Cerebral small vessel disease-related protease HtrA1 processes latent TGF-β binding protein 1 and facilitates TGF-β signaling

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High temperature requirement protein A1 (HtrA1) is a primarily secreted serine protease involved in a variety of cellular processes including transforming growth factor β (TGF-β) signaling. Loss of its activity causes cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), an inherited form of cerebral small vessel disease leading to early-onset stroke and premature dementia. Dysregulated TGF-β signaling is considered to promote CARASIL pathogenesis, but the underlying molecular mechanisms are incompletely understood. Here we present evidence from mouse brain tissue and embryonic fibroblasts as well as patient skin fibroblasts for a facilitating role of HtrA1 in TGF-β pathway activation. We identify latent TGF-β binding protein 1 (LTBP-1), an extracellular matrix protein and key regulator of TGF-β bioavailability, as a novel HtrA1 target. Cleavage occurs at physiological protease concentrations, is prevented under HtrA1-deficient conditions as well as by CARASIL mutations and disrupts both LTBP-1 binding to fibronectin and its incorporation into the extracellular matrix. Hence, our data suggest an attenuation of TGF-β signaling caused by a lack of HtrA1-mediated LTBP-1 processing as mechanism underlying CARASIL pathogenesis.

small vessel disease | proteolysis | extracellular matrix | LTBP-1

Cerebral small vessel disease (SVD) accounts for roughly one fifth of all strokes worldwide and is recognized as a major cause of cognitive decline and dementia (1). Familial forms of SVD have successfully been used as model conditions for mechanistic studies on SVD (2–4). CARASIL (cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy), a recessive SVD, is characterized by juvenile and recurrent strokes, extensive brain white matter lesions, and premature dementia (5). The disease is caused by mutations in the *HTRA1* (high temperature requirement protein A1) gene (6) encoding an evolutionarily conserved serine protease (7). CARASIL mutations typically result in loss of HtrA1 activity, suggesting impaired substrate processing as a disease mechanism (6, 8, 9).

HtrA1 has been shown to degrade a number of substrates, most of which are situated in the extracellular compartment, thus suggesting the extracellular space as primary location of HtrA1 function. HtrA1-mediated proteolysis has been implicated in various disease processes such as neurodegeneration (10, 11), age-related macular degeneration (12, 13), carcinogenesis (14) and arthritis (15). Only recently, identification of *HTRA1* as the CARASIL-causing gene has highlighted its role in the vascular system and in TGF-β signaling, a well-defined regulator of angiogenesis and vascular homeostasis (16, 17) known to be implicated in several vascular conditions including Marfan Syndrome, Loeys–Dietz Syndrome, and hereditary hemorrhagic telangiectasia (18).

Initial studies on CARASIL reported increased levels of the TGF-β prodomain (also called latency-associated peptide, LAP) and of TGF-β target genes in the cerebral vasculature of affected patients. This finding led to the proposition that up-regulation of the TGF-β pathway drives CARASIL pathogenesis (6, 8). However, the initial results were mostly obtained using overexpressing cells and autopsy material from advanced cases. Also, there is some controversy as to how HtrA1 interferes with TGF-β signaling. Proposed mechanisms include HtrA1-mediated extracellular cleavage of mature TGF-β (19, 20), cleavage of TGF-β receptors (21), and intracellular degradation of LAP (8).

To better define the mechanistic link between HtrA1 and TGF-β and to identify physiological HtrA1 substrates relevant for CARASIL we investigated the effects of HtrA1 deficiency on the TGF-β pathway in brain tissue and embryonic fibroblasts from *HTRA1* knockout mice and in skin fibroblasts from a CARASIL patient. Unexpectedly, we observed a consistent reduction of TGF-β activity in both murine and human material suggesting a facilitating role of HtrA1 in TGF-β signaling. We further identified latent TGF-β

Significance

Cerebral small vessel disease (SVD) is a major cause of stroke and dementia. Hereditary forms, such as cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), may provide insights into key molecular mechanisms and pathways. The serine protease HtrA1, whose activity is impaired in CARASIL, has been proposed to attenuate TGF-β signaling leading to increased pathway activity in diseased arteries. We analyzed HtrA1-deficient mouse brain tissue and mouse and CARASIL patient fibroblasts and found a reduction in signaling activity on various pathway levels suggesting a facilitating role of HtrA1. Moreover, we identified LTBP-1 as a novel HtrA1 substrate and provide evidence for its functional modulation by HtrA1-dependent proteolysis. Our data suggest down-regulation of TGF-β signaling as a key mechanism underlying CARASIL pathogenesis.

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binding protein-1 (LTBP-1), a matricellular factor with a major role in TGF-β bioactivation (16, 22) as a novel physiological HtrA1 substrate and report on the functional consequences of its processing. Our findings point to a down-regulation of the TGF-β pathway in CARASIL pathogenesis and suggest LTBP-1 as a key HtrA1 substrate.

Results

Loss of HtrA1 Function Results in Reduced TGF-β Signaling. To study the consequences of HtrA1 deficiency on the TGF-β pathway we made use of a knockout mouse model generated by gene trapping. Insertion of the trapping vector into the first intron of the *HTRA1* gene resulted in the generation of a truncated transcript lacking exons 2–9 (Fig. S1 *A* and *B*). Loss of HtrA1 expression was demonstrated in various tissues on mRNA level by real-time PCR (Fig. 1*A*) and in mouse brain on protein level by combined immunoprecipitation/immunoblotting (Fig. 1*B*). TGF-β signaling was analyzed by immunohistological analysis of SMAD2/3 phosphorylation, an intracellular indicator of TGF-β activity, using mouse brain sections. Unexpectedly, we observed a significant decrease in HtrA1-deficient mice (Fig. 1*C*), which was confirmed by Western blot analysis of brain lysates (Fig. S2). We further analyzed mRNA levels of connective tissue growth factor (CTGF), a well known TGF-β target gene (23), and detected a ∼50% reduction in the brains from young and aged *HTRA1* knockout animals (Fig. 1*D*).

To further substantiate these findings, fibroblasts were isolated from wild-type or HtrA1-deficient mouse embryos and immortalized by serial passaging yielding several independent mouse embryonic fibroblast (MEF) lines. HtrA1 expression or deficiency was confirmed by real-time PCR (Fig. S3*A*) and by immunoblotting of culture medium (Fig. S3*B*). To analyze the TGF-β pathway, we first quantified TGF-β1 in MEF culture medium using an ELISA and observed a ∼40% reduction in *HTRA1* knockout cells (Fig. 2*A*, *Left*). Levels of bioactive TGF-β determined by a cellular reporter assay based on secreted alkaline phosphatase (SEAP) (24) (Fig. S4 *A* and *B*) were also decreased (Fig. 2*A*, *Right*). We next analyzed phosphoSMAD2/3 levels by immunoblotting and similar to brain tissue observed a strong reduction in HtrA1-deficient cells (Fig. 2*B*). Moreover, these cells expressed markedly decreased mRNA levels of the TGF-β target genes CTGF and plasminogen activator inhibitor 1 (PAI-1) (Fig. S3*C*). In summary, these results indicated reduced TGF-β pathway activity in HtrA1-deficient mice.

To confirm these findings in human cells, we used skin fibroblasts isolated from a CARASIL patient of Pakistanian origin, who showed typical signs of cerebral small vessel disease as well as lumbar spondylosis and alopecia and was found to carry a previously unreported HtrA1 mutation (A173T). In culture medium of patient fibroblasts, both total and bioactive TGF-β levels were decreased by ∼80% in comparison with cells from a control individual (Fig. 2*C*). Moreover, SMAD2/3 phosphorylation could not be detected in lysates from these cells (Fig. 2*D*) and CTGF as well as PAI-1 mRNA levels were strongly reduced (Fig. 2*E*). These findings confirmed our data obtained in mice suggesting a facilitating role of HtrA1 in TGF-β signaling. Because the observed effects were in contrast to previously reported observations and could not be explained by processing of the proposed HtrA1 substrates mature TGF-β (19, 20), LAP (8), or TGF-β receptors (21), we set out to search for alternative candidate substrates.

HtrA1 Efficiently Processes the N-Terminal Region of LTBP-1. In a first step toward the identification of novel HtrA1 substrates, we determined the localization of HtrA1 in MEF cultures, by immunoblotting of conditioned medium as well as cytosolic and extracellular matrix (ECM) fractions obtained by sequential protein extraction. Although HtrA1 was barely detectable in cell lysates, high levels were present in the medium demonstrating efficient secretion (Fig. 3*A*). Of note, substantial amounts could also

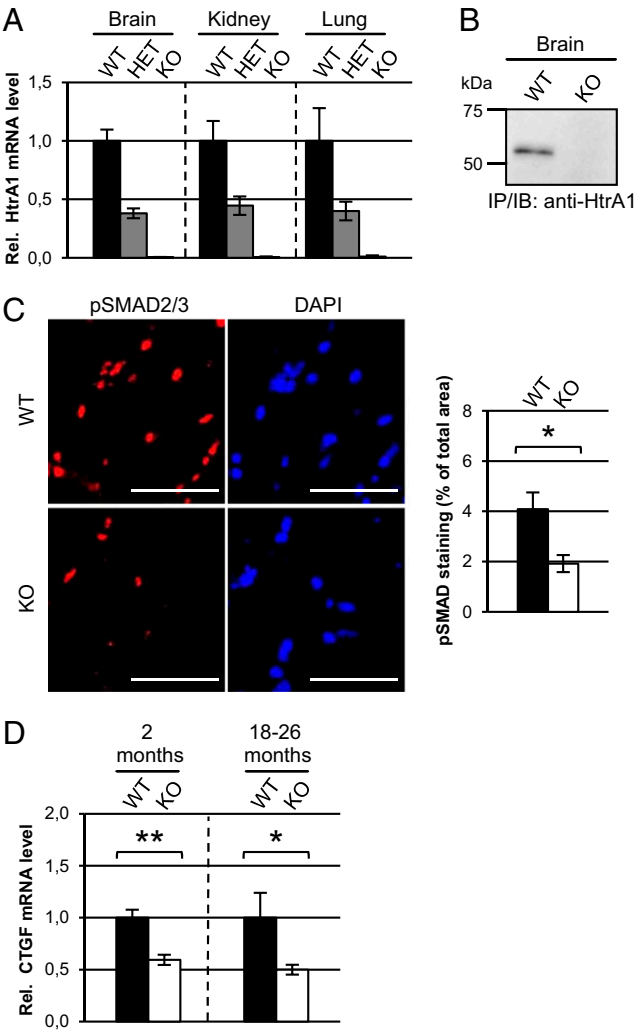

Fig. 1. TGF-β signaling is impaired in HtrA1-deficient mouse brains. (*A*) Relative HtrA1 mRNA levels in mouse brain, kidney and lung were determined by real-time PCR using β-actin for normalization (WT: wild-type; HET: heterozygous; KO: *HTRA1* knockout). The mean value measured for WT mouse tissues was set to 1. $n = 3$. (*B*) Mouse brain lysates were subjected to HtrA1 immunoprecipitation (IP) using rabbit anti-HtrA1 antiserum, followed by HtrA1 immunoblotting (IB) using anti-HtrA1 (23E1) antibody. (*C*) Brain sections of 20- to 26-mo-old mice were stained with phosphoSMAD2/3 antibody (red) and nuclei were visualized with DAPI (blue). (Scale bar: 50 μm.) Quantification of the immunopositive area in corresponding brain regions of *HTRA1*-KO and *HTRA1*-WT mice is shown as percentage of the total image area. $n = 6$. (*D*) Relative CTGF mRNA levels from 2 and 18- to 26-mo-old mouse brains were determined by real-time PCR. 2 mo: $n = 4–7$; 18–26 mo: 7–10. Results are expressed as mean ± SEM; $*P < 0.05$, $**P < 0.01$.

be observed in the ECM fraction, characterized by the presence of fibronectin, prompting us to consider ECM-associated proteins with a role in TGF-β signaling as promising HtrA1 substrates.

Members of the latent TGF-β binding protein (LTBP) family are part of the large latency complex (LLC) that anchors TGF-β to the ECM (22). LTBP-1 is the best characterized member of this family and undergoes proteolytic processing resulting in activation of TGF-β (25–28). We therefore investigated LTBP-1 as a potential HtrA1 substrate using an in vitro proteolysis assay involving treatment of culture medium from LTBP-1-transfected cells with increasing concentrations of purified, recombinant HtrA1. Immunoblotting revealed a dose- and time-dependent conversion of full-length LTBP-1 to a fragment of lower molecular

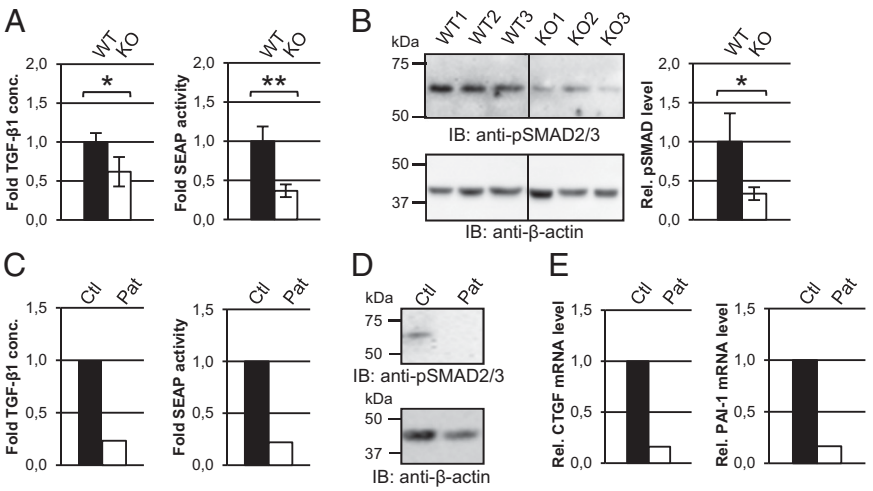

Fig. 2. Decreased TGF-β signaling in HtrA1-deficient and patient fibroblasts. (*A*) Culture medium from MEF cell lines was used to measure TGF-β1 protein concentration by ELISA (*Left*) and bioactive TGF-β levels by a cellular bioassay using secreted alkaline phosphatase (SEAP) as readout (*Right*). After normalization to total protein amounts in the medium HtrA1-WT values were set to 1. (*B*) MEF lysates were analyzed by immunoblotting using an anti-phosphoSMAD2/3 or an anti-β-actin antibody. PhosphoSMAD signals were quantified densitometrically and normalized to β-actin. (*C*) Culture medium from skin fibroblasts derived from a control individual (Ctl) or from a CARASIL patient (Pat) was collected. Total (*Left*) and bioactive TGF-β1 levels (*Right*) were measured. (*D*) Human fibroblast lysates were analyzed by immunoblotting using an anti-phosphoSMAD2/3 or an anti-β-actin antibody. (*E*) CTGF and PAI-1 mRNA levels were measured by real-time PCR and normalized to β-actin. Results are representative of at least two independent experiments.

weight (Fig. 3*B* and Fig. S5*A*). Processing was detected at HtrA1 concentrations as low as 1 nM, whereas degradation of LAP or mature TGF-β required much higher concentrations (100–1,000 nM, Fig. 3*B*). The generation of a stable LTBP-1 cleavage product indicated limited proteolysis rather than global degradation (Fig. S5*B*). To determine the effect of HtrA1 loss-of-function mutations, LTBP-1 proteolysis was analyzed using conditioned supernatants from HEK293 cells transfected with constructs encoding wild-type HtrA1, the active site mutant S328A or various CARASIL mutants including A173T (Fig. S6). Whereas medium containing wild-type HtrA1 produced a cleavage pattern similar to that obtained with purified protease, medium from cells expressing mutant HtrA1 left LTBP-1 mainly intact despite efficient secretion (Fig. 3*C*). We, therefore, conclude that LTBP-1 represents an efficient, previously unknown substrate for HtrA1.

To localize the cleavage region, we used epitope-tagged and truncated LTBP-1 expression constructs (Fig. 4*A*). Detection of cleavage products derived from full-length LTBP-1 containing epitope tags at both ends revealed a C-terminal fragment similar to that observed with the untagged LTBP-1 construct (Fig. 4*B*, *Left*) and an array of poorly defined 40–60 kDa N-terminal fragments (Fig. 4*B*, *Right*) indicating proteolysis within the N-terminal region. Accordingly, we found no cleavage of an N-terminal deletion variant (ΔN-LTBP-1) (Fig. 4*C*, *Left*), whereas a C-terminally truncated variant (ΔC-LTBP-1) was clearly processed (Fig. 4*C*, *Center*). To map the precise cleavage site, we purified ΔC-LTBP-1 and exposed it to purified HtrA1 resulting in the appearance of two major cleavage products, with the larger one corresponding in size and immunoreactivity to the fragment observed before (Fig. 4*C*, *Right*). Analysis of both fragments by Edman degradation revealed identical N-terminal sequences demonstrating cleavage between the Val^{224}-Ala^{225} and Ala^{225}-Ala^{226} bonds of LTBP-1. Hence, HtrA1 cleaves within a region located upstream of the known protease-sensitive hinge region of LTBP-1 (Fig. 4*A*).

To analyze LTBP-1 processing by endogenous HtrA1, we performed the cleavage assay with concentrated culture medium from MEF cells containing HtrA1 concentrations in the range of 5–25 nM. Wild-type cell culture medium converted LTBP-1 to a fragment similar to that generated by purified or HEK293 cell-derived HtrA1, whereas medium from HtrA1 knockout cells left LTBP-1 intact (Fig. 5*A*). Processing was abolished by the selective HtrA1 inhibitor NVP-LBG976 (10), but persisted in the presence of the metalloprotease inhibitor EDTA confirming the specificity of the cleavage (Fig. 5*B*). Altogether these observations point to HtrA1 as a major LTBP-1-processing protease and indicate an impairment of LTBP-1 cleavage in the absence of HtrA1.

HtrA1-Mediated LTBP-1 Proteolysis Prevents Its Association with Fibronectin and the ECM. HtrA1-mediated processing occurs within a region mediating LTBP-1 interaction with fibronectin

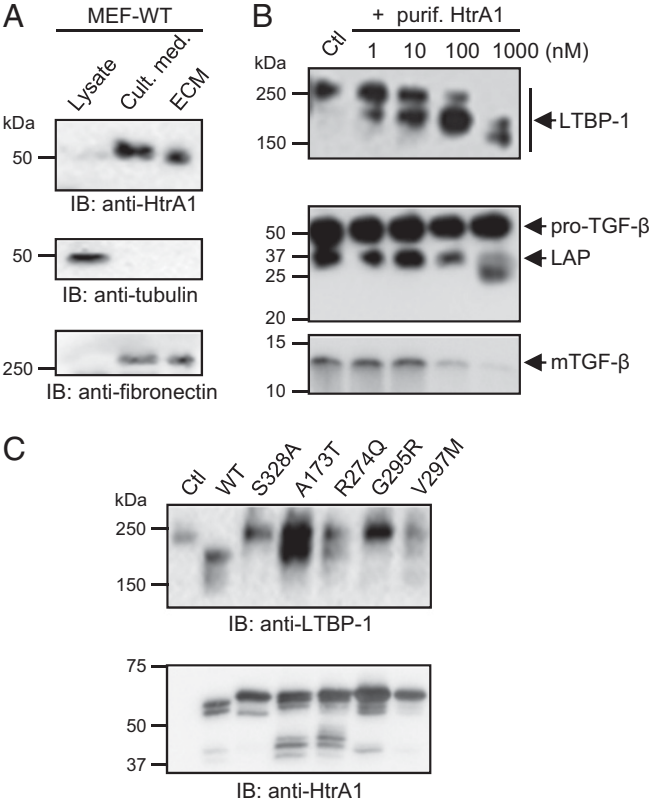

Fig. 3. HtrA1 efficiently processes LTBP-1. (*A*) Upon sequential extraction, HtrA1 expression was analyzed by immunoblotting (IB) in MEF lysate, culture medium (Cult. med.) and extracellular matrix (ECM). All fractions were also probed with anti-tubulin and anti-fibronectin antibodies. (*B*) Medium from LTBP-1- (*Upper*) or TGF-β1- (*Lower*) expressing HEK293 cells was treated with the indicated concentrations of purified HtrA1 or with PBS (Ctl) followed by immunoblotting. pro-TGF-β: TGF-β proform; LAP: latency-associated peptide; mTGF-β: mature TGF-β. (*C*) LTBP-1-containing medium was exposed to medium from vector-transfected cells (Ctl) or cells expressing either wild-type HtrA1 (WT) or one of four different CARASIL mutants. LTBP-1 (*Upper*) and HtrA1 (*Lower*) were detected by immunoblotting.

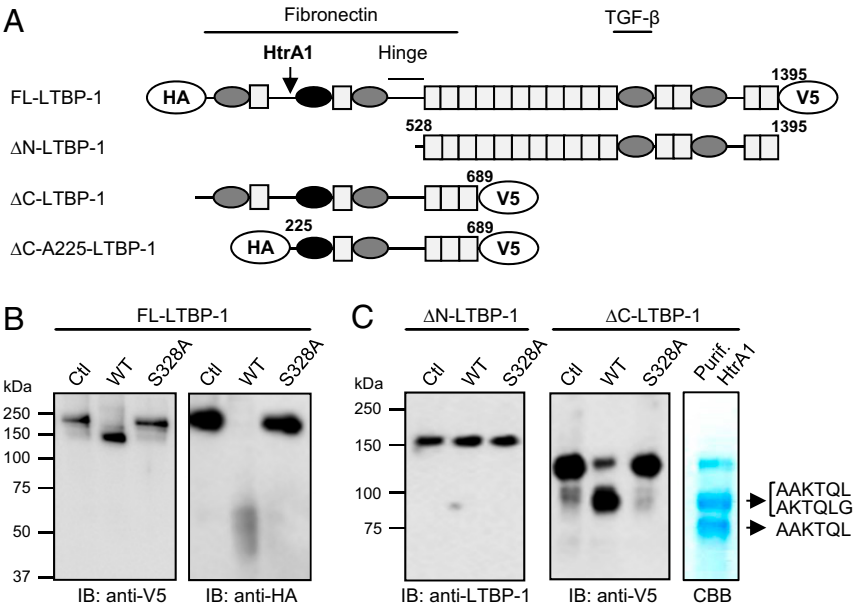

Fig. 4. Mapping of the HtrA1 cleavage site within LTBP-1. (*A*) Domain organization of LTBP-1 including cysteine-rich domains (gray ovals), a hybrid domain (black oval) and EGF-like domains (rectangles). The protease-sensitive hinge region as well as binding regions for fibronectin and TGF-β are shown. The HtrA1 cleavage site is indicated by an arrow. Numbers represent amino acids. The various LTBP-1 constructs used in this study are depicted. HA: Hemagglutinin tag; V5: V5 tag. (*B*) Medium from HEK293 cells expressing full-length (FL-) LTBP-1 was exposed to medium from vector- (Ctl), HtrA1 WT-, or HtrA1 S328A-transfected cells and LTBP-1 analyzed by immunoblotting. (*C*) HEK293 cell-derived ΔN-LTBP-1 (*Left*) or ΔC-LTBP-1 (*Center*) was treated with control, HtrA1 WT-, or HtrA1 S328A-containing medium and immunoblotting was performed. Purified ΔC-LTBP-1 (*Right*) was treated with purified HtrA1 and upon SDS/PAGE and Coomassie brilliant blue (CBB) staining two proteolytic fragments were isolated and subjected to Edman degradation. The resulting amino-terminal sequences are indicated. Note that the smaller fragment (~75 kDa) could not be detected by an anti-V5 antibody indicating carboxyl-terminal processing.

(Fig. 4*A*) (29, 30), a crucial step during LTBP-1 incorporation into the ECM (31, 32). To investigate the consequences of HtrA1-dependent LTBP-1 processing on this interaction, we established an in vitro solid-phase assay measuring binding of HEK293 cell-derived LTBP-1 to immobilized purified fibronectin (Fig. S7*A*). In this assay, LTBP-1 showed a dose-dependent binding (Fig. S7*B*), that was restricted to fibronectin among a set of adhesive matrix proteins (Fig. S7*C*). An association was also observed with ΔC-LTBP-1, but not with ΔN-LTBP-1 lacking the fibronectin binding region (Fig. S7*D*). Treatment of full-length LTBP-1 or ΔC-LTBP-1 with purified HtrA1 or HtrA1-containing culture medium before the binding assay drastically decreased their binding to fibronectin, an effect that was not detected upon treatment with culture medium containing the active site mutant S328A (Fig. 6*A*). Similarly, ΔC-A225-LTBP-1, a truncated form mimicking the proteolytic fragment generated by HtrA1 (see Fig. 4*A*), showed an equally reduced binding suggesting a role of HtrA1-mediated proteolysis in regulating the LTBP-1–fibronectin interaction (Fig. S7*E*).

Next, we adapted our binding assay to measure the interaction of LTBP-1 with the ECM produced by primary murine aortic smooth muscle cells (AoSMC). After 7 d in culture, these cells have deposited a matrix containing an extensive fibronectin network (Fig. S7*F*). Following matrix production, AoSMCs were fixed, exposed to LTBP-1–containing cell culture medium and binding was quantified as described before. In contrast to ΔN-LTBP-1, full-length LTBP-1 and ΔC-LTBP-1 showed efficient binding (Fig. S7*G*), which was significantly reduced upon prior treatment with purified HtrA1 (Fig. 6*B*). These findings indicated that HtrA1-mediated LTBP-1 cleavage prevents its association with the ECM.

Finally, we transfected MEF cells with either ΔC-LTBP-1 or ΔC-A225-LTBP-1 and evaluated their integration into the ECM. Sequential extraction revealed that both LTBP-1 forms were present in comparable levels in the tubulin-rich cellular fraction and in the culture medium (Fig. 6*C*) demonstrating equally efficient expression and secretion. However, ΔC- LTBP-1 showed a much stronger accumulation in the matrix fraction than ΔC-A225-LTBP-1. Immunofluorescence analysis confirmed the impaired association of the ΔC-A225-LTBP-1 variant with the ECM (Fig. 6*D*).

Discussion

By analyzing brain tissue and embryonic fibroblasts from *HTRA1* knockout mice as well as primary fibroblasts from a CARASIL patient we found loss of HtrA1 function to be associated with down-regulation of the TGF-β pathway. We further identified LTBP-1, a key extracellular regulator of TGF-β bioactivation (22), as a novel HtrA1 substrate. These findings shed light on the role of HtrA1 in TGF-β signaling and advance our understanding of the molecular mechanisms underlying CARASIL pathogenesis.

A physiological relevance of LTBP-1 processing by HtrA1 is strongly suggested by the following observations: First, proteolysis of LTBP-1 in vitro occurred at HtrA1 concentrations as low as 1 nM, which is in the physiological range (33). In contrast, cleavage of mature TGF-β and LAP required 10- to 100-fold higher protease concentrations making it unlikely that they represent biologically relevant substrates. Second, we provide direct evidence for substrate cleavage by endogenous, MEF-derived HtrA1, whereas previous studies used purified or overexpressed HtrA1. Of note, overexpression may cause protease retention in the endoplasmic reticulum, where Shiga et al. (8) observed LAP degradation. Third, we found LTBP-1 processing to be prevented by HtrA1 deficiency, by a specific HtrA1 inhibitor and by CARASIL loss-of-function mutations arguing for the specificity of the

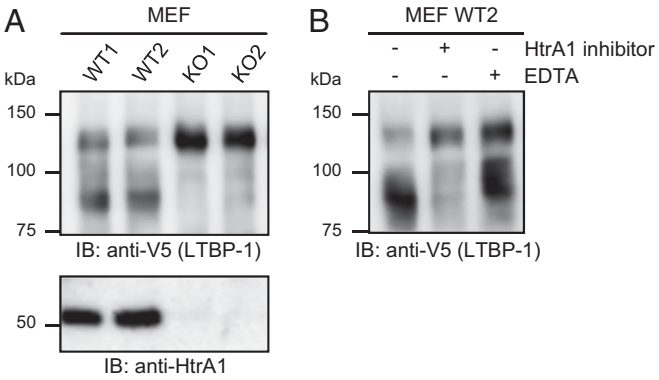

Fig. 5. LTBP-1 processing by endogenous HtrA1. (*A*) Concentrated culture medium from wild-type (WT) or *HTRA1*1 knockout (KO) MEF lines was incubated with ΔC-LTBP-1-containing medium. LTBP-1 and HtrA1 were analyzed by immunoblotting using anti-V5 and anti-HtrA1 (16C8) antibodies. (*B*) ΔC-LTBP-1 was exposed to medium from the HtrA1-WT2 line in the absence or presence of a selective HtrA1 inhibitor or EDTA, before LTBP-1 was immunodetected.

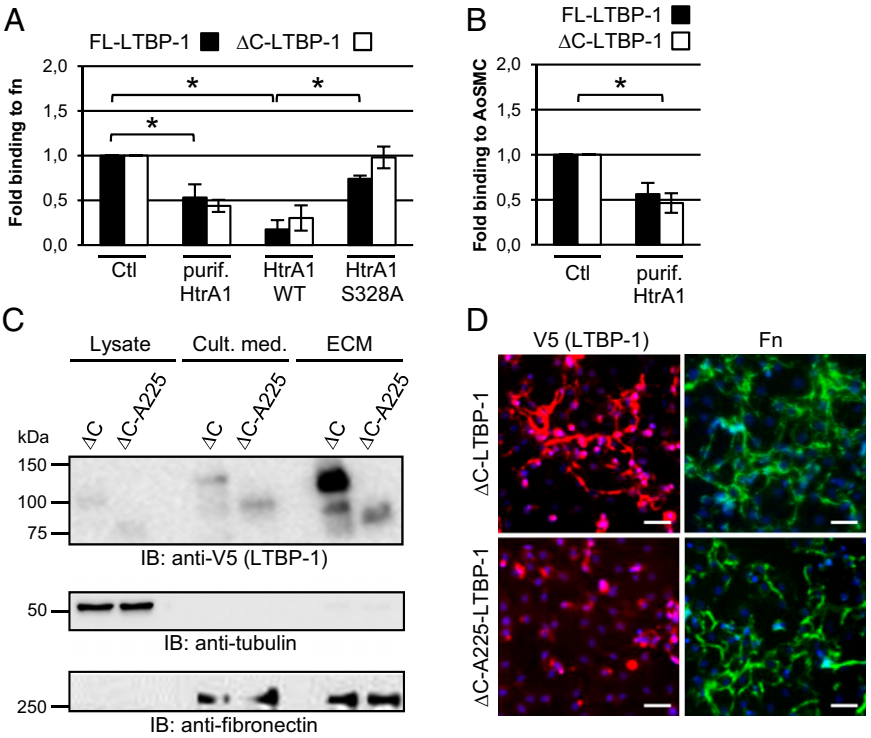

Fig. 6. Regulation of the LTBP-1 interaction with ECM by HtrA1-mediated proteolysis. (*A*) HEK293 cell-derived full-length LTBP-1 (FL-LTBP-1) or ΔC-LTBP-1 was incubated with PBS or medium from vector-transfected cells (Ctl), with 250 nM purified HtrA1 or with medium containing HtrA1-WT or -S328A, before LTBP-1 binding to immobilized fibronectin was measured and values obtained by control treatment were set to 1. (*B*) HEK293 cell-derived FL-LTBP-1 or ΔC-LTBP-1 was incubated with PBS (Ctl) or 250 nM purified HtrA1 before binding to fixed AoSMC cultures was measured. Results are expressed as mean ± SEM of 4–6 experiments; $*P < 0.05$. (*C*) Cell culture medium (Cult. med.) as well as cellular (Lysate) and matrix (ECM) fractions from MEF cells transfected with plasmids encoding ΔC-LTBP-1 or ΔC-A225-LTBP-1 were analyzed by anti-V5 (LTBP-1), anti-tubulin or anti-fibronectin immunoblotting. (*D*) Transfected MEF cells were subjected to immunofluorescence microscopy using anti-V5 (LTBP-1) or anti-fibronectin antibodies. (Scale bar: 50 μm.)

cleavage. At least in cultured fibroblasts, HtrA1 appears to represent the predominant LTBP-1 processing protease. Fourth, the efficient secretion of HtrA1 and incorporation into the ECM of cultured fibroblasts demonstrates spatial proximity of protease and substrate. Finally, we found LTBP-1 to undergo limited processing rather than global degradation. Site-specific LTBP-1 proteolysis has been shown to result in its detachment from ECM and in TGF-β release and activation (25–28). In accord with this finding, we observed decreased LTBP-1 incorporation into ECM upon HtrA1-mediated cleavage and an attenuation of TGF-β activity in HtrA1-deficient tissues and cells. Our findings in fibroblasts suggest a dysregulation of TGF-β signaling also in noncerebral tissue, a systemic effect is nevertheless unlikely because TGF-β pathway alterations were not observed in several other tissues and serum from *HTRA1* knockout mice.

Our findings contrast with previous studies that have reported an increase in TGF-β signaling in CARASIL brains (6, 8). However, they were restricted to two autopsy cases with limited information on histopathological samples and it cannot be excluded that the observations reflect fibrotic changes of blood vessels in advanced disease stages. On the other hand, *HTRA1* knockout mice and patient fibroblasts, the model systems used in our study, do not cover all clinical and molecular aspects of CARASIL. *HTRA1* knockout mice are viable, have a normal life expectancy and display no obvious disease symptoms. Although a detailed characterization of these mice is in progress, studies of other monogenic SVDs have revealed that human pathology is difficult to recapitulate in mouse models (34). Despite these limitations we nevertheless consider the consistency of results obtained from two different species as good evidence for their disease relevance. Future studies will have to address the discrepancies in TGF-β signaling activity and to validate HtrA1-mediated LTBP-1 processing as a mechanism driving CARASIL pathogenesis.

Our findings add to current evidence suggesting a role of the TGF-β signaling pathway for normal vascular function. Of note, mutations in components of this pathway or its interacting partners result in severe vascular defects, as illustrated by Loeys-Dietz syndrome, hereditary hemorrhagic telangiectasia, and Marfan syndrome (18). Specifically, our results reinforce a role of ECM-mediated TGF-β sequestration which is considered a crucial step in the temporal and spatial regulation of TGF-β activity (35). TGF-β incorporation into the ECM is mediated by interactions between LTBPs and two major structural ECM constituents, fibrillin-1 and fibronectin (36). Importantly, Marfan syndrome has not only been attributed to fibrillin-1 mutations, but also shown to be associated with altered TGF-β signaling (37).

Interestingly, dysregulated TGF-β signaling has also been implicated in sporadic cerebral small vessel disease (38). In addition, data from a recent study conducted in our laboratory indicate a role of LTBP-1 in the pathogenesis of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (39), another monogenic SVD showing considerable phenotypic overlap with CARASIL.

In conclusion, our findings provide an unexpected link between the TGF-β signaling pathway and CARASIL which might also be of relevance to the more common forms of cerebral small vessel disease.

Materials and Methods

Mice. Homozygous *HTRA1*$^{-/-}$ mice (strain HtrA1$^{Gt(OST394864)Lex}$) were generated in collaboration with Taconic (Hudson) by gene trapping. See *SI Materials and Methods* for details.

Cell Culture. Mouse embryonic fibroblasts were isolated from 14.5-d post coitus embryos and immortalized by serial replating. Human fibroblasts were obtained from skin biopsies of a CARASIL patient bearing a homozygous *HTRA1* mutation (A173T) and a healthy control individual. Mouse aortic smooth muscle cells (AoSMC) were isolated from C57BL/6 mice.

Biochemical Assays. Primers used for PCR are listed in Table S1. Details of the protein purification, the solid-phase binding and proteolysis assays, and immunoblotting/immunofluorescence procedures are described in *SI Materials and Methods*.

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