complex (SLC) is a non-covalently linked tetramer of 2 LAP (latency-associated peptide) (40 kDa) and 2 TGFβ (12 kDa). LTBP1 + SLC together form the large latent complex (LLC) through covalent bonds between LAP and LTBP1. LLC interacts with the ECM through non-covalent interactions of LTBP with fibrillins and fibronectin.
B Immunoblot analysis of LAP in mammary whole tissue lysates from Wt and Hpn−/− mouse. The 90 kDa band represents unprocessed pro-TGFβ, and the 40 kDa band corresponds to mature TGFβ LAP peptide.
C Schematic representation of the mouse experiment, showing orthotopic transplantation of Wt-Wap-Myc tumor cells into syngeneic Wt and Hpn−/− recipients.
D Immunoblot analysis of phospho-Smad2/3, total Smad2/3, and GAPDH (loading control) expression in Wt tumors transplanted into either Wt or Hpn−/− recipients (N = 10 tumors each).
E Western blot analysis of phospho-Smad2/3, total Smad2/3, hepsin, and β-tubulin (loading control) in MCF10A-pIND20-HPN cells treated with 1 µg/ml doxycycline for 48 hours (DOX; overexpression of hepsin) and the TGFβR1 inhibitor galunisertib (TGFβR1i, 10 µM) as indicated.
F Immunoblot analysis of conditioned medium (LAP peptide) and cell extracts (hepsin and vinculin) from control (DOX) or hepsin overexpressing (DOX+ MCF10A-pIND20-HPN supplemented with 100 ng/ml latent-TGFβ1 (small latent complex). Ponceau was used as a loading control for the cell culture medium.
G TGFβ1 ELISA assay to detect active and total TGFβ levels in conditioned medium from MCF10A-pIND20-HPN pL6-TGFβ1 cells with (DOX+) or without (DOX−) hepsin overexpressing. The TGFβ1 ELISA assay detects active TGFβ levels (first 2 columns), but heat treatment of the conditioned medium activates all TGFβ, thus effectively measuring total TGFβ (right two columns). Data are presented as scatter plot (mean denoted by the black line), and paired Student’s t-test was used for statistical analyses.
H Silver-stained protein gel with samples from an in vitro protease activity assay with recombinant SLC and hepsin. SLC was incubated with increasing concentrations of recombinant hepsin (numbers indicate the concentration of hepsin in nM). Western blots in (B, E, F) are representative of at least three repeats.
(Figs 4D, lower panel, and EV3F–J). While the in vitro cleavage assays exposed only one direct hepsin cleavage site in fibronectin, we note that hepsin overexpression induced a smear-like pattern of extracellular fibronectin cleavage products in MCF10A-plND20-HPN cell culture supernatant (Fig 4B). Thus, it is likely that other proteases downstream of hepsin also contributed to hepsin-dependent fibronectin degradation.

Next, we investigated whether hepsin also downmodulates fibronectin in vivo. To that end, we analyzed a panel of tissue samples from our wild-type (Wt) and Hpn−/− mice. The results showed that Hpn−/− mammary glands, and also skin, expressed more fibronectin protein than mammary glands from Wt littermates (Fig 5A). Similar effects were not observed in other examined tissues (Fig 5A). Importantly, the increased fibronectin protein abundance in the Hpn−/− mammary glands was not accompanied by increased Fn1 mRNA expression (Fig EV3K). An inverse correlation between fibronectin and hepsin was also observed in mammary glands from another hepsin knockout mouse strain (Wu et al., 1998) (Fig 5B) and in prostate tissue from mice genetically engineered to overexpress hepsin specifically in the prostate gland (Klezovitch et al., 2004) (Fig 5C).

Together, our findings demonstrate that hepsin downregulates fibronectin protein levels in cultured cells and in vivo.

**Hepsin regulates TGFβ1 signaling via fibronectin**

If our hypothesis that hepsin activates TGFβ signaling through degradation of fibronectin is correct, downregulation of fibronectin should be sufficient to increase TGFβ signaling. We assessed this using four different experiments. We silenced fibronectin in MCF10A-plND20-HPN cells and assessed TGFβ signaling using Western blot for pSmad2/3. Consistent with our hypothesis, downregulation of fibronectin levels in these cells was sufficient to increase pSmad2/3 levels (Figs 6A and EV4A shows uncut immunoblot). In an MCF10A cell line overexpressing TGFβ-V5, silencing of fibronectin released the fully processed V5-tagged TGFβ (12 kDa) into the culture medium and also upregulated Smad2/3 phosphorylation (Figs 6B and EV4B shows uncut immunoblot). In addition, we performed a TGFβ1 reporter assay for which we created the MCF10A-plND20-HPN pL6-TGFβ1 (SRE) TGFβ1 reporter cell line, harboring a construct with luciferase under the control of a Smad-response element (SRE). In line with our hypothesis that fibronectin modulates TGFβ signaling, silencing fibronectin, as well as induction of hepsin expression, in this cell line increased TGFβ1 reporter activity (Figs 6C and EV4D). Lastly, we verified that knockdown of fibronectin leads to TGFβ release into the culture supernatant using a breast cancer cell line (Hs578T) that expresses high levels of fibronectin (Wu et al., 1998) (Fig 6C) and DOX-induced hepsin expression (Fig 6F). Similar to the experiments with silencing FN1 (Fig 6B) or DOX-induced hepsin expression (Fig 6E), this 0.01 ng/ml of TGFβ1 induced robust induction of pSMAD2/3 levels in this cell line (Fig EV4F), thus providing evidence that the changes in SRE reporter activity and pSMAD2/3 levels are in concordance.

Together, our results demonstrate that hepsin-mediated TGFβ signaling depends on fibronectin, consistent with a role for hepsin in fibronectin degradation and subsequent activation of latent-TGFβ (see a schematic model in Fig 6G).

**Discussion**

In this study, we identified the protease hepsin as a promoter of TGFβ signaling in the mammary gland and mammary tumors. We provide evidence that hepsin promotes TGFβ signaling through downregulation of fibronectin levels and subsequent release of latent-TGFβ from the ECM storage compartment.

The oncogenic role of hepsin overexpression has been well-established (Klezovitch et al., 2004; Tervonen et al., 2016), but the mechanisms through which hepsin promotes initiation and progression of tumors are still largely unclear. The especially frequent hepsin overexpression in breast cancer (up to 70% (Tervonen et al., 2016)) warrants further studies of the role of hepsin in mouse models of breast cancer. To facilitate such studies, we generated a new hepsin knockout mouse in the inbred FVB/N strain using CRISPR/Cas9 gene editing, since many common mouse models of breast cancer designed to study multistage carcinogenesis, such as MMTV-PyT (directs polyomavirus middle T antigen to mammary tissue), C3(1)-Tag (drives SV40 large T antigen to prostate and mammary tissue), and Wap-Myc (directs Myc to luminal mammary epithelial cells), have been generated in this strain. We anticipate that these Hpn−/− mice will facilitate future studies aimed to clarify the role of hepsin in multistage tumorigenesis through syngraft approaches, as presented here (Fig 3C).

The FVB/N Hpn−/− mice displayed hearing loss and reduced liver size, consistent with the phenotypes reported for earlier hepsin knockout mouse models (Guipponi et al., 2007; Li et al., 2020), demonstrating the validity of our knockout model. In addition to these phenotypes, we observed increased epithelial branching in Hpn−/− mammary glands. This novel mammary gland phenotype resembles the phenotype reported for Tgfb1−/− females, which show a branching phenotype at the age of 6 weeks (Ewan et al., 2002; Ingman & Robertson, 2008). Tgfb1−/− mice develop multiple severe phenotypes, such as neurodegeneration, developmental defects, and autoimmunity (Shull et al., 1992; Kulkarni et al., 1993; Dickson et al., 1995; Proetzel et al., 1995; Sanford et al., 1997; Brionne et al., 2003). The absence of these phenotypes in Hpn−/− knockout mice shows that hepsin is not required for overall TGFβ function in vivo but it is
Figure 4. Hepsin promotes the proteolytic processing of fibronectin.

A Coomassie-stained protein gels with cell lysates and concentrated culture supernatants of MCF10A-pIND20-HPN cells, with (DOX+) or without (DOX−) hepsin overexpression. M indicates the media only control. R and NR above the gels indicate reducing and non-reducing conditions, respectively. Numbered arrowheads indicate areas of the gel that were analyzed by mass spectrometry; corresponding proteins differently expressed in hepsin overexpressing cells are listed on the left. The image with two lanes on the right is a copy of the indicated area with supernatant under reducing conditions, highlighting the part from which fibronectin was identified. Red boxes indicate the lanes that were analyzed by mass spectrometry.

B Immunoblot analyses of fibronectin expression in concentrated culture supernatant from MCF10A-pIND20-HPN cells with (DOX+) or without (DOX−) hepsin overexpression. Ponceau staining of the Western blot is shown as the loading control.

C Immunoblot analysis of fibronectin, hepsin, and β-tubulin (loading control) in cell lysates from MCF10A-pIND20-HPN cells with (DOX+) or without (DOX−) hepsin overexpression. The graph shows the quantification of fibronectin levels. Student’s t-test was used for statistical analyses. Data are represented as mean ± SD.

D Silver-stained protein gel with samples from an in vitro protease activity assay with recombinant hepsin and either full length purified plasma fibronectin (upper panel) or the 70 kDa most N-terminal fragment of fibronectin (lower panel). Full-length fibronectin (1 μg) or the 70 kDa fibronectin fragment (1 μg) was incubated with increasing concentrations of recombinant hepsin. Arrowheads indicate full-length fibronectin, the 70 kDa fragment, the 50 kDa cleavage fragment generated by hepsin, and hepsin. The schematic figure shows the full-length fibronectin protein and the 70 kDa N-terminal fragment. The red arrow indicates the location of the putative hepsin cleavage site. RGD indicates the RGD-binding domain in fibronectin. Experiments in (B, C, D) are representative of at least three repeats.

Source data are available online for this figure.
plausible that hepsin is either a tissue-specific regulator of TGFβ function or that other proteases compensate the loss of hepsin outside the mammary gland. This selective role of hepsin in the regulation of extracellular TGFβ signaling opens up possibilities to modulate the TGFβ pathway in a mammary tissue-selective manner with the existing neutralizing antibodies or small molecule inhibitors of hepsin (Ganesan et al., 2012; Koschubs et al., 2012; Pant et al., 2018b; Damalanka et al., 2019).

TGFβ signaling is regulated by the ECM, in which three components are directly involved in the storage of latent-TGFβ: LTBP, fibrillin, and fibronectin (ten Dijke & Arthur, 2007). Interestingly, some human conditions caused by dysregulation of TGFβ signaling are linked either to genetic alterations of TGFβ-binding fibrillins, or proteolytic alterations of LTBP, indicating that release of the growth factor complex from the ECM may regulate pathway activation and provide a niche for a tissue-specific regulatory layer (Nepont et al., 2003; Ge & Greenspan, 2006; Quarto et al., 2012; Beaufort et al., 2014).

One important mechanism to activate latent-TGFβ is mediated by integrins (Robertson & Rifkin, 2016), which can bind LAP/LTBP and activate TGFβ by either pulling latent-TGFβ apart through mechanical forces generated by the actin cytoskeleton that are transmitted to the fibronectin-containing ECM by the integrins or presenting latent-TGFβ to metalloproteases for proteolytic cleavage (Fontana et al., 2005; Mamuya & Duncan, 2012). In addition to integrin-mediated TGFβ activation, various integrin-independent TGFβ activation pathways are known, for example, through reactive oxygen species, pH, and proteolytic cleavage (Robertson & Rifkin, 2016). Our data identify hepsin as a novel activator of TGFβ signaling that mediates the release of latent-TGFβ from the ECM (Fig 2A–G) through degradation of fibronectin (Fig 6A–F) and does not activate TGFβ signaling through direct or indirect cleavage of LAP (Fig 3F–H). In biological settings, the activation of latent-TGFβ is likely to occur through an interplay of different mechanisms, for example, depending on the tissue, physiological, or pathophysiological context.

The most established function of fibronectin is to mediate the interaction between cells and various ECM components, therefore acting as a “glue” and a structural component (Mouw et al., 2014). The N-terminal part of fibronectin, for example, directly interacts with fibrillins, which is critical for the maturation of ECM fibers and incorporation of latent-TGFβ into ECM stores (Dallas et al., 2005). Therefore, cleavage of fibronectin, especially the N-terminus, which we demonstrate is directly cleaved by hepsin in vitro, may affect ECM storage of TGFβ and thus TGFβ signaling. It is likely, however, that other proteases also play a role in hepsin-mediated TGFβ signaling in vivo as hepsin can activate other proteases, such as MMPs (Reid et al., 2017; Wilkinson et al., 2017) and serine proteases (Andreassen et al., 1997; Moran et al., 2006; Reid et al., 2017), reported to regulate the cleavage of fibronectin (Moran et al., 2006; Borgoño et al., 2007; Doucet & Overall, 2011; Zhang et al., 2012). Such proteolytic cascades may result, for example, from hepsin-induced downregulation of the general serine protease inhibitor HAI-1 (Tervonen et al., 2016). Studies with other proteases capable of cleaving fibronectin have demonstrated that accumulation of fibronectin may lead to alterations of collagen fibers in vivo and thus changes in the overall ECM structure (Taylor et al., 2015). Whether changes in fibronectin levels, due to the deregulation of hepsin, result in gross structural changes in overall ECM architecture remains to be investigated.

**Figure 5.** Hepsin reduces fibronectin levels in vivo.

A Immunoblot analysis of fibronectin in indicated tissues isolated from Wt, Hpn+/− and Hpn−/− mouse (representative of at least three repeats). *As the same tissue lysates were used as in Fig 1F, the same GAPDH blot is used here as the loading control.

B Immunoblot analysis of fibronectin and β-tubulin (loading control) in lysates from Wt and Hpn−/− mouse mammary tissue (N = 5 animals each) from another hepsin knockout mouse model (Wu et al., 1998).

C Immunoblot analysis of fibronectin, hepsin, and β-tubulin (loading control) in prostate lysates of control mice or mice with prostate-specific hepsin overexpression (Klezovitch et al., 2004). N = 2 mice each, the two prostate lobes were run separately (A and B).
Figure 6. Hepsin-mediated TGFβ1 signaling through the downregulation of fibronectin.

A Immunoblot analysis of fibronectin, pSmad2/3, total Smad2/3, and GAPDH (loading control) in cell lysates from MCF10A-pIND20-HPN cells without hepsin overexpression. The graph depicts quantification of pSmad2/3 normalized to total Smad2/3 levels, compared to siCtrl (N = 3 biological repeats).

B Immunoblot analysis of cell lysates (pSmad2/3, total Smad2/3, and vinculin as loading control) and concentrated cell culture supernatant (anti-V5 for TGFβ1) from MCF10A-pIND20-HPN pL6-TGFβ1-V5 cells without hepsin overexpression. Ponceau staining is shown as a loading control for the Western blot with concentrated culture supernatant. The graph depicts quantification of pSmad2/3 normalized to total Smad2/3 levels, compared to siCtrl (N = 3 biological repeats).

C TGFβ1 luciferase reporter assay of control or fibronectin silenced MCF10A-pIND20-HPN pL6-TGF β1 reporter cells with (DOX+) or without (DOX−) hepsin overexpression (N = 7 biological repeats; Y-axis shows fold change in relative light units (RLU)).

D Immunoblot analysis of cell lysates (fibronectin and β-tubulin (loading control)) and concentrated cell culture supernatant (TGFβ) from Hs578T cells with knockdown of fibronectin. Ponceau staining is shown as the loading control for the Western blot with concentrated culture supernatant. A representative blot from three biological repeats is shown.

E Immunoblot analysis of fibronectin, pSmad2/3, total Smad2/3, hepsin, and GAPDH (loading control) in cell lysates from MCF10A-pIND20-HPN cells with (DOX+) and without (DOX−) hepsin overexpression, and with or without fibronectin silencing (siFN1). The graph depicts quantification of pSmad2/3 normalized to total Smad2/3 levels (N = 3 biological repeats).

F TGFβ1 luciferase reporter assay of control or fibronectin silenced MCF10A-pIND20-HPN pL6-TGFβ1 reporter cells, with (DOX+) and without (DOX−) hepsin overexpression. (N = 6 biological repeats; Y-axis shows fold change in relative light units (RLU)).

G Model figure depicting how hepsin regulates TGFβ signaling. Under Wt conditions, hepsin promotes degradation of fibronectin, which releases latent-TGFβ from ECM stores, thus resulting in the induction of TGFβ signaling. In hepsin knockout mammary glands, TGFβ signaling is compromised as latent-TGFβ cannot be released from ECM stores (Created with BioRender.com). Statistical analyses in (A, B, C, E, F) were done using Student’s t-test. Data in (A, B, C, E, F) are presented as dot plots where black lines represent the mean.

Source data are available online for this figure.
In conclusion, we show that hepsin mediates the release of latent-TGFβ from the ECM storage specifically in the mammary tissue, which leads to increased TGFβ signaling. This suggests a novel role for hepsin as a mediator between ECM proteolysis and TGFβ growth factor signaling.

Materials and Methods

Reagents

Table 1 lists purified proteins used in this study.

| Protein                                           | Company | Catalog |
|---------------------------------------------------|---------|---------|
| Purified plasma fibronectin                        | Merck   | F0895   |
| Fibronectin proteolytic fragment from human plasma, 70 kDa | Merck   | F0287   |
| Fibronectin proteolytic fragment from human plasma, 30 kDa | Merck   | F9911   |
| Fibronectin 40 kDa α chymotryptic fragment (heparin-binding region) | Merck   | F1903   |
| Fibronectin proteolytic fragment from human plasma, 45 kDa | Merck   | F0162   |
| Fibronectin 120 kDa α chymotryptic fragment (cell attachment region) | Merck   | F1904   |
| hLatent-TGFβ1                                      | CST     | #5154   |
| Recombinant human hepsin protein, CF               | R&D     | 4776-SE |

Cell lines

All cell lines were regularly tested for mycoplasma contamination. The SUM52PE cell line was a generous donation from Dr. SJ Cook and Dr. PR Gavine (Chell et al., 2013) and was cultured in Hams F12 (Invitrogen), insulin 5 µg/ml, hydrocortisone 1 µg/ml, L-glutamine, and antibiotics. All other cell lines were obtained from American Type Culture Collection (ATCC). HMEC-hTERT cell media was MCDB 170 (US Biological) supplemented with insulin 5 µg/ml, bovine pituitary extract (BPE) 70 µg/ml, hydrocortisone 0.5 µl/ml, EGF 5 ng/ml, human transferrin 5 µg/ml, and isoproterenol 0.01 µM and 5% FBS (Biowest). Cell lines MCF7, T47D, MCF10A, and Hs578T were cultured as described in Tervonen et al., 2016. MCF12A was cultured in DMEM/F12 (Invitrogen) supplemented with 10% FBS (Biowest) and 5% FBS (Lonza) with 10% FBS (Lonza) with 10% FBS (Biowest). MDA-MB-436 and MDA-MB-453 were cultured in Medium (Lonza) with 10% FBS (Biowest).

siRNA knockdown

Fibronectin knockdown was performed with siRNA pool (siTOOL Biotech) and HIPerFect (Qiagen) transfection reagent, with 100 nM siRNA final concentration. Hs578T cells were switched to serum-free media after transfection, for supernatant analysis. Supernatants were concentrated as described elsewhere (see Mass Spectrometry).

Virus transduction, TGFβ reporter cell lines

Cell line transduction was carried out by incubating plated cells with virus-containing supernatant O/N in the presence of 8 µg/ml of polybrene (MOI = 1). We generated the TGFβ reporter cell lines by transducing MCF10A cells with SRE construct harboring lentivirus particles (MOI=10). Reporter construct virus particles were obtained commercially (Keralast #FCT228). The luciferase signal was detected by using Pierce™ Firefly Luciferase Glow Assay Kit (Thermo Scientific, PI16177) according to the manufacturer’s instructions. Readings were obtained with FLUOstar OMEGA multiplate reader (BMG LabTech).

Cloning

The pLenti6-TGFβ1-V5 overexpression construct was generated via Gateway cloning from the ORFeome library by the Genome Biology Unit supported by HiLIFE and the Faculty of Medicine, University of Helsinki, and Biocenter Finland.

Tissue, organ weight, and body composition analysis

Carmine alum staining (Sigma, #C1022-5G) of whole mammary glands was performed in inguinal mammary glands from 6-week-old females were spread on glass slips and fixed with Kahle’s fixative (Partanen et al., 2019) O/N at room temperature followed by one wash with 70% ethanol and gradual change to Milli-Q water. Glands were stained with carmine alum O/N, gradually transferred into 100% ethanol, and cleared in xylene. Upon clearing glands were transferred into 100% ethanol, followed by 100% glycerol. The branching was quantitated using ImageJ 1.46r.

H&E staining was performed as described previously (Partanen et al., 2012).

Body composition for liquid, fat, and lean mass percentage and organ weight was measured in 6-week-old mice with Bruker minispec LF50 Body Composition Analyzer (Bruker). Statistical significance was derived from a one-sided t-test. All the groups had at least N = 3.

RNASeq

RNA was extracted from up to 6-week-old mouse mammary glands using hard-tissue beads (Precellys) and TRizol (Thermo Fisher Scientific). RNA was then DNase treated and purified using RNeasy Mini Kit from Qiagen. Before the library preparation for sequencing, 1 µg of total RNA was treated with NEBNext rRNA Depletion Kit (Human/Mouse/Rat) that removes the ribosomal RNA from the total RNA. The ribosomal depleted RNA was purified with RNeasy mini Elute columns (Qiagen). The absence of rRNA and the quantity of mRNA were measured with Agilent TapeStation 4200. Samples
were sequenced with Illumina NextSeq500 (Illumina, San Diego, CA, USA) at the Biomedicum Functional Genomics Unit, University of Helsinki, using NEBNext Ultra Directional RNA Library Prep Kit. The sequencing was performed as single-end sequencing for read length 75 bp. GSEA analysis and statistical analysis were performed in GSEA 4.0.3 software (Subramanian et al., 2005). Gene set clustering was analyzed with Cytoscape (Merico et al., 2010). Genomic PCR products were Sanger sequenced (Sanger sequencing at the Finnish Institute for Molecular Medicine, HiLIFE, University of Helsinki and Biocenter Finland) using the same reverse primer used also for genomic PCR (above).

**Proteins and hepsin cleavage**

Recombinant human hepsin was incubated at various concentrations with 1 μg/μl substrate proteins (latent-TGFβ1, fibronectin fragments) for 1 h, 37°C in PBS, separated on gradient gels and silver stained (GE Healthcare, GE17-1150-01). Silver staining was performed by the Meilahti Clinical Proteomics Core Facility.

**Antibodies and inhibitors**

The inhibitors and antibodies used in this study were Galunisertib (Selleckchem, S2230), anti-sheep HRP (Upstate cell signaling solutions, #12-342), anti-goat HRP (Millipore, AP106P), anti-rabbit HRP (Selleckchem, S2230), anti-sheep HRP (Upstate cell signaling solutions) for 1 h, 37°C in PBS, separated on gradient gels and silver stained (GE Healthcare, GE17-1150-01). Silver staining was performed by the Meilahti Clinical Proteomics Core Facility.

**Animal models and experiments**

All work and sample isolation from 11-month-old Hpn−/− (Wu et al., 1998), 6-month-old LBHepsin (Kasper et al., 1998) mice were performed at Lerner Research Institute, Cleveland Clinic, USA; Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, USA, respectively. All mice used for the experiments in Helsinki were housed in individually ventilated cages under the optimal conditions of temperature and humidity. The experiments were conducted according to 3R principles, and animal welfare was regularly monitored.

Wap-Myc mammary/tumor cell isolations were performed like previously described (Partanen et al., 2012; Tervonen et al., 2016). Experiments were approved by The National Animal Ethics Committee of Finland (ESAVI/3678/04.10.07/2016).

One day before transplantation, Wap-Myc epithelial cells were isolated and transduced with the plducer21-HPN (Tervonen et al., 2016), lentiviral construct, MOI = 10. On transplantation day, 105 cells were injected into the cleared fat pads of 3-week-old recipient mice. The experimental mice started receiving doxycycline in drinking water, supplemented with 5% sucrose, 3 days after the transplantation, the control mice received drinking water with sucrose only. Tumors were induced at week 8 by two sequential pregnancies. Mice were sacrificed when the tumor diameter reached 1 cm.

Transplantation experiment of Wt Wap-Myc tumor cells and into Wt and Hpn−/− recipients: 3-week-old Wt FVB recipient mice were obtained from Janvier Labs. Cells were isolated 1 day before transplantation from Wt Wap-Myc tumors and kept in floating culture O/N in growth media (Partanen et al., 2012). On transplantation day, viability was estimated with trypan blue, 105/gland cells were injected into cleared fat pads of 4-week-old recipient mice (2 glands/mouse). Mice were sacrificed when the tumor diameter reached 2 cm.

**Generation of Hpn knockout mice by CRISPR/Cas9 editing**

gRNAs for in vitro CRISPR/Cas9 genome editing were in vitro transcribed from mouse genome-wide arrayed lentiviral CRISPR/Cas9 gRNA libraries (Sigma-Aldrich/Merck). Sanger gRNA ID 182530, target sequence with PAM was 5’-AGGTGGCAGCTCTCAATTTG-3’ in exon 4 (exon ID: ENSMUSE00000198671). For in vitro transcription, primers were from Sigma-Aldrich/Merck and the sequence for gRNA synthesis forward primer was 5’-TAATACGACTCACTATAGAGTTGGCAGCTCTCAATTTG-3’ and reverse primer 5’-TTCTAGCTCTAAAAAGGAAGGTGCCAGCTCTCAATTTG-3’. The gRNA was produced with GeneArt™ Precision gRNA Synthesis Kit (Thermo Fischer Scientific) according to the manufacturer’s instructions. Injections into mouse zygotes were performed with the purified gRNA and Cas9 mRNA. Altogether, 209 zygotes were injected, 178 transferred into founding mothers (FVB strain), and 100 live pups were born. Mice were genotyped from ear samples taken after weaning by genomic PCR that was performed with the following primers: forward 5’-TGTCTACGAAAAAGGAAGGTGCCAGCTCTCAATTTG-3’ and reverse 5’-GGAGAACAGGCGGGTTGTAAAGGAAGGTGCCAGCTCTCAATTTG-3’ flanking the gRNA-targeting sequence (product length was 493 bp). PCR mix contained a final concentration of 2 mM dNTP mix (Bioline, London, UK), 200 nM of primers (oligos from Sigma-Aldrich/Merck), 1 mM MgCl2, 1 × Phusion HF PCR Buffer (Thermo Fischer Scientific), Phusion High-Fidelity DNA polymerase (Thermo Fischer Scientific), and 2 ng/μl DNA template. Genomic PCR products were Sanger sequenced (Biomedicum Sequencing Unit and Sequencing Unit, FIMM, HiLIFE life science research infrastructures, University of Helsinki and Biocenter Finland) using the same reverse primer used also for genomic PCR (above). We found that 15 mice (16% of live-born F0 cohort) were mutant for Hpn. We selected F0 male with 50 bp deletion in Hpn exon 4 as a founding father of the strain. This male was paired with wild-type females to obtain F1 generation. The strain was maintained as heterozygotes (no apparent phenotype), which mated to produce wild-type and Hpn−/− littermates.

**Acoustic startle reflex (ASR) test**

The test is based on Willott et al (2003). Shortly, mice were placed in a transparent plastic tube (Ø 4.5 cm, length 8 cm) that was put in the startle chamber (Med Associates) with a background white noise of 65 dB and left undisturbed for 5 min. Acoustic startle stimuli (20-ms white noise bursts) were presented in random order with 8–15 s between the subsequent trials. Altogether, 36 trials with the following noise intensities were randomly applied: 68, 72, 75, 78, 82, 86, 90, 100, and 110 dB. The startle response was recorded for 65 ms starting with the onset of the startle stimulus. The maximum startle amplitude recorded during the 65-ms sampling window was used as the dependent variable and averaged over four trials with given
stimulus intensity. The test was performed by Mouse Behavioral Phenotyping Facility (MBPF) at Neuroscience Center, Helsinki Institute of Life Science (HiLIFE), University of Helsinki and Biocenter Finland (N).

**Immunoblot, antibody array, RNA expression analysis, silver staining, and ELISA**

Immunoblots were quantitated using ImageJ. Statistical significance was determined via a one-sided t-test, unless specified otherwise in the figure legends. All lysates for immunoblot analysis were prepared by lysing cells in PBS-1% Triton X-100 buffer supplemented with Protease Inhibitor Cocktail and Phosphatase inhibitor (Roche). Thereafter, the resulting suspension was incubated on ice for 10 min, centrifuged, and the supernatant collected. Tissue lysates were made in the mentioned lysis buffer by using Precellys 24 tissue homogenizer (Bertin instruments) and the Precellys kit (#K03961-1-003.2). Proteins were separated on gradient SDS gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad #1704158). Transfer quality was evaluated with Ponceau staining (Sigma, #P7170). For antibody array experiments, the whole mammary lysates were prepared from 6-week-old virgin females according to the manufacturer’s instructions (R&D Systems) and analyzed with Mouse XL Cytokine Antibody Array Kit (RPPA). Signal intensity was quantitated with MATLAB. The proteins from LPB-hepsin prostate samples were extracted from ventral lobes in RIPA buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1% NP-40, 1% Na deoxycholate, 0.1% SDS, 1 mM EDTA, protease/phosphatase inhibitors). The active and total TGFβ ELISA was performed with Human TGF-beta 1 Quantikine ELISA Kit (R&D Systems) according to manufacturer’s instructions, except for the activation step, which was substituted with heat activation (Brown et al., 1990). In order to detect the absolute levels of TGFβ and not only the active form, we performed heat mediated activation, which liberates the active TGFβ from the small latent complex not detectable by the assay (Brown et al., 1990). Readings were obtained with FLUOstar OMEGA multplate reader (BMG Labtech) at 450 nm.

**Mass spectrometry**

Culture supernatants were concentrated with Amicon Ultra-0.5 ml 3 K Centrifugal Filters according to the manufacturer’s instructions (Millipore). Lysates and supernatants were boiled with lysis buffer with (R)/without β-mercaptoethanol (NR) and separated on gradient gels (Bio-Rad).

Gels were stained o/n RT with Coomassie stain (10% acetic acid, 50% ethanol, Coomassie Brilliant Blue-R250 in MQ), followed by destaining (10% acetic acid, 50% ethanol in MQ).

Bands that were altered by hepsin overexpression (DOX+/DOX−) were excised and analyzed by MALDI-TOF as described before (Vaara et al., 2014), and the results were plotted against the proteome of the Mammalian taxon (66651 sequences). Mass spectrometric analysis was performed by the Meilahti Clinical Proteomics Core Facility.

**Data availability**

The raw RNAseq data for the pND21-Wap-Myc tumors GSE164510 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164510), Hpm wild-type, and knockout mammary glands GSE164509 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164509) are publically available at the GEO repository.

**Expanded View** for this article is available online.

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**Author contributions**

DB conceptualized; provided resources; curated the data; involved in formal analysis; investigated; contributed to methodology; and wrote the original draft, review, and editing. SMP provided resources; curated data; validated; investigated; contributed to methodology; and wrote the original draft, review, editing, and illustrations. PM conceptualized; provided resources; curated the data; involved in formal analysis; investigated the study; contributed to methodology; and wrote the original draft. IS involved in formal analysis; visualized the study; and wrote the original draft. KB curated the data; involved in the original draft. K.B. curated the data; involved in formal analysis; investigated the study; contributed to methodology; and wrote the original draft. JE provided resources and methodology. JP conceptualized the study; involved in funding acquisition; visualized the study; contributed to methodology; and wrote the original draft. JE-H provided resources and methodology. OK, VV, SL, and QW provided resources and wrote the original draft. OM, SK, and PL provided resources, curated the data, involved in funding acquisition, and wrote the original draft. JP conceptualized the study; involved in funding acquisition; visualized the study; contributed to methodology; and wrote the original draft, review, and editing. TT conceptualized the study; provided resources; curated the data; involved in formal analysis; supervised the study; involved in funding acquisition; investigated the study; contributed to methodology; and wrote the original draft. H-AH provided resources and methodology. PM provided resources; curated the data; involved in formal analysis; supervised the study; contributed to methodology; and wrote the original draft. TR provided resources and methodology. SK, and PL provided resources, curated the data, involved in funding acquisition, and wrote the original draft. OM, SK, and PL provided resources, curated the data, involved in funding acquisition, and wrote the original draft. JP conceptualized the study; involved in funding acquisition; visualized the study; contributed to methodology; and wrote the original draft, review, and editing. JK conceptualized the study; contributed to methodology; and wrote the original draft, review, and editing. TT conceptualized the study; provided resources; curated the data; involved in formal analysis; supervised the study; involved in funding acquisition; investigated the study; contributed to methodology; and wrote the original draft. H-AH provided resources and methodology.

**Conflict of interest**

Dr. Klefström’s research projects received funding from AbbVie, Orion Pharma, and Roche/Genentech. Dr. Klefström has served as a member of a scientific advisory board or consultant to AbbVie, Astra-Zeneca, MSD, Orion Pharma, Pfizer, Roche/Genentech, and UPM Biomedicals. Dr. Pouwels has consulted for Biomedicum Genomics. Other authors declare no conflicts of interest.
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