The secreted protease Adamts18 links hormone action to activation of the mammary stem cell niche

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Estrogens and progesterone control breast development and carcinogenesis via their cognate receptors expressed in a subset of luminal cells in the mammary epithelium. How they control the extracellular matrix, important to breast physiology and tumorigenesis, remains unclear. Here we report that both hormones induce the secreted protease Adamts18 in myoepithelial cells by controlling Wnt4 expression with consequent paracrine canonical Wnt signaling activation. Adamts18 is required for stem cell activation, has multiple binding partners in the basement membrane and interacts genetically with the basal membrane-specific proteoglycan, Col18a1, pointing to the basement membrane as part of the stem cell niche. In vitro, ADAMTS18 cleaves fibronectin; in vivo, Adamts18 deletion causes increased collagen deposition during puberty, which results in impaired Hippo signaling and reduced Fgfr2 expression both of which control stem cell function. Thus, Adamts18 links luminal hormone receptor signaling to basement membrane remodeling and stem cell activation.
The breast is the only organ to develop mostly after birth. Milk ducts arborize from the nipple and grow into a specialized subcutaneous stroma called the mammary fat pad in mice. The ductal wall comprises a bi-layered epithelium with the inner luminal cells and outer myoepithelial cells. The epithelium is separated from the stroma by specialized extracellular matrix (ECM), the basement membrane (BM). The ovarian hormones, estrogens and progesterone, are key drivers of mammary gland development and also influence breast carcinogenesis. Both estrogen receptor α (ER) and progesterone receptor (PR) are members of the nuclear receptor family and are readily detected by immunohistochemistry (IHC) in a subset of luminal cells. Activation of hormone receptor signaling in cells with high hormone receptor expression, termed sensor cells, triggers the expression of paracrine factors such as amphiregulin and Rankl, which are required for mammary epithelial cell proliferation as well as Wnt4 and Cxcl12, which activate stem/progenitor cells. Mammary stem and progenitor cells have been identified and characterized based on cell surface markers and functional assays. However, the precise cellular and biochemical components of the stem cell niche and its endocrine regulation remain poorly defined.

Evidence has been provided that mammary ECM can reprogram non-mammary cells to form mammary glands, suggesting that it contains critical cues for epithelial development. Hedgehog signaling acts via Gli2 downstream of growth hormone receptor signaling in fibroblasts to trigger changes in paracrine signaling and ECM proteins that affect stem cell function. This suggests that stromal fibroblasts are part of the niche under direct endocrine control by growth hormone. Stromal changes accompany different morphogenic processes induced by epithelial hormone signaling and are a hallmark of breast carcinogenesis. Indeed, high radiographic density, which reflects an increase in fibrillar collagen content in the breast stroma, is the single most important factor for breast cancer and correlates with progesterone exposure. How ECM and stroma are controlled by the major endocrine drivers of breast development and carcinogenesis, epithelial ER and PR signaling, remains elusive.

Adamts18 is an orphan member of the A Disintegrin-like And Metalloproteinase domain with Thrombospondin type 1 Motifs (ADAMTS) family of secreted Zn-dependent metalloproteinases, which comprises 19 members. Like other zinc metalloproteinases, ADAMTS catalytic activity depends on zinc ion binding within the active site; unique to ADAMTS18 is an ancillary domain containing thrombospondin type 1 repeats. ADAMTS proteases are synthesized as precursors with an N-terminal propeptide, which is excised by pro-protein convertases such as furin. Some ADAMTSs process ECM components such as fibrillar collagen, while others are implicated in turnover of the chondroitin sulfate proteoglycans aggrecan and versican, and ADAMTS13 uniquely cleaves von-Willebrand factor to maturity. We have previously reported that Adamts18 is required for eye, lung and female reproductive tract and kidney development in the mouse. It is highly homologous to Adamts16, which has a role in renal development and fertility, and can cleave fibrinectin. Here, we show that Adamts18 provides a mechanistic link between epithelial steroid hormone receptor signaling and changes in the ECM, in particular the BM, that regulate mammary epithelial stemness.

**Results**

**Adamts18 expression is driven by the PR/Wnt4 axis.** To elucidate the mechanisms, by which PR signaling in luminal mammary epithelial cells may elicit ECM changes, we sought genes induced in vivo by progesterone treatment that fulfilled two criteria: (1) They encoded secretory proteins and (2) They showed delayed induction by progesterone as expected of any indirect PR target which is expressed by myoepithelial cells and can hence directly interact with the BM. Adamts18 induction was detected at 16 hours (h) and 78 h but not at 4 h and 24 h but not 18 h following progesterone stimulation. RT-PCR analysis of fluorescence activated cell sorting (FACS)-sorted cells from adult mammary gland extracts showed a 7-fold enrichment of Adamts18 mRNA in myoepithelial cells (Lin- CD24+ CD49f+ ) over luminal (Lin- CD24- CD49f- ) cells (Fig. 1a), in line with recent single cell RNA sequencing data, confirming expression in myoepithelial cells.

Analysis of Adamts18 transcript levels at different stages of mammary gland development revealed low prepubertal expression that increased 2.7- and 8.6-fold in 4- and 6- and 8-week-old females, respectively; expression rose further during pregnancy with a peak at mid-pregnancy day 10.5/12.5 (Fig. 1b). RNAscope in situ hybridization for Adamts18 transcripts combined with immunofluorescence (IF) for the myoepithelial marker α-smooth muscle actin (Sma) confirmed myoepithelial-specific expression of Adamts18 in pubertal and adult mammary ducts (Fig. 1c, d). The increased Adamts18 expression during pregnancy was not attributable to generalized but rather to myoepithelial-specific upregulation of expression (Fig. 1e). Thus, Adamts18 expression in the mammary epithelium is developmentally regulated, and its mRNA is enriched in myoepithelial cells, making it an attractive candidate to mediate ECM changes downstream of epithelial hormone action.

Next, we tested whether endocrine factors contribute to developmental Adamts18 expression. First, we mimicked pubertal estrogen stimulation by injecting ovariectomized 21-day-old mice with 17-β-estradiol. Within 18 h of injection, Adamts18 transcript levels in extracts from total mammary glands increased 1.76-fold (Fig. 1f). Second, we asked whether changes in progesterone levels as they occur during estrous cycles affect Adamts18 transcript levels and obtained mammary gland extracts from mice in estrus and diestrus. Progesterone plasma levels determined by liquid chromatography-mass spectrometry were on average 2.8-fold higher in diestrous than in estrus (Fig. 1g). Adamts18 transcript levels in the mammary glands were 1.6-fold higher in diestrous over estrous (Fig. 1h). Thus, physiological Adamts18 expression correlates with plasma progesterone levels, suggesting that it is progesterone-responsive. The subtle increases in transcript levels are consistent with myoepithelial cells representing a minor fraction of the mammary cell types and hence of the total RNA in the whole tissue extracts we analyzed.

To determine whether epithelium-intrinsic PR signaling is required for Adamts18 mRNA expression, mammary epithelia from WT.VEGF+PR−/−EGFP+ mice were grafted to contralateral fat pads of WT recipients surgically cleared of the endogenous epithelium and allowed to grow out for six weeks. At sacrifice, reconstitution was validated by fluorescence stereomicroscopy of the engrafted glands. Adamts18 transcript levels in the mammary glands successfully reconstituted with PR−/− epithelium were on average 27% of those in the contralateral controls (Fig. 1i). Thus, epithelial PR expression is required for Adamts18 mRNA expression.

Wnt4 is a plausible candidate to induce Adamts18 expression in myoepithelial cells because it is a PR target transcribed exclusively in PR+ luminal cells and activates canonical Wnt signaling in the myoepithelial cells, which express Adamts18. We analyzed expression of various Wnt signaling components expressed in the mammary epithelium by RT-PCR in contralateral glands engrafted with WT.VEGF+PR−/−EGFP+ mammary epithelia. Among the Wnt genes, only Wnt4 transcript levels were significantly lower in the mutant grafts, furthermore...
the transcript levels of the stem cell marker Lgr5 and the Wnt co-receptor Lrp6 were decreased (Fig. 1j). Consistent with canonical Wnt signaling activation downstream of PR/Wnt4 controlling Adamts18 expression, TCF4 binding sites were reported in the Adamts18 promoter by ChIP-seq analysis\(^{27}\). To assess whether canonical Wnt signaling controls Adamts18 expression in vivo, we analyzed Adamts18 expression in mammary glands with hyperactive canonical Wnt signaling in the myoepithelium\(^6\) due to the presence of an MMTV-Wnt1 transgene\(^{24}\). Ectopic Wnt1 expression was readily detected in transgenic glands and expression of the canonical Wnt signaling target, Axin2, was increased 5-fold over the non-transgenic control while Adamts18 mRNA levels were increased 7-fold (Fig. 1k). RNAscope for Adamts18 transcripts combined with IF for Sma showed the increased expression specifically in myoepithelial cells (Fig. 1l).

To test whether Wnt4 was furthermore required for Adamts18 expression, we engrafted contralateral cleared fat pads with WT.

The graph and figure legends are as follows:

**Fig. 1 Adamts18 expression in the mouse mammary gland.** a Dot plot showing Adamts18 mRNA expression normalized to Hprt in FACS-sorted CD24+/CD49f− (luminal), CD24+/CD49f+ (myoepithelial) and CD24−/CD49f− (stromal) cells. Data represent mean ± SD from \(n = 3\) independent experiments. Student t-test, two-tailed. b Bar plot showing Adamts18 mRNA levels normalized to Hprt in mammary glands at different developmental stages. Each bar represents pool of 3 mice, mean ± SD for technical replicates. c−e Representative micrographs showing Adamts18 mRNA localization in mouse mammary gland during puberty (c), adulthood (d) and pregnancy day 12.5 (e). Red dots represent Adamts18 in situ hybridization signal, green: α-Sma, blue: DAPI, arrows show myoepithelial cells; scale bar, 50 μm. f Relative Adamts18 transcript levels normalized to Krt5 in mammary glands from 6 control and 5 E2-treated mice. Data represent mean ± SD, unpaired Student t-test, two-tailed. g Dot plot showing plasma progesterone levels determined by LC/MS during diestrus (\(n = 10\)) or estrus (\(n = 9\)). Data represent mean ± SD, Student t-test, two-tailed. h Dot plot showing Adamts18 mRNA levels normalized to Krt5 in mammary glands from mice shown in g. Data represent mean ± SD, Student t-test, two-tailed. i Dot plot showing Adamts18 mRNA normalized to Hprt in 6 contralateral mammary glands transplanted with WT.EGFP\(^+\) or PR−/−.EGFP\(^+\) epithelium. j Bar graph showing relative transcript expression of different Wnt signaling components normalized to Hprt in contralateral glands of 8 mice transplanted with WT.EGFP\(^+\) and PR−/−.EGFP\(^+\) epithelia. Each data point represents one gland, mean ± SD, paired Student t-test, two-tailed. k Dot plots showing relative transcript levels of Wnt1, Axin2 and Adamts18 normalized to Hprt in mammary glands from 5 WT and 3 MMTV-Wnt1 virgin mice. Data represent mean ± SD, Student t-test, two-tailed. l Representative micrographs of Adamts18 mRNA localization, (red) dots, in mammary glands from 3 WT and 3 MMTV-Wnt1 females, α-Sma (green) and DAPI (blue); arrows show myoepithelial cells. Scale bar, 50 μm. m Dot plots showing mRNA levels of Wnt4 and Adamts18 normalized to Hprt in contralateral glands of 3 mice transplanted with WT.EGFP\(^+\) and Wnt4−/−.EGFP\(^+\) epithelium harvested at 8.5-day of pregnancy. *\(p<0.05\); **\(p<0.01\); ***\(p<0.001\); ****\(p<0.0001\).
EGFP+ and Wnt4−/−.EGFP+ mammary epithelia and harvested the transplanted glands on day 8.5 of pregnancy when Wnt4-dependent canonical Wnt signaling activity peaks. Levels of Wnt4 expression in the mutant grafts were 1% of WT levels and Adams18 expression was reduced to 35% of WT levels (Fig. 1m). Thus, increased canonical Wnt signaling induces Adams18 expression and both PR and Wnt4 are required for Adams18 mRNA expression. This indicates that myoepithelial Adams18 expression is downstream of the luminal PR/Wnt4 axis.

Potentially, our conclusion could be confounded by lineage differentiation and cell specification defects resulting from PR and Wnt4 deletions. In light of the finding that both PR−/− and Wnt4−/− epithelial cells can differentiate into milk secreting alveolar cells, major cell specification defects are improbable. Nevertheless, we examined the possibility of a lineage differentiation defect by determining the ratio of luminal and myoepithelial cells in the two mutants. FACS analysis of lineage-depleted WT and PR−/− mammary cells showed no significant difference in the two cell lines (Supplementary Fig. 1a). As the Wnt4−/− mice die on embryonic day 13, we resorted to transplanting WT,EGFP+ and Wnt4−/−.EGFP+ mammary epithelia derived for embryonic mammary buds to contralateral fat pads and quantified the percentage of Sma+ epithelial cells by IF. The percentage of epithelial cells was decreased from 34% in the WT to 26% in the Wnt4−/− epithelium (Supplementary Fig. 1b). To gain more insights into the lineage deregulation, we went on to compare FACS-sorted GFP+ luminal and myoepithelial cells from conditionally Wnt4-deleted (MMTV::Cre−;Wnt4fl/fl;mt/m) and control (MMTV::Cre−;Wnt4wt/wt;mt/m) epithelia by Affymetrix microarray analysis. The number of genes differentially expressed between the two genotypes was almost twice as high in the myoepithelial than in the luminal cell populations (Supplementary Fig. 1c–e). Hence, despite a lineage defect, there are major gene expression changes in the myoepithelium. Gene set enrichment analysis (GSEA) of the differentially expressed genes revealed that signatures reflecting the activity of the canonical Wnt signaling target, Myc, and the expression of its target genes were decreased in the Wnt4−/− myoepithelial but not luminal cells (Supplementary Fig. 1f). Together these findings are consistent with the model that Wnt4 secreted by luminal cells activates canonical Wnt signaling in the myoepithelial cells. Wnt4 was the most significantly down-modulated gene in the luminal compartment (Supplementary Fig. 1d). While expression of Cytokeratin 5 (Krt5) a gene typically enriched in myoepithelial cells, was increased in the Wnt4−/− luminal cells no cell type-related gene signatures were identified (Supplementary Fig. 1d).

In the myoepithelial cell population, the secreted Wnt signaling inhibitor, Wif1, was the most significantly down-modulated gene suggesting the existence of a negative feedback loop in intraepithelial homeostasis (Supplementary Fig. 1e). The stem and progenitor cell markers, Sox9 and Lgr5, were decreased (Supplementary Fig. 1e). Adams18 was also among the down-modulated genes but failed to reach statistical significance (Supplementary Fig. 1e). GSEA revealed furthermore a decreased stem cell signature and an increase in Tgf-β targets in the Wnt4−/− myoepithelial cells (Supplementary Fig. 1g). Reactome pathway analysis revealed a protein interactome centered around cell-cell junction and cell junction organization as well as cell-cell communication (Supplementary Fig. 1h). Taken together, while the deletion of Wnt4 results in a stem cell defect with some consequent cell lineage defect, the gene is expressed in the luminal compartment and its deletion affects transcription mostly in the myoepithelial compartment where Adams18 is expressed.

Mammary gland development in Adams18−/− mice. To assess the functional importance of Adams18 in mammary gland development, we generated mice homozygous for an allele lacking exons 8 and 9, which encode the Zn-binding catalytic site and analyzed their inguinal mammary glands at critical developmental stages by whole mount stereomicroscopy. In prepubertal, 14-day-old WT and Adams18−/− littermates, the ductal system was rudimentary and of similar size in both genotypes (Fig. 2a). Consistently, extent of fat pad filling (Fig. 2b) and the number of branching points were comparable in prepubertal, 14-day-old, WT and Adams18−/− littermates (Fig. 2c). In pubertal, 4–6-week-old, WT females, milk ducts grew by characteristic dichotomous branching, extended beyond the sublumbar lymph node, and had enlarged tips, terminal end buds (TEBs) characteristic of this stage (Fig. 2d). In the Adams18−/− littermates, ducts barely reached the lymph node (Fig. 2d). The extent of fat pad filling was reduced by 50% (Fig. 2e), the number of branching points by 60% (Fig. 2f) and the number of TEBs by 40% compared to the WT counterparts (Fig. 2g). In adult, 14-week-old, females, the milk ducts reached the edges of fat pads in both genotypes. In WT females, ductal complexity was increased through side branching whereas ducts of the Adams18−/− littermates were simple (Fig. 2h) and the number of branching points was 58% of WT (Fig. 2i). Thus, Adams18 is required for ductal development both during puberty and adulthood.

Histological examination of mammary glands from 6-week-old mice revealed structurally normal ducts with intact luminal and myoepithelial layers in both genotypes (Fig. 2j). To address whether the observed delay in ductal elongation was due to increased cell death and/or decreased cell proliferation, we stained sections from pubertal glands for cleaved-caspase 3 and phosphorylated histone H3 (pH3). The proportion of cleaved caspase3+ cells did not differ significantly (Fig. 2k) but the pH3-index in Adams18−/− mammary epithelia was reduced to 64% of WT levels (Fig. 2l, m). Thus, the delayed ductal elongation is due to decreased cell proliferation.

Adams18 function in the mammary epithelium. Adams18−/− pups show a transient growth delay, which may indirectly affect mammary gland development. In addition, subfertility associated with abnormalities in the female reproductive tract, such as dorsoverental vagina or imperfect vagina of Adams18−/− females precluded analysis of mammary gland development during pregnancy. To discern the epithelial-intrinsic role of Adams18 in ductal growth at later developmental stages, we grafted mammary epithelium from WT,EGFP+ and Adams18−/−.EGFP+ females to contralateral inguinal glands of 3-week-old WT female mice surgically diestved of their endogenous epithelium. To unequivocally distinguish the engrafted epithelium from host epithelium that could have been inadvertently left behind during surgery, the donor cells constitutively expressed an enhanced green fluorescent protein (EGFP) under control of a chicken β-actin promoter. Six weeks after engraftment, outgrowths derived from WT donors filled the host fat pads whereas the contralateral Adams18−/− epithelia failed to do so (Fig. 3a) and the branching points were decreased by 33% (Fig. 3b). Twelve weeks after engraftment, both WT,EGFP+ and Adams18−/−.EGFP+ outgrowths filled the host fat pads but side branching was decreased in Adams18−/−.EGFP+ epithelial grafts (Fig. 3c). Flow cytometry of dissociated glands showed a 30% reduction in EGFP+ cells (Fig. 3d) consistent with decreased cell proliferation resulting in lower epithelial cell numbers and delayed branching. Thus, the mammary branching phenotype in Adams18−/− females is intrinsic to the mammary epithelium.
At 14.5 days of pregnancy, epithelia of both genotypes showed widespread alveoli both by fluorescence stereomicroscopy and histology (Fig. 3e). At day 1 of lactation, alveoli were fully distended (Fig. 3f) suggesting normal lactogenic function. However, at both time points, spaces between EGFP+ epithelial structures were larger in Adamts18−/−_EGFP+ grafts than in the WT counterparts consistent with reduced side branching at earlier stages (Fig. 3e–g).

In line with the morphologic analysis and the decreased number of MECs, transcript levels of lactogenic differentiation markers such as Lalba, Wap, and CsnA were lower in mutant glands compared to WT controls but failed to reach statistical significance when normalized to the epithelial marker Krt18 (Fig. 3h). Thus, while epithelial cell numbers are decreased in the absence of Adamts18, the protease is not required for cytodifferentiation.

Adamts18 expression has been reported in the stromal compartment and was confirmed by semi quantitative RT-PCR analysis of WT fat pads engrafted with Adamts18−/−_EGFP epithelium showing 25% of the Adamts18 transcript levels detected in WT recombinants (Supplementary Fig. 2a). To determine the functional importance of this stromal expression, WT.EGFP+
mammary epithelium was transplanted into cleared inguinal mammary fat pads of 3-week-old \( \text{Adams}^{1/2} \) and WT mice. The recombined tissues were contralaterally transplanted unto the abdominal muscles of adult WT females. Fluorescence stereomicroscopy 6 weeks later showed that WT donor epithelium filled both WT and \( \text{Adams}^{1/2} \) fat pads to comparable extent (Supplementary Fig. 2b, c). Twelve weeks after surgery, in both WT and \( \text{Adams}^{1/2} \) fat pads the implanted epithelia had developed side branches to comparable extent (Supplementary Fig. 2d). Thus, stromal \( \text{Adams}^{1/2} \) expression is not required for ductal branching.

As epithelial ER and PR signaling drive pubertal dichotomous branching and estrous cycle-induced side branching, respectively\(^{29,33} \), we asked whether receptor expression was affected by...
Adams18 inactivation. IHC of sections from contralateral glands engrafted with WT.EGFP+ and Adams18−/−.EGFP+ epithelia revealed comparable proportions of ER+ (Fig. 3i) and PR+ cells (Fig. 3j) indicating that Adams18 is not required for ER or PR protein expression.

The role of Adams18 in mammary epithelial self-renewal. Delayed pubertal ductal outgrowth and reduced side branching together with normal alveologenesis and cytodifferentiation were previously observed in Wnt4−/− epithelia26 and shown to result from a stem cell defect6. To test whether Adams18 deletion also affects mammary stem cells (MaSCs), we analyzed cells from dissociated WT and Adams18−/− mammary glands by FACSTM using CD24 and CD49f detection after depletion for lineage positive cells (Fig. 4a). The number of lineage-depleted cells obtained from mammary glands of 14-week-old females was one third less in Adams18−/− compared to WT (Fig. 4b). The percentage of both luminal (Lin− CD24+ CD49f+) and myoepithelial (Lin− CD24low CD49flow) cells was not significantly altered in Adams18−/− glands but the stromal cell fraction (Lin− CD24− CD49f−) increased by 19% in the mutant glands (Fig. 4c). Mammary progenitors, which give rise to colonies and are called colony forming cells (CFCs) represented <1% of the lineage negative cells in both genotypes whereas the number of MaSCs (Lin− CD24medCD49fhigh) also defined as mammary repopulating units (MRUs) was decreased by 43% in Adams18−/− glands (Fig. 4a, d).

To functionally evaluate stem cell frequency in WT and Adams18−/− mammary epithelia, we injected serially diluted single cells from WT.EGFP+ and Adams18−/−.EGFP+ mammary glands to contralateral cleared fat pads of 3-week-old myoepithelial/basal characteristics37, as a model. We ectopically transfected them respectively, by the same assay6. We searched for its binding partners. In light of the progressive to cease completely upon the 5th transplant (Fig. 4f, h, i). Histological analysis of the 4th generation transplants by H&E revealed no obvious difference (Fig. 4g). Thus, Adams18 is required for the regeneration capacity of the mammary epithelium, albeit to a lesser extent than PR and Wnt4, whose deletion blocks reconstitution at the 4th and 3rd generation, respectively, by the same assay6.

The basement membrane is part of the stem cell niche. To address the mechanisms by which Adams18 affects stem cell activity, we searched for its binding partners. In light of the myoepithelial cell-specific expression of the protease, we chose the human breast epithelial cell line, MCF10A, which has myoepithelial/basal characteristics37, as a model. We ectopically expressed V5-tagged ADAMTS18 in these cells, immune precipitated it from the conditioned medium, and analyzed co-immunoprecipitated proteins by mass spectrometry. We discovered 238 proteins cumulatively in 3 independent experiments (Supplementary Data 1), of which 31 were identified in ≥2 experiments (Fig. 5a). Transforming Growth Factor Beta-Induced (TGFBI), a secreted molecule that contains RGD domains similar to fibronectin and laminin and inhibits cellular adhesion to the ECM, was among the 12 proteins identified in all 3 experiments38. Bioinformatic analysis with MetaCore showed that top enriched MetaCore processes related to ECM organization and hemidesmosome assembly (Fig. 5b, Supplementary Table 1). The top localizations of the putative ADAMTS18 interactors were ECM, laminin-5 complex, and BM (Fig. 5c, Supplementary Table 2). Together, these findings support the hypothesis that Adams18 function relates to the ECM, and more specifically, to the connection between epithelium and BM. This implies that the BM may be part of the stem cell niche.

To seek in vivo evidence for a role of the BM as part of the stem cell niche we turned to mice deficient for ColI8a1 because this heparin-sulfate proteoglycan is specifically localized to BMs39. Whole mount stereo-microscopy and morphometric analysis showed that ColI8a1−/− females like their Adams18−/− counterparts had delayed ductal elongation and fewer TEBs compared to their WT littermates (Fig. 5d). Adams18 and ColI8a1 double-deficient (DKO) mice showed a further decrease in TEB numbers, fat pad filling, and branching points at 6 weeks compared to single knockouts (Fig. 5e) indicating that Adams18 and ColI8a1 have additive roles in ductal elongation. To assess whether this genetic interaction affects stem cell function, we serially transplanted the DKO epithelium. While the contralateral WT epithelium reconstituted glands over 5 transplant cycles, the DKO epithelium failed to reconstitute by the 3rd generation (Fig. 5f–h). Thus, Adams18 and ColI8a1 cooperate in mammary stem cell control, providing in vivo evidence for a role of the BM in stem cell function, likely as part of the stem cell niche.

Adams18 modulates the ECM. To probe for structural alterations in the ECM related to Adams18 deletion, we used picrosirius red to stain Adams18−/− and WT pubertal mammary glands. Fibrillar collagen was increased around the ducts and TEBs in Adams18−/− relative to WT (Fig. 6a). Immunoblotting of protein lysates from pubertal WT and Adams18−/− glands and quantification showed that levels of the important BM components, laminin and collagen IV increased 1.7- and 3.9-fold, respectively, in Adams18−/− glands (Fig. 6b, c). Levels of the major fibrillar collagen, collagen I, were increased 6.2-fold (Fig. 6b, c). Assembly of nascent collagen I, laminin and collagen IV matrices rely on initial assembly of fibrils composed of the prominent ECM glycoprotein fibronectin, the first ECM protein to be expressed during tissue development and wound healing40,41. Fibronectin levels were 3.2-fold higher in the mutants than in WT (Fig. 6b, c). IF showed increased staining intensity for all these proteins around ducts and TEBs in Adams18−/− relative to WT pubertal glands (Fig. 6d). The staining was restricted to the BM for laminin and collagen IV but extended to the interstitial ECM for collagen I and fibronectin. Thus, in the absence of Adams18, major ECM/BM components accumulate in the pubertal mammary gland in line with an important role for Adams18 in ECM/BM remodeling.

Interestingly, analysis of mammary glands from 14-week-old WT and Adams18−/− littermates showed that protein levels of laminin, collagens I and IV as well as fibronectin did not differ significantly between the two genotypes (Fig. 6e, f). This shows that Adams18 is critical for ECM/BM modulation during pubertal ductal elongation and suggest that this specific developmental window determines mammary stem cell function.

Adams18 cleaves fibronectin. In contrast with the increased fibronectin protein levels, its mRNA levels were unaltered in the

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pubertal Adamts18−/− mammary glands (Fig. 6g) suggesting that the observed increased staining could result from translational or posttranslational changes attributable to lack of Adamts18. As fibronectin is the prime component of nascent ECM fibers and a substrate of the Adamts18 homolog Adamts16, we tested whether it is equally an Adamts18 substrate. We purified the secreted active form of ADAMTS18 from HEK-293T cells and incubated it with N-terminal 70 kDa bronectin. The exogenous fibronectin fragment migrated slightly faster when co-incubated with EDTA and was undetectable in the presence of ADAMTS18 after 24 h. When the digest was supplemented with EDTA, which chelates the bivalent metal ions required for ADAMS activity, no change in fibronectin abundance was seen (Fig. 6h). Additionally, HEK-293T cells expressing ADAMTS18 or a control vector were incubated without or with the 70 kDa recombinant fibronectin. By western blot, the medium of cells expressing ADAMTS18 showed a readily detectable 30 kDa bronectin fragment (Fig. 6i) similar to that detected after...
cleavage by Adams16\textsuperscript{21}. The amount of cleaved fibronectin increased 16-fold in the presence of ADAMTS18 (Fig. 6j). Thus, the presence of ADAMTS18 leads to fibronectin proteolysis, which may influence abundance of other ECM proteins and indirectly regulate growth factor availability and signaling.

Stem cell signaling in Adams18\textsuperscript{−/−} glands. Our findings pointed to the observed stem cell defect being secondary to changes in the ECM/BM. To elucidate the mechanisms by which altered ECM affected stem cell signaling, we transcriptionally profiled 3 pairs of contralateral glands engrafted with either WT.\textsuperscript{EGFP\textsuperscript{+}} or Adams18\textsuperscript{−/−}.\textsuperscript{EGFP\textsuperscript{+}} epithelia using RNA-seq. PCA analysis was used to identify and visualize possible batch effects due to sources of variation in the mice used (Supplementary Fig. 3a). After removing these effects by applying 2-way ANOVA analysis was used to identify and visualize possible batch effects due to sources of variation in the mice used (Supplementary Fig. 3b). Expression of Adams18, Egr2, and Ctgf was tested and found reduced in all 3 Adams18\textsuperscript{−/−} samples after read
count normalization (Supplementary Fig. 3c). Overall, in Adamts18−/− transplanted glands, expression of 313 genes decreased (FC < 0.8, p < 0.05) and that of 273 genes increased (FC > 1.25, p < 0.05) (Fig. 7a). Analysis of the differentially expressed genes by pathway enrichment analysis using both ReactomePA42 and ClusterProfiler43 showed that cell junctions and ECM were affected, in particular various collagens and laminins (Supplementary Fig. 3d–g). More specifically, out of 40 significant GO terms, 11 were related to the ECM and 10 to Fgfr signaling, a pathway critical for stem cells44,45 (Supplementary Table 3). Two of the 40 terms related to Hippo-Yap/Taz signaling another pathway critical for stem cell differentiation, which is upstream of Fgfr246. When we specifically interrogated the genes whose expression decreased, Reactome pathway analysis revealed Yap/Taz-mediated gene expression (Fig. 7b) and a protein interactome centered around cell-cell communication and cell-cell junctions as well as ECM, laminin and collagen complexes and assembly (Fig. 7c) that partly overlap with the Wnt4 specific interactome (Supplementary Fig. 1h).

In light of the increased ECM deposition, the differential expression of various ECM-related genes as well as the involvement of the Yap/Taz signaling pathway, we evaluated integrin expression in the Adamts18−/− glands. We extracted 27 Integrin genes, α and β Integrin subunits, from the RNAseq analysis and generated a heatmap (Supplementary Fig. 3h). No integrin-related gene was significantly altered by adjusted p-value,
but Itga3, Itgb4, and Itgb7 were significantly altered by p-value. Analysis of their expression levels by qRT-PCR at puberty in 3rd mammary glands from 6 pairs of 5-week-old, pubertal WT and Adamts18−/− littermates; n = 5. Scale bar, 100 μm. b Representative western blot analysis on 3rd mammary glands from 5-week-old, pubertal WT and Adamts18−/− littermates; n = 4. β-actin loading control, MW marker in red. c Dot plots showing relative protein levels of laminin, collagen I, collagen IV, and fibronectin normalized to actin in 4 pubertal WT and Adamts18−/− littermates. Paired Student t-test, two-tailed; *p < 0.01. d, Fluorescent micrographs showing IF on 4th mammary gland sections from 5-week-old, pubertal WT and Adamts18−/− littermates for laminin, collagens I and IV as well as fibronectin (green) and DAPI nuclear stain (blue), n = 3. Arrows point to ECM density around TEBS or ducts; scale bar, 100 μm. e Representative western blot analysis on 3rd mammary glands of 14-week-old WT and Adamts18−/− littermates; n = 3. β-actin loading control, MW marker in red. f Dot plots showing relative protein levels of laminin, collagen I, collagen IV, and fibronectin normalized to actin in 3 adult WT and Adamts18−/− littermates. Paired Student t-test, two-tailed; n.s. not significant. g Dot plot showing relative transcript levels of Fni normalized to Hprt in 3rd mammary glands from 6 pairs of 5-week-old WT and Adamts18−/− littermates. Paired Student t-test, two-tailed, n.s. not significant. h Representative Western blot analysis of 3 independent experiments in which fibronectin (FN)−70K was incubated with purified active Adamts18 in the presence or absence of EDTA and/or protease inhibitor (PI). Anti-FN antibody specific to the N-terminal heparin-binding domain. i Western blot analysis of FNI−70K incubated with ADAMTS18 overexpressing HEK-293T cells in the presence or absence of EDTA. j Bar graph showing levels of cleaved FN in supernatants from control transfected and Adamts18 overexpressing HEK-293T cells in 2 independent experiments.

but Itga3, Itgb4, and Itgb7 were significantly altered by p-value. Analysis of their expression levels by qRT-PCR at puberty in mammary glands from WT and Adamts18−/− mice showed increased Itga3 and Itgb4, two integrins previously implicated in mammary stem cell function17,18 and part of laminin 5 receptors, to be significantly down modulated in the mutants (Fig. 7d).

Together these findings suggest that Adamts18 is required for activation of the Hippo pathway, which in turn induces Fgfr2 expression, activation of which is critical for stem cell function. Consistent with this scenario, the 3 Hippo target genes, Ctgf, Fgfr2, and Gata346,49 were reduced to 73%, 68% or 78% of WT levels, respectively, in additional transplants in the absence of Adamts18 (Fig. 7e). Double-IF for Yap and the myoepithelial marker α-smooth muscle actin (Sma) showed expected nuclear localization of Yap in WT myoepithelial cells (Fig. 7f)50. In the contralateral Adamts18−/−.EGFP+ epithelia the signal intensity of Yap was decreased in myoepithelial cells (Fig. 7f). Quantitative image analysis revealed that the mean nuclear intensity of the Yap staining in the mutant epithelium was 58% of the contralateral WT.EGFP+ transplanted glands (Fig. 7g).

To further support our claim that BM modulation by Adamts18 involves the Yap/Taz signaling pathway, we assessed the expression levels of downstream targets, Cited-1, Ctgf, Fgfr2, Gata3 in pubertal WT, Col18a1−/−, Adamts18−/−, and DKO mice. Additionally, we assessed the expression levels of Itga3 and Itgb4 altered in Adamts18−/− mice. In line with our previous findings (Fig. 7h), we found the Yap/Taz targets to be significantly down modulated in pubertal Adamts18−/− and the DKO. Col18a1−/− glands displayed downmodulation in Adamts18, Cited-1, and Ctgf. This suggests that modulation of the BM composition by Adamts18 leads to activation of Yap/Taz signaling with increased Fgfr2 expression and signaling which results in stem cell activation.

ADAMTS18 in the human breast. Our data indicate that Adamts18 translates the hormonal stimuli received by luminal cells into activation of stem cells via changes to the BM in the mouse mammary gland. To assess whether this signaling axis may also operate in the human breast, we generated a polyclonal antibody to ADAMTS18 and validated it on MCF10A overexpressing V5 tagged human ADAMTS18 with or without a short hairpin RNA (shRNA) to knock down overexpressed ADAMTS18 (Supplementary Fig. 4). IHC of reduction mammary gland sections from different patients exposed to progesterone showed increased Adamts18 expression of downstream targets, Ctgf, Fgfr2, and Gata347,48 and part of laminin 5 receptors, to be significantly down modulated in the mutants (Fig. 7d). IHC of reduction mammary gland sections from 5-week-old, pubertal WT and Adamts18−/− littermates; n = 5. Scale bar, 100 μm. b Representative western blot analysis on 3rd mammary glands of 14-week-old WT and Adamts18−/− littermates; n = 3. β-actin loading control, MW marker in red. f Dot plots showing relative protein levels of laminin, collagen I, collagen IV, and fibronectin normalized to actin in 3 adult WT and Adamts18−/− littermates. Paired Student t-test, two-tailed; n.s. not significant. h Representative Western blot analysis of 3 independent experiments in which fibronectin (FN)−70K was incubated with purified active Adamts18 in the presence or absence of EDTA and/or protease inhibitor (PI). Anti-FN antibody specific to the N-terminal heparin-binding domain. i Western blot analysis of FNI−70K incubated with ADAMTS18 overexpressing HEK-293T cells in the presence or absence of EDTA. j Bar graph showing levels of cleaved FN in supernatants from control transfected and Adamts18 overexpressing HEK-293T cells in 2 independent experiments.

Discussion

Here, we have addressed the longstanding puzzle of how epithelial ER and PR signaling connect to ECM changes that accompany both normal breast development and breast carcinogenesis. We show that the gene encoding Adamts18 is expressed in the myoepithelial downstream of Wnt4 secretion induced by ER/PR signaling luminal sensor cells (Fig. 9). The myoepithelial cells respond by canonical Wnt signaling activation and link luminal hormone receptor signaling to stromal changes with functional consequences. Our finding that altered BM composition affects MaSCs shows that the BM is a central part of the stem cell niche and a critical determinant of stem cell function.

The precise nature of the BM and interstitial ECM changes that alter signaling remain to be determined. Numerous factors, such as tissue stiffness and growth factor availability, directly or indirectly controlled by Adamts18 may be critical. The observed changes in the abundance of collagen I, collagen IV, laminin, fibronectin, and glycoproteins, like collagen XVIII, may be secondary to the reduced fibronectin clearance but Adamts18 may also be directly involved in their processing; other family members have glycoprotein substrates14. Increased laminin expression was also observed in Adamts18−/− adipose tissue52 and embryonic brains53 with effects on early adipocyte differentiation and spine and synapse formation. A detailed analysis of kidney and lung development in WT and Adamts18−/− mice revealed that expression of the enzyme by branching tips is important for branching and organ size54.

We identified enhanced Yap/Taz nuclear localization and increased Fgfr2 signaling as potential mechanisms underlying
stem cell activation downstream of Adamts18 activity (Fig. 9). Whether Yap/Taz activation is central to increased Fgfr2 signaling and/or whether biochemical changes in the BM result in increased ligand availability was not addressed in our study. Yap/Taz signaling is typically activated by extracellular cues such as increased stiffness. Our gene expression analysis did not provide direct indications for this; whether the increased expression of muscle-related genes may also impinge on Yap/Taz or whether another stiffness independent mechanism is important, remains to be explored. We speculate that Adamts18-induced modifications of the ECM affect integrin-mediated, F-actin dependent cell-ECM adhesion and contraction, which promote cellular mechanical tension and Yap/Taz activation. As such, the progesterone/Wnt4/Adamts18 axis provides an entry point for further studies of epithelial-BM interactions.

The regulatory axis we identified genetically in the mouse mammary gland likely operates in the human breast with implication for breast cancer prevention and treatment. Exposure to progesterone as it occurs recurrently during menstrual cycles has been shown to induce WNT4 expression and can increase...
Fig. 7 Adamts18 impinges on transcription and regulates cell signaling. a Volcano plot showing genes, which are differentially expressed between contralateral glands transplanted with Adamts18−/− and WT epithelia; n = 3, Kolmogorov-Smirnov test, all highlighted genes have p-values < 0.05. Genes with log2(FC) >0.5 in red and log2FC <0.5 in blue. Names of selected genes are indicated. b Enrichment map plot of Reactome pathway analysis (ReactomePA) on genes downregulated in 3 pairs of contralateral glands engrafted with WT and Adamts18−/− epithelia in 3 independent experiments with 3 different donors. c CNE plot of ReactomePA of genes down regulated in contralateral glands transplanted with WT and Adamts18−/− epithelia. d Bar graphs showing relative transcript levels of Adamts18, Ibag3, Ibag4, and Ibag1, normalized to Hprt in 5 pubertal host mice bearing contralateral transplants of WT and Adamts18−/− epithelia. Data represent mean ± SD. Unpaired Student t-test, two-tailed. e Bar graphs showing relative transcript levels of Fgf2, Ctgf, and Gata3 normalized to Hprt in contralateral glands transplanted with WT and Adamts18−/− epithelia, n = 6. f Representative IF for SMA (green) and YAP (red) counterstained with DAPI (blue) of 4th mammary gland sections from 5-week-old WT and Adamts18−/− littermates; n = 3. Arrows indicate YAP positive nuclei of myoepithelial cells. g Dot plot showing quantification of relative mean intensity of nuclear YAP detected in myoepithelial cells of 5-week-old WT and Adamts18−/− littermates; n = 3. Each point represents an individual TEB. h Bar graphs showing relative transcript levels of Adamts18, Col11a1, Cited-1, Ctgf, Fgf2, Gata-3, Ibag3, and Ibag-4, normalized to Hprt in pubertal WT, ColI1a1−/−, Adamts18−/−, and DKO; n = 9, 8, 4, and 4, respectively. Data represent mean ± SD, one-way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, n.s. not significant.

ADAMTS18 expression, as we show here. The resulting BM/ECM remodeling may contribute to the increased breast cancer risk associated with recurrent menstrual cycles. Furthermore, the increased risk of postmenopausal women exposed to combined hormone replacement therapy with ethinyl estradiol and progesterone increased risk of postmenopausal women exposed to combined hormone replacement therapy with ethinyl estradiol and progesterone makes it an excellent target for antibody-mediated therapy. As such, targeting ADAMTS18 appears as a feasible strategy for primary and secondary prevention unlikely to elicit major side effects.

Methods

Mice. All mice were maintained and handled according to Swiss guidelines for animal safety and experiments were performed in accordance with protocols approved by the Service de la Consommation et des Affaires Vétérinaires of Canton de Vaud, Switzerland, with a 12-h-light-12-h-dark cycle, controlled temperature and food and water ad libitum. 129SV/C57BL6, mT/mG60, and de Vaud, Switzerland, with a 12-h-light-12-h-dark cycle, controlled temperature and food and water ad libitum. 129SV/C57BL6, mT/mG60, and 129SV/C57BL6/OlaHsd mice from Harlan Laboratories. Adamts18−/−, Col11a1−/−, and MMTV-Cre (lineA)62, Wnt1−/−63, Wst5−/−63, Wst6−/−63, and Tg(Act-EGFP)61 mice were maintained in C57BL/6/OlaHsd background.

Patient sample processing. The cantonal ethics committee approved the study (183/10). Breast tissue was obtained from women undergoing reduction mammoplasties with no previous history of breast cancer. All human subjects provided functional in adult mice31. Furthermore, in its extracellular location ADAMTS18 makes it an excellent target for antibody-mediated therapy. As such, targeting ADAMTS18 appears as a feasible strategy for primary and secondary prevention unlikely to elicit major side effects.

Methods

Mice. All mice were maintained and handled according to Swiss guidelines for animal safety and experiments were performed in accordance with protocols approved by the Service de la Consommation et des Affaires Vétérinaires of Canton de Vaud, Switzerland, with a 12-h-light-12-h-dark cycle, controlled temperature and food and water ad libitum. 129SV/C57BL6, mT/mG60, and NOD.Cg-Pkdcsdil I2gztom1 Wj[Sl] (NSG) mice were purchased from Jackson Laboratories and C57BL/6/OlaHsd mice from Harlan Laboratories. Adamts18−/−, Col11a1−/−, and MMTV-Cre (lineA)62, Wnt1−/−63, Wst5−/−63, Wst6−/−63, and Tg(Act-EGFP)61 mice were maintained in C57BL/6/OlaHsd background.

Patient sample processing. The cantonal ethics committee approved the study (183/10). Breast tissue was obtained from women undergoing reduction mammoplasties with no previous history of breast cancer. All human subjects provided
Histology. Inguinal mammary glands were fixed in 4% PFA in phosphate-buffered saline (PBS, pH 7.2) overnight at 4 °C, embedded in paraffin and cut into 4 μm sections. Hematoxylin and eosin or sirius red staining were performed according to standard protocols. For immunostaining, sections were de-waxed, rehydrated and subjected to antigen retrieval with 10 mM citrate buffer, pH 6.0 for 20 min at 95 °C. Sections were counterstained with Mayer’s hematoxylin. For fluorescence microscopy, nuclei were counterstained with DAPI (Sigma). IF images were acquired on a LEICA M205FA with a Leica DFC 340FX camera. Fat pad filling and branching points were determined using ImageJ software.

RNA in situ Hybridization. Adamts18 ISH was performed using RNAscope (Advanced Cell Diagnostics, Newark, CA) following the manufacturer’s protocol. Briefly, 4 μm sections were deparaffinized and hybridized to a mouse Adamts18 probe set (452251; Advanced Cell Diagnostics) using a HybEZ oven (Advanced Cell Diagnostics) and the RNAscope 2.5 HD Detection Reagent Kit (322360; Advanced Cell Diagnostics) and stained with anti-SMA after the RNAscope procedure.

Transplantation. Fat pads were transplanted onto the abdominal muscle wall of adult WT females. Single cell suspensions of mammary epithelial cells in 20% matrigel were injected and 1 mm³ of epithelial fragments were transplanted to cleared fat pads. Intraductal injection of human breast epithelial cells was performed via cleaved teat.

Mammary gland whole-mounts. Mammary gland whole-mounts were performed as described, and stereomicrographs were acquired with a LEICA MZ FLIII stereomicroscope and Leica MC170 HD. Fluorescence stereomicrographs were acquired on a LEICA M205FA with a Leica DFC 340FX camera. Fat pad filling and branching points were determined using ImageJ software.

Single cell preparation. Reduction mammaoplasty microstructures were incubated with 1% collagenase A (Roche, final concentration of 1.0 mg/mL) in (DMEM)/F12 Dulbecco’s modified Eagle’s medium containing 1% penicillin/streptomycin (cat. 15070-063; Thermo Fisher Scientific) and 1% fungizone (cat. 15290-018; Thermo Fisher), overnight at 37 °C. Cells were dissociated to single cells with 0.25% trypsin-15070-063; Thermo Fisher Scientific), overnight at 37 °C. Cells were dissociated to single cells with 0.25% trypsin and passed through 40 μm cell strainer. To isolate human cells from humanized mammary glands, single cells were incubated with mouse cell depletion cocktail (Miltenyi Biotec, 130-104-694) and passed through LS columns (130-042-401) on MACS separator according to manufacturer’s protocol (Miltenyi Biotec).

Hormone measurements. Progesterone hormone levels in the plasma were measured using LC-MS (Q-Exactive, ThermoFisher Scientific). Fluorescence activated cell sorting. Single cell suspensions of mammary glands from 15- to 25-week-old virgin females were processed as described and sorted on a FACS Aria (Becton Dickinson).

Hormone treatments. Low consistency silicon elastomer (MED-4011) two parts (part A, MP3745/E81949 and part B, MP3744/E81950) were mixed with hormone powder, incubated at 37 °C overnight as described, and implanted subcutaneously. Three-week-old mice were ovarioctomized and injected subcutaneously 10 days later with 17-β-estradiol 5 ng/g of body weight (Sigma–Aldrich, St. Louis, MO) using 5 mg/ml in 100% ethanol stock or vehicle. Mammary glands were harvested 18 h after injection.
RT-PCR: Mammary glands were homogenized with TRIzol reagent (Invitrogen), total RNA was isolated with miRNeasy Mini Kit (Qiagen), cDNA was synthesized with random p(dN)6 primers (Roche) and MMLV reverse transcriptase (Promega), real-time PCR analysis in triplicates was performed with SYBR Green FastMix (Quanta) reaction mix. Primers used for RT-PCR, see Supplementary Table 5.

Protein extraction and western blot: Total proteins from the 3rd mammary glands of 5- and 14-week-old WT and Adamts18−/− littermate mice were extracted in Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris–HCl and 0.1% SDS) with a tissue disruptor on ice. 500 µl of buffer was used for 100 mg tissue and debris was removed by centrifugation. Transfected MCF-7 and MCF-10A were lysed with RIPA lysis buffer supplemented with protease inhibitors and protein concentration measured with a BCA kit (Pierce). The immune precipitates were subjected to SDS-PAGE, the gel was stained with SYDCLX membrane scanner with Li-COR and band intensities quantified by ImageJ.

AP-MS analysis for ADAMTS18 binding proteins: MCF-10A cells were spin-infectected with an ADAMTS18 lentivirus containing a V5 tag or LacZ control virus. Cells were cultured to confluence in 10 cm dishes. Proteins were extracted with RIPA lysis buffer supplemented with protease inhibitors and protein concentration measured with a BCA kit (Pierce). ADAMTS18 was immunoprecipitated from 1 mg of protein using anti-V5 antibody conjugated agarose beads (Sigma A7345). The immune precipitates were subjected to SDS-PAGE, the gel was stained with colloidal Coomassie blue (Biorad), bands were excised and subjected to reduction/alkylation followed by tryptic digestion and LC-MS/MS proteomic analysis. Detected peptides were mapped against the human protein database, label-free protein quantification was performed and protein lists were constructed in Scaffold 4 Proteomics Software using a minimum of 2 peptides to identify the proteins with a peptide false discovery rate (FDR) of 0.1% and protein FDR of 0.3%.

Cloning: A3C-ADAMTS18-867aa cDNAs were amplified from cDNA library prepared from eyes and fused to FLAG-tag and His-tag at N-terminus by PCR and cloned into λ integrase and HinflIII restriction sites of pcDNA3.1/Hygromycin expression vector (Invitrogen). Plasmids were purified with HighPure midiprep kit (Invitrogen).

Fibronectin cleavage: 500 ng purified 70 K fibronectin (Sigma) were mixed with 50 ng of purified A3C-ADAMTS18 in digestion buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 5 µM ZnCl₂), incubated 24 h at 37 °C in presence or absence of EDTA (25 mM) and PI (Pierce), and analyzed by WB with ABC16048, AB16049, ADAMTS18 (Eurogetech) and β-actin (Sigma ab185101) antibodies. IRDye conjugated secondary antibodies were detected with Odyssey CLx membrane scanner with Li-COR and band intensities quantified by ImageJ.

Bioinformatic analysis: For details of RNA-seq and microarray analyses on research design is available in the Nature Research Reporting Summary linked to this article.

Statistics: Prism 6 software (GraphPad) used for statistical analyses and the statistical tests with their reported p-values are indicated in each figure. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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Competing interests
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