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Abstract

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Cathepsin D enhances breast cancer invasion and metastasis through promoting hepsin ubiquitin-proteasome degradation

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Abstract

Hepsin is required for the growth and maintenance of normal morphology, as well as for cell motility and development, initiation of blood coagulation and pro-inflammatory immune response. Here we showed that Cathepsin D (CtsD) as a novel protein is involved in the regulation of hepsin. CtsD destabilizes hepsin by promoting its ubiquitylation and subsequent proteasomal degradation in breast cancer cells. Breast cancer tissue microarray also indicated that hepsin expression was negatively correlated with CtsD by immunohistochemistry. Overexpression of CtsD promoted breast cancer cell migration, invasion and metastasis by enhancing the expression of intercellular cell adhesion molecule-1 (ICAM-1) *in vitro* and *in vivo*. These effects were inhibited by ectopic hepsin expression. Taken together, our data reveal a critical CtsD-hepsin signaling axis in migration and metastasis, which may contribute to a better understanding of the function and molecular mechanism in breast cancer progression.

Keywords: CtsD, hepsin, G2E3, ICAM-1, breast cancer

Introduction

Hepsin is a type II transmembrane serine protease frequently overexpressed in most tissues and different tumors, including breast cancer [1]. *In vitro* studies suggest hepsin is required for the growth and maintenance of normal morphology [2], as well as for cell motility [3] and development[4], initiation of blood coagulation [5] and pro-inflammatory immune response [6]. Low mRNA and protein expression of hepsin not only are positively associated with advanced breast cancer tumor malignancy, but also predict poor breast cancer survival [7]. However, contrary results also reported that hepsin overexpression was significantly associated with tumor stage, lymph node metastasis in patients with breast cancer [1]. Down-regulation of hepsin significantly reduced cell proliferation and invasion in breast cancer cells (BCC) [1]. It raised the question on the function and mechanism of hepsin in breast cancer. Therefore, the studies to understand the exact physiological role and mechanism of hepsin in breast cancer may help in resolving the contradictory results.

Cathepsins were originally identified as lysosomal proteases, but recent work highlighted their atypical roles in the extracellular space, cytoplasm and nucleus [8]. Among these, Cathepsin D (CtsD) is overproduced by BCC and the pro-enzyme is abundantly secreted in the tumor microenvironment [9]. Indeed, CtsD stimulates BCC proliferation, fibroblast outgrowth, angiogenesis, breast tumor growth and metastasis formation [10-17]. CtsD is also an independent marker of poor prognosis for breast cancer associated with metastasis [18, 19]. Secreted CtsD enhances proteolysis in the breast tumor microenvironment by degrading the cysteine cathepsin inhibitor cystatin C [20] and promotes mammary fibroblast outgrowth by binding to LDL receptor-related protein-1 (LRP1) [21].

In this study, we demonstrate that CtsD promotes hepsin ubiquitylation and its subsequent proteasomal degradation in BCC. Moreover, our data reveal a critical CtsD-hepsin signalling axis in modulating migration and metastasis, which may contribute to a better understanding of the molecular mechanism in breast cancer progression.

Materials and Methods

Cell culture and reagents

293T and human BCC lines, MCF-7, T47D, ZR-75-1 and MDA-MB-231 cells were maintained in 5% CO_2 at 37°C. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco; USA). Cell transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Recombinant human ICAM-1 (rhICAM-1) was purchased from Raybiotech (USA). ICAM-1 inhibitor A-205804 was purchased from Selleck (USA).

Clinical samples

Breast cancer samples for real-time PCR were obtained from the Department of General Surgery, ZhongShan Hospital, Fudan University, Shanghai, China. The use of human tissue samples and clinical data was approved by the Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University. All donors provided written informed consent to donate their samples. All methods were taken in accordance with the approved guidelines of School of Medical graduate Fudan University.

Tissue microarray slides of clinical tumor samples in this study were obtained from the National Engineering Research Center important project on bioarray sample set, which includes 190 cases of breast cancer cases randomly selecting from the Shanghai Outdo Biotech Biobank. Their age ranged from 27 to 85 years (mean, 53.4±11.7 years). The diagnosis of breast cancer was confirmed by pathologic examination. Staging data were according to the seventh edition of the AJCC Cancer Staging Manual [22]. Our study was approved by the Research Ethics Committee of Shanghai Outdo Biotech. Informed written consents were obtained from all of the patients and this study was carried out in compliance with the Declaration of Helsinki.

Plasmid construction and RNA interference

Hepsin was amplified by PCR from human cDNA library and inserted into pcDNA3.1A/V5-His vector. CtsD and CtsB were amplified by PCR from human cDNA library and inserted into pcDNA3.1/myc vector. C9-CtsS and c9-CtsL were purchased from Addgene (USA). GFP-G2E3 was purchased from Origene (USA). SiRNA1 (5'- GUGGACCAGAACAUCUUCUTT -3' 5'-AGAAGAUGUUCUGGUCCACTT -3') or siRNA2

(5'-CUCUGUCCUACCUGAAUGUTT-3' 5'- ACAUUCAGGUAGGACAGAGTT -3' for human CtsD genes (Genepharma, China) were used to transfect T47D and ZR-75-1 cells at concentrations of 50 nM, according to the manufacturer's instructions. SiRNA (5'-GGUGUACAAUAUGCAAUAATT-3' 5'-UUAUUGCAUAUUGUACACCTT -3') for human G2E3 gene (Genepharma, China) were used to transfect T47D and ZR-75-1 cells at different concentrations, according to the manufacturer's instructions. A non-targeting siRNA (5'-uucuccgaacgugucacgutt-3' 5'- acgugacacguucggagaatt -3' (Genepharma, China) was used as a negative control. 72h after transfection, cellular proteins were analyzed by western blotting.

In vivo tumorigenesis assay

Female SCID mice (4–6 weeks old) were purchased from Slac (Shanghai, China) and maintained under specific pathogen-free conditions. All procedures were approved by the Institutional Animal Care and Use Committee at the Medical Center of Fudan University and conformed to the legal mandates and federal guidelines for the care and maintenance of laboratory animals. Mice were injected with the MCF-7-luc cells and corresponding stable clones with CtsD expression or CtsD/hepsin co-expression (5×10^5 cells per mouse) via tail vein injection. Lung metastasis was monitored by the lumina K bioluminescence imaging system. Plasma sICAM-1 secretion was detected using commercially available ELISA kit (Raybiotech**,** USA) according to the manufacturer's protocol.

Immunohistochemical staining and scoring

Immunohistochemistry was performed using a two-step procedure following the protocol recommended by Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+ (Dako, Denmark). Proteins were detected with anti-CtsD antibody (Cell Signal Technology, USA), anti-hepsin and anti-G2E3 antibodies (Abcam, UK). Depending on the staining area, the score of protein expression was conducted: 0, 0-5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, > 75%, and the staining intensity was categorized as follows: no staining scored 0, weakly staining scored 1, moderately

staining scored 2 and strongly staining scored 3, respectively. Composite expression score (CES) is calculated from intensity and area measurements for immunostaining (CES = 4*(intensity score - 1) + area score), yielding a series of results ranging from 0 to 12.

Protein identification by mass spectrometry

V5-hepsin immunoprecipitated material was separated by SDS-PAGE and Coomassie brilliant blue staining. Protein identification was performed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and tandem mass spectrometry (MS-MS). The protein band of interest was trypsin-digested. MALDI-TOF and MS-MS analysis of the trypsin digests were performed on a Voyager reflector instrument (Applied Biosystems, USA) and a Q-STAR (PerSeptive Biosystems, USA) in positive ion mode. Protein sequence data base searching was performed with MS-Fit and Mascot.

Western blotting

For immunoblotting, total protein extracts were prepared using lysis buffer (1% NP-40, 500 mM NaCl, 5 mM EDTA, 50 mM NaF, 20 mM Tris-HCl [pH 7.5], 1 mM Na_3NO4 and 10 μM Na_2MnO_4) in the presence of proteinase inhibitor mixture (Roche Applied Science, Switzerland). Protein samples were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). Blots were blocked with PBS containing 5% milk and 0.1% Tween and then incubated with primary antibody. Primary antibodies were anti-V5 antibodies (Invitrogen, USA); anti-HA and anti-hepsin antibodies (Santa Cruz Inc, USA); anti-CtsD, anti-β-actin, anti-cyclin B1, anti-EMT related proteins (Cell Signal Technology, USA); anti-C9 and anti-G2E3 antibodies (Abcam, UK); anti-GFP and anti-myc antibodies (Proteintech, USA).

Cell Surface Protein Labeling

The cells expressing hepsin were labeled with 1 mM EZ Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific, USA) in PBS (pH 8.0) at 4°C for 5 min. The reaction was quenched with 100 mM glycine in PBS. The cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100, and a protease inhibitor mixture. 30ul Streptavidin-Sepharose beads (Thermo Fisher

Scientific, USA) were added to the cell lysate, and the mixture was rotated at 4°C for 2h. After washing, the beads were boiled in sample buffer. Proteins were analyzed by western blotting using primary antibodies.

Immunoprecipitation

For co-immunoprecipitation experiments, cell lysates were incubated with 2 μg relevant antibody at 4°C for 2h. Pre-equilibrated protein G-agarose beads (Roche**,** Switzerland) were added and incubated overnight, collected by centrifugation, and then gently washed three times with the lysis buffer. The bound proteins were eluted and analyzed using western blots.

For immunoprecipitation of cell surface V5-hepsin or endogenous hepsin, cells were incubated with anti-V5 or anti-hepsin antibody for 60 min on ice. After three washes in ice-cold PBS to remove unbound antibody, cells were lysed in lysis buffer. Post-nuclear supernatants were incubated with protein G-agarose beads for overnight at 4°C to capture antibody-bound hepsin. After three washes with the lysis buffer, samples were eluted in SDS sample buffer for 10 min at 95°C, separated by SDS-PAGE and for western blotting.

In vitro migration and invasion assays

Transwell migration and invasion assays were performed in 24-well transwell plates according to the manufacturer's instructions (Millipore, USA). The bottom of transwell chamber was coated without or with BD Matrigel Basement Membrane Matrix (BD Biosciences, USA) for migration or invasion. MCF-7 and MDA-MB-231 cells in basic culture medium without serum were seeded in the upper chamber, while the bottom chambers were filled with culture medium containing 20% FBS as a chemo-attractant. Migration and invasion of cells were determined 40h later. Non-invading cells on the upper side of the chamber were removed from the surface of the membrane by scrubbing. The infiltrating cells were fixed with 4% paraformaldehyde and stained with crystal violet, and cell numbers were counted from five fields. Each experiment was repeated three times.

Real-time polymerase chain reaction (PCR)

Total RNA in the clinical samples was isolated using TRIzol reagent (Gibco BRL and Life Technologies**,** USA) according to the manufacturer's instruction. Total mRNA was converted to cDNA using AMV reverse transcriptase (Takara, Japan). Real-time PCR was carried out to amplify cDNA using SYBR Premix Ex Taq (Takara, Japan). Hepsin and CtsD mRNA levels were evaluated using specific primers after normalization with glyceraldehyde3-phosphate dehydrogenase (GAPDH). The primer sequences were: hepsin, 5'-GTCTGCAATGGCGCTGACTTC-3' (sense) and 5'-TCCGAGAGATGCTGTCCTCACA-3' (anti-sense); CtsD, 5'-GCAAACTGCTGGACATCGCTTG-3' (sense) and 5'-CCATAGTGGATGTCAAACGAGG-3' (anti-sense); GAPDH, 5'-GTCTCCTCTGACTTCAACAGCG-3' (sense), and 5'-ACCACCCTGTTGCTGTAGCCAA-3' (anti-sense).

Cytokine antibody arrays

The level of secreted cytokines was analyzed in the supernatants of tumor cells by using a human inflammation antibody array (RayBiotech, USA). Transfected cells at a density of 1×10^6 cells/ml were plated in serum-free medium for 48h; supernatants were collected and analyzed following the manufacturer's recommendations.

Enzyme-linked immunosorbent assay (ELISA)

MCF-7 and MDA-MB-231 cells were cultured in 24-well plates. After transfection for 48h, the supernatants were collected and human IL-6 and IL-1β secretion was measured using commercially available ELISA kits (RD, USA) according to the manufacturer's protocol. Human PDGF-BB and human sICAM-1 secretion was detected in the same way (Raybiotech, USA) according to the manufacturer's protocol. The absorbance value was determined at 450 nm using Bio-Tek ELX800 microplate reader (Winooski, USA) within 30 min.

Statistical analysis

Experimental data were presented as means ±S.D. of at least three independent replicates. The correlation among the mRNA and protein obtained by real-time PCR and immunohistochemistry was determined using Spearman's rank correlation test in SPSS version 19.0. and R version 3.3.3. Groups were compared using a two-tailed

Student's t-test. *p<0.05, **p<0.01 and ***p<0.001 were considered significant.

Results

Identification of CtsD as a binding protein of hepsin

First, immunoprecipitates obtained with IgG or anti-V5 hepsin antibody were resolved on an SDS-polyacrylamide gel. Coomassie Blue staining of the gel revealed a ~45 kDa band in V5-bound immunoprecipitates (Supplementary Fig. 1). Mass spectrometry was used for identification of the protein in V5-bound immunoprecipitates. From the peptide sequencing, a total of 4 experimental masses matched theoretical masses from hepsin (Table 1), and Figure. 1A showed the sequencing of 11 consecutive amino acids. Because CtsD is a marker of poor prognosis in breast cancer, so we detected CtsD and hepsin expression in different BCC and normal human breast epithelial (MCF-10A) cell line. Western blot analysis revealed that the BCC exhibited elevated CtsD and reduced hepsin expression, albeit to varied extent, as compared with MCF-10A (Fig. 1B). The expression of CtsD and hepsin protein was also examined in a tissue microarray containing 30 pairs of breast tissues by IHC staining analysis. The IHC density of hepsin exhibited a decrease in breast cancer tissues compared with their matched peritumor tissues ($P < 0.05$) (Supplementary Fig. 2). On the contrary, the IHC density of CtsD exhibited a significant increase in breast cancer tissues compared with their matched peritumor tissues ($P < 0.001$) (Supplementary Fig. 2). To determine whether CtsD interacts with hepsin, we performed co-immunoprecipitation experiments in transfected MCF-7 and MDA-MB-231 cells with V5-hepsin and myc-CtsD. CtsD was readily detected in association with hepsin in lysates (Fig. 1C). To establish whether CtsD binds hepsin at the cell surface, we labelled surface hepsin with V5 or hepsin-specific antibody at 4°C (Fig. 1D). The interaction of exogenous hepsin and CtsD was confirmed in surface of MCF-7 and MDA-MB-231 cells (Fig. 1E). The endogenous CtsD and hepsin interaction was also observed in T47D and ZR-75-1 cells which showed moderate CtsD and hepsin expression (Fig. 1F).

CtsD regulates hepsin expression in BCC

As CtsD can interact with hepsin in human BCC, it is possible that accumulated CtsD protein contributes to modulating the expression of hepsin in BCC. Hepsin is a transmembrane protein, so we examined the membrane expression of hepsin by biotin labeling of cell surface proteins and western analysis (Fig. 2A). As shown in Fig. 2B, ectopic expression of CtsD significantly down-regulated the cell surface hepsin protein level in a dose-dependent manner. Used as a control, β-actin was detected in cell lysates, but not in labeled surface proteins (Fig. 2B). As another control, the biotin-labeled membrane hepsin did not change when the cells were transfected with CtsB, S and L (Fig. 2C). Moreover, knock-down of CtsD induced the up-regulation of hepsin expression in T47D and ZR-75-1 cells (Fig. 2D). CtsD overexpression is implicated in human breast cancer and supports tumor growth and metastasis formation. To examine the correlation between hepsin and CtsD in clinical breast cancer, CtsD and hepsin protein levels were analyzed by immunohistochemical staining on tissue microarray slides (TMA). The TMA contains 190 cases of breast tumour specimens, including 101 luminal and 89 triple-negative breast cancer (TNBC). Immunohistochemical analysis presented a strong negative correlation between CtsD and hepsin expression in clinical breast cancer samples (Supplementary Fig. 3). Correlation analysis also revealed that hepsin expression was negatively correlated with CtsD expression both in luminal breast cancer ($P<0.001$, $r=-0.433$) (Figure. 2E) and TNBC ($P<0.001$, $r=-0.473$) (Figure. 2F).

CtsD promotes hepsin degradation by ubiquitination

To determine whether downregulation of hepsin protein by CtsD occurs at transcriptional level, real-time PCR was performed. **R**esults showed that CtsD transfection had no effect on hepsin mRNA level in MCF-7 cells (Fig. 3A), indicating CtsD regulates hepsin expression mainly at the posttranscriptional level. Real-time PCR results also showed that hepsin mRNA level has no correlation with the CtsD in 32 primary human breast cancer samples (P=0.607, r=-0.094) (Fig. 3B). In Fig. 2B, we had confirmed the impact of CtsD on the protein level of hepsin. We next determined whether CtsD could affect the hepsin stability. 36h post transfection with the empty vector or myc-CtsD, the half-life of hepsin was evaluated by cycloheximide (CHX) chase experiment. As shown in Fig. 3C, hepsin protein was found to display a shorten half-life under myc-CtsD transfected condition, compared to that in control condition, implying that CtsD promoting the degradation of hepsin *in vitro*. To determine whether CtsD induced hepsin degradation is mediated by proteasomal pathway, we treated T47D and ZR-75-1 cells with proteasome inhibitor MG132 and found MG132 significantly blocked hepsin degradation induced by CtsD (Fig. 3D), suggesting CtsD might target hepsin protein to proteasomal degradation. Ubiquitination is a key process that leads to proteasome degradation of cellular proteins. So we employed ubiquitylation ladder assay in order to observe a change in hepsin ubiquitylation. MCF-7 and MDA-MB-231 cells were transfected with HA-tagged ubiquitin, V5-hepsin expression plasmids, and empty vector or myc-CtsD construct for 48h and treated with MG132 or vehicle for 5h (Fig. 3E).

Polyubiquitylation signal of hepsin was detected upon CtsD transfection, moreover, ubiquitination of hepsin was further enhanced in the presence of MG132 (Fig. 3E). These results demonstrate that CtsD down-regulates hepsin expression by promoting its ubiquitination and the subsequent proteasome-mediated degradation.

G2E3 targets hepsin for degradation

Three types of enzymes are required in the process of ubiquitylation: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-ligases (E3s), among which, only E3s define the target specificity of ubiquitylation reaction. To further investigate the mechanism of CtsD induced hepsin proteasomal degradation, we identified it E3 ligase via mass spectrometry, using an anti-V5 antibody for immunoprecipitation in V5-hepsin transfected 293T cells. Among these predicted interacting proteins, we identified G2/M phase-specific E3 ubiquitin-protein ligase (G2E3) as a putative E3 ligase for hepsin. Overexpressed GFP-G2E3 in T47D and ZR-75-1 cells resulted in a significant decrease in hepsin protein, which was totally reversed by adding MG132 to the culture medium (Fig. 4A). As shown in Fig. 4B, ectopic expression of CtsD significantly up-regulated the G2E3 protein level in a dose-dependent manner. Since G2E3 is a critical modulator of cell cycle progression especially during G2/M phase, we analyzed G2E3 expression in transfected MCF-7 and MDA-MB-231cells synchronized at G2/M stage. Cyclin B1 (a specific marker for G2/M stage) expression was increased during the G2/M stage, however, G2E3 expression was not affected in CtsD-transfected cells (Supplementary Fig. 4). Using small interfering RNAs (siRNAs) targeting human G2E3 gene, we showed that blocking G2E3 expression could instantly inhibited hepsin degradation induced by CtsD in a dose-dependent manner (Fig. 4C). Consistently, CHX chase experiment showed that G2E3 knockdown could block the decreased half-life of hepsin induced by CtsD (Fig. 4D). In addition, we performed ubiquitin ladder experiment to visualize ubiquitylation in MCF-7 and MDA-MB-231 cells. Consistent

with Fig. 3E, polyubiquitylation signal of hepsin was detected upon CtsD induced, while HA-tagged ubiquitin could no longer or less be detected after knocking down G2E3 (Fig. 4E). Among total 190 BCC samples used, immunohistochemical and correlation analysis between hepsin, CtsD and G2E3 staining revealed that hepsin expression was negatively correlated with G2E3 expression in luminal breast cancer ($P<0.001$, $r=-0.389$) and TNBC ($P<0.001$, $r=-0.417$); CtsD expression was positively correlated with G2E3 expression in luminal breast cancer ($P<0.001$, $r=0.477$) and TNBC ($P<0.001$, $r=0.571$) (Supplementary Fig. 5A and B).

CtsD promotes the migration and invasion of breast cancer by downregulation of hepsin in vitro.

To further evaluate the prognostic value of CtsD and hepsin in breast cancer, we explored the correlation between protein expression and clinical stage. As shown in Supplementary Fig. 6, high CtsD expression was associated with advanced clinical stage (III), and high hepsin expression was associated with early clinical stage (I) both luminal and TNBC samples. Moreover, *in vitro* transwell assays demonstrated that overexpression of CtsD promoted the migration and invasion of BCC, the additional expression of hepsin inhibited the effect (Fig. 5A, B and Supplementary Fig. 7). Migration and invasion of breast cancer represent the major reason for its poor prognosis. The increased motility and invasive properties of tumor cells occur during epithelial–mesenchymal transition (EMT). CtsD expression induced downregulation of epithelial markers (E-cadherin and Claudin-1) and the upregulation of mesenchymal molecules (N-cadherin, Vimentin and snail). Expression of exogenous hepsin in CtsD-overexpression cells largely rescued the expression of E-cadherin and Claudin-1 (Fig. 5C). In addition, the change of tumor microenvironment has been proposed to contribute to the development of cancer. Our previous study showed that hepsin could up-regulate the secretion of IL-6 and IL-1β in hepatocytes [23]. We therefore assessed the global effect of hepsin on the pattern of cytokines using cytokine antibody arrays in MCF-7 cells. Among the relatively highly expressed cytokines, IL-6 and IL-1β were lightly increased, ICAM-1 and PDGF-BB were

decreased greatly (Fig. 5D). ELISA also confirmed the changed secretion of these factors upon hepsin transfection, respectively (Supplementary Fig. 8A, Fig. 5E). To our surprise, CtsD promoted the secretion of ICAM-1 and had no effect on the secretion of other factors (Supplementary Fig. 8B, Fig. 5E). We next explored the role of ICAM-1 in modulation of migration and invasion in MCF-7 cells. As shown in Supplementary Fig. 9 and Fig. 5F, rhICAM-1 promoted the migration and invasion of hepsin transfected MCF-7 cells *in vitro*. On the contrary, the inhibitor A-205804 decreased the migration and invasion in CtsD transfected MCF-7 cells. Moreover, the addition of rhICAM-1 partially inhibited E-cadherin and claudin-1 upregulation and increased Vimentin, N-cadherin and snail expression in hepsin transfected MCF-7 cells (Fig. 5G). ICAM-1 inhibitor A-205804 exerted the opposite function in CtsD transfected MCF-7 cells (Fig. 5G).

Hepsin rescues CtsD induced breast cancer metastasis in vivo.

To directly assess whether CtsD promotes metastasis *in vivo*, we intravenously injected CtsD-overexpression MCF-7-luc cells into female SCID mice and subjected these mice to bioluminescent imaging. CtsD-overexpression cells exhibited an increasing number of lung nodules (Fig. 6A and B), implying that CtsD can promote BCC metastasis *in vivo*. At 35 days post-injection, all control mice bore with an average of 30 visible metastatic nodules per mouse (Fig. 6C and D). In contrast, mice injected with CtsD-overexpression cells were moribund due to massive lung metastases with an average of 80 visible metastatic nodules per mouse. Histologic analyses supported the macroscopic observations and discovered a large number of metastatic lesions produced by CtsD-overexpression cells (Fig. 6C and D). Consistent with the function of hepsin *in vitro*, expression of exogenous hepsin in CtsD-overexpression cells largely inhibited the formation of lung metastasis, and average 20 visible metastatic nodules were observed per mouse (Fig. 6A-D). Mouse ICAM-1 could be detected in plasma at the level of approximately 4.5 ng/ml due to the secretion of this chemokine from tumor cells into tumor microenvironment and blood (Fig. 6E), and ICAM-1 expression was remarkably increased in plasma of mice bearing CtsD-overexpression cells (Fig. 6E). Expression of exogenous hepsin in

CtsD-overexpression cells largely inhibited the production of ICAM-1 (Fig. 6E).

Discussion

CtsD is a crucial protein that plays an essential role in local recurrence, distant metastasis, worse prognosis, and shorter disease-free and overall survival of breast cancer. In this study, we found that overexpression of CtsD increases the level of G2E3, so promotes the ubiquitination and degradation of hepsin. A tight correlation between CtsD, hepsin and G2E3 on human luminal and TNBC specimens confirms their potential regulation. More critically, our study provides several new insights into the involvement of CtsD and hepsin in breast cancer metastasis.

The ubiquitin ligase G2E3 is a novel modulator of the response to DNA-damaging treatment. “G2E3” stands for “G2-specific E3 ligase” since it was originally reported as a putative ubiquitin ligase with maximum mRNA levels in the G2/M phase of the cell cycle [24]. However, G2E3 expression did not change in CtsD transfected MCF-7 and MDA-MB-231 cells at G2/M stage (Supplementary Fig. 4). It suggested that cell cycle shows no significant effect on the G2E3 expression regulated by CtsD. The removal of G2E3 decreased the proliferation rate of cancer cells and increased apoptosis [25]. The role of G2E3 could potentially be to ubiquitinate its substrate. In this study, we discovered that G2E3 affected the stability of hepsin by means of post-translational modifications. As the physiological target proteins of G2E3, G2E3 mediates the proteasomal degradation of hepsin. It should be noted that T47D and ZR-75-1 cells showed moderate CtsD and hepsin expression, and BT-549 cell had lower CtsD and hepsin expression (Fig. 1B). Thus, in addition to ubiquitination, the expression of hepsin on BCC might be modulated by other factors and signaling pathways. Our previous research showed that different regulators may affect the function of hepsin by means of protein-protein interactions [23] and subcellular localization [26]. Evidently, these results expanded the regulated mechanism of hepsin.

ICAM-1 is a member of the immunoglobulin superfamily and is expressed on many cell types, including endothelial cells, leukocytes and cancer cells, where it can be up-regulated in response to various proinflammatory cytokines or stimuli [27]. ICAM-1 expression in cancer cells has been correlated with progression to a more aggressive state and has been shown to mediate various cancer-related biological processes such as survival, migration, extravasation, homing and metastasis [28-32]. Recently, a soluble form of ICAM-1 has been described and its elevated levels have been associated with advanced gastric, colonic, gall bladder, pancreatic and renal carcinomas [33-36]. In this study, we identified tumor-derived ICAM-1 as the key factor involved in CtsD-hepsin mediated regulation of breast cancer metastasis *in vitro* and *in vivo* (Supplementary Fig. 9, Fig. 5F and Fig. 6E). In addition to obviously decreased ICAM-1, we also found that the expression of pro-inflammatory chemokines IL-6 and IL-1β (Supplementary Fig. 8A), which have been shown to mediate tumor-promoting cross-talks between tumor and tumor microenvironment [37-39], were slightly regulated upon hepsin overexpression. However, hepsin showed the inhibitory effect on BCC migration and metastasis (Supplementary Fig. 9 and Fig. 5F). Therefore, it is likely that hepsin modulating breast cancer migration and metastasis is under the tight regulation of a balance between stimulating and inhibiting factors in tumor microenvironment. Interestedly, CtsD only promoted the secretion of ICAM-1 and had no effect on the secretion of other factors (Fig. 5E and Supplementary Fig. 8B). Of course, we cannot exclude the possibility that more tumorigenic factors were modulated by CtsD and involved in the progression of breast cancer. The elucidation of the interplay of these factors upon CtsD-hepsin axis might shed light on the treatment of breast cancer in the clinic.

Consistent with data obtained from the *in vitro* assays, our clinical result proved that hepsin expression was negatively associated with clinical stage in breast cancer (Supplementary Fig. 6). On the contrary, Xing et al. reported that hepsin overexpression was positively associated with tumor stage, lymph node metastasis in patients with breast cancer [1]. Contrary results have also been reported in other types

of tumor [40-44]. In addition, several hepsin single nucleotide polymorphism (SNP) genotypes were proved to be associated with breast cancer survival in patients treated with radiotherapy [45]. Some SNPs in non-coding regions of the hepsin gene may contribute to prostate cancer susceptibility in men of European origin [46, 47]. Similar correlations were found in a population of Korean men [48]. According to these results, we speculated that hepsin expression might either promote or suppress tumors depending on the phase of tumorigenesis and the change of tumor microenvironment.

Although the above-mentioned uncertainties still await future studies, our data have confirmed the notion that CtsD enhances breast cancer invasion and metastasis through promoting hepsin ubiquitin-proteasome degradation.

Authorship contributions

CY. Z. designed the experiments and wrote the paper; CY. Z, MM. Z, SS. S performed the experiments. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Identification of CtsD as a binding protein of hepsin. (A) The ~45 kDa band was subjected to mass spectrometric analysis and identified as a unique peptide of hepsin. (B) Proteins extracted from 8 different human BBC and MCF-10A were subjected to western blot with the indicated antibodies. CtsD and hepsin expression levels were quantified by densitometric analysis (Totallab 2.01). (C) MCF-7 and MDA-MB-231 cells were transiently transfected with V5-hepsin and myc-CtsD plasmids. The lysates were immunoprecipitated with IgG or anti-V5 antibody, followed by western blot analysis with anti-myc antibody. 10% whole cell lysate (input) was probed for the expression of exogenous hepsin and CtsD. (D) Cell surface immunoprecipitation workflow. (E) MCF-7 and MDA-MB-231 cells were transiently transfected with V5-hepsin and myc-CtsD plasmids. Cells were treated as described (D) and immunoprecipitates were analyzed by WB. (F) T47D and ZR-75-1 cells were treated as described (D) and immunoprecipitates were analyzed by WB. IP, immunoprecipitation; and WB, western blotting.

Figure 2. CtsD down-regulates hepsin expression in BBC. (A) Experimental workflow of cell surface label. (B) Cell surface label and western blot analysis of hepsin expression in T47D and ZR-75-1 cells transfected with indicated doses of myc-CtsD. (C) Relative hepsin cell surface expression in indicated plasmids transfected T47D and ZR-75-1 cells. Lower bands indicated CtsD, B, S, L protein expression, respectively. (D) T47D and ZR-75-1 cells were transfected with 50 nM control siRNA or CtsD siRNA for 72h. Cell surface hepsin was detected with western blot. (E, F) Levels of hepsin showed an inverse correlation with those of CtsD in clinical luminal breast cancer and TNBC.

Figure 3. CtsD promotes hepsin degradation. (A) MCF-7 cells were transfected with myc-CtsD or empty vector. Hepsin mRNA was determined by real-time PCR and normalized against GAPDH. Error bars represent ±S.D. of triplicate experiments. The

two-tailed Student's *t*-test was used. NS denotes no significance. (B) Human breast cancer samples were lysed and directly subjected to real-time PCR. The correlation between hepsin and CtsD mRNA was determined using Spearman's rank correlation test. (C) T47D and ZR-75-1 cells were transfected with myc-CtsD or empty vector. After 36h, cells were treated with 50uM CHX for indicated times. The cell surface proteins were analyzed by western blotting. Hepsin expression levels were quantified by densitometric analysis (Totallab 2.01), statistically analyzed from three independent experiments and presented on the lower panel. *P<0.05; **P<0.01; ***P<0.001. (D) T47D and ZR-75-1 cells were transfected as indicated and treated with MG132 or Dimethyl Sulphoxide (DMSO) for 5h. The cells were labeled and analyzed by western blot analysis. (E) Overexpression of CtsD promoted hepsin ubiquitination *in vitro*. MCF-7 and MDA-MB-231 cells were transfected with V5-hepsin, myc-CtsD and HA-ubiquitin constructs. 48h after transfection, cells were treated with DMSO or 50uM MG132 for another 5h. Cells were lysed, then the immunoprecipitated complex was separated and blotted with anti-HA antibody. Ub indicated ubiquitin modification.

Figure 4. G2E3 targets hepsin for degradation. (A) G2E3 overexpression decreases hepsin protein. T47D and ZR-75-1 cells were transfected as indicated, followed by MG132 treatment. Cell lysates were directly subjected to western blotting. (B) Western blot analysis of G2E3 expression in T47D and ZR-75-1 cells transfected with indicated doses of myc-CtsD. (C) G2E3 knockdown rescues hepsin protein reduced by CtsD. Three different concertation of G2E3 siRNA were transfected, respectively, into T47D and ZR-75-1 cells. Protein levels of hepsin were determined by western blotting. G2E3 knockdown efficiency was analyzed by western blot. (D) G2E3 knockdown increases hepsin stability. T47D and ZR-75-1 cells were transfected with myc-CtsD combinations of control siRNA or siG2E3, respectively. CHX chase experiment was performed and hepsin protein was determined by western blotting (upper panel). The lower panel shows relative protein amounts of different groups. Error bars represent ±S.D. of triplicate experiments. The two-tailed Student's t-test

was used. **P<0.01; ***P<0.001. (E) G2E3 knockdown blocks CtsD induced-ubiquitylation of hepsin. MCF-7 and MBA-MD-231 cells were transfected as indicated. Ubiquitylation assay was conducted. The efficiency of G2E3 knockdown was validated by western blot.

Figure 5. CtsD promotes the migration and invasion of breast cancer by downregulation of hepsin *in vitro*. (A) Graphic representation of cell migration described in Supplementary Figure 7. Data are the percentage of vector control values. (B) Graphic representation of cell invasion described in Supplementary Figure 7. Data are the percentage of vector control values. (C) CtsD was overexpressed in MCF-7 and MDA-MB-231 cells. A rescue experiment with overexpression of hepsin in CtsD expressing cells was also performed. The level of E-cadherin, claudin-1, N-cadherin, vimentin and Snail1 was analyzed by western blot. (D) Serum-free culture supernatants from MCF-7 cells were analyzed by cytokine arrays. (E) The effect of hepsin and/or CtsD on the expression of ICAM-1 in supernatants by ELISA. (F) Graphic representation of cell migration and invasion described in Supplementary Figure 9. Data are the percentage of migrating cells as the mean±S.D. of three separate experiments. (G) The effect of rhICAM-1 or inhibitor A-205804 on hepsin or CtsD transfected MCF-7 cells was detected. The level of E-cadherin, claudin-1, N-cadherin, vimentin and Snail1 was analyzed by western blot. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

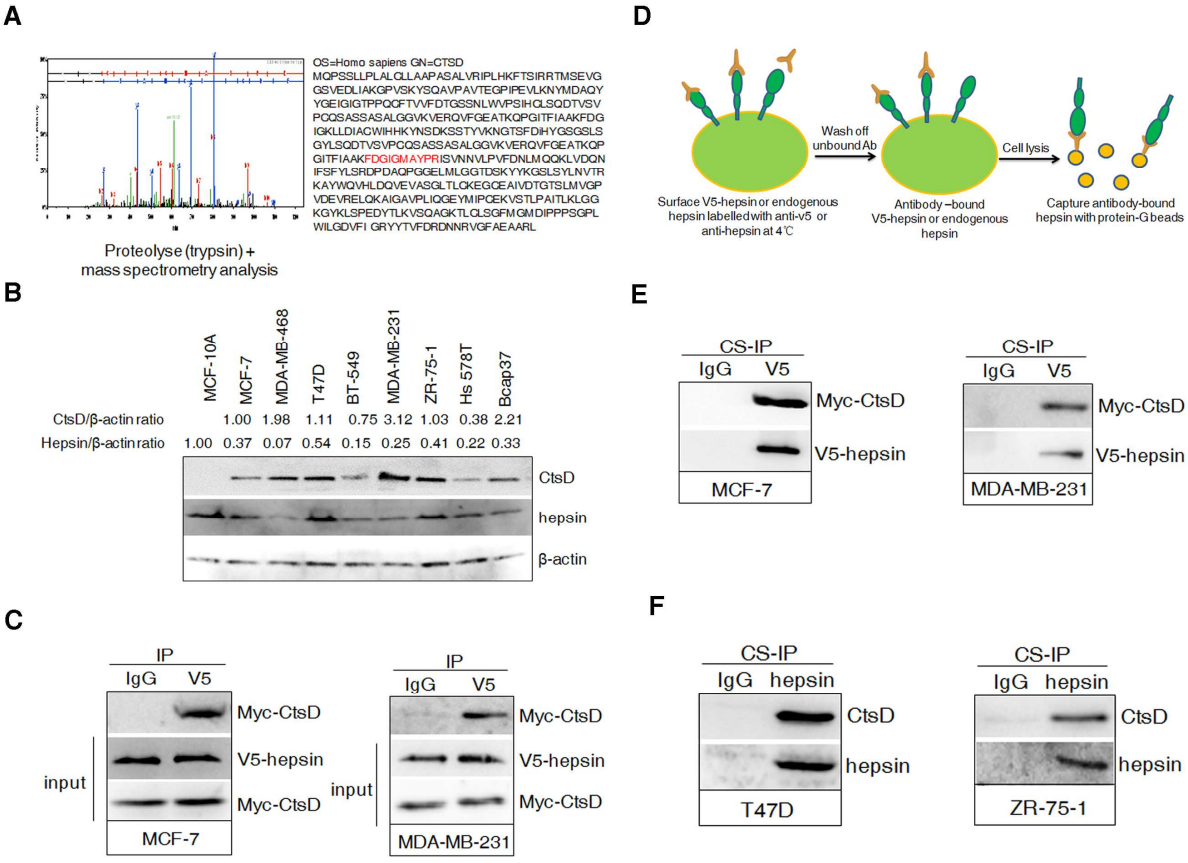
Figure 6. Overexpression of CtsD promotes tumour metastasis *in vivo*. (A) MCF-7-luc cells stably transfected with control, CtsD plasmid or CtsD/hepsin plasmids together were injected through tail vein into female SCID mice. Lung metastasis was assessed every week by bioluminescence imaging. Presented images are representative of each experimental group. (B) Normalized bioluminescence signals from lung metastasis in mice (n=6) as experiment outlined above. Data are presented as mean±S.D. (C) Representative images of lung lesions from experimental groups in A. (D) Graphic representation of lung weight and numbers of metastatic

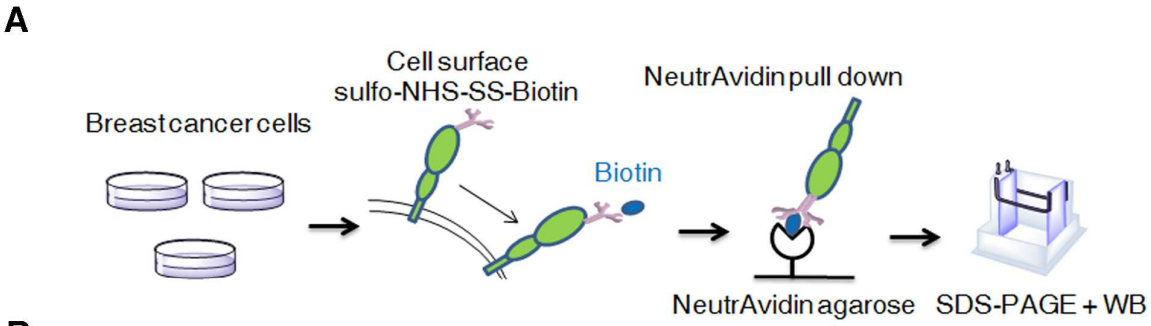
nodules from mice in experimental groups described in A. Data are presented as mean±S.D. (E) Mouse ICAM-1 levels in plasma were measured by ELISA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 1

Identification of hepsin from obtained peptide masses. the Δ mass between measured and matched masses

Measured masses	Matched masses	Δ mass	Peptide sequence consistent with mass
1238.6134	1238.6118	0.0016	FDGILGMAYPR
534.759	1534.7549	0.0041	TMSEVGGSVEDLIAK
1897.0283	1897.0197	0.0086	YSQAVPAVTEGPIPEVLK
1958.0242	1958.0295	-0.0053	ISVNNVLPVFDNLMQQK

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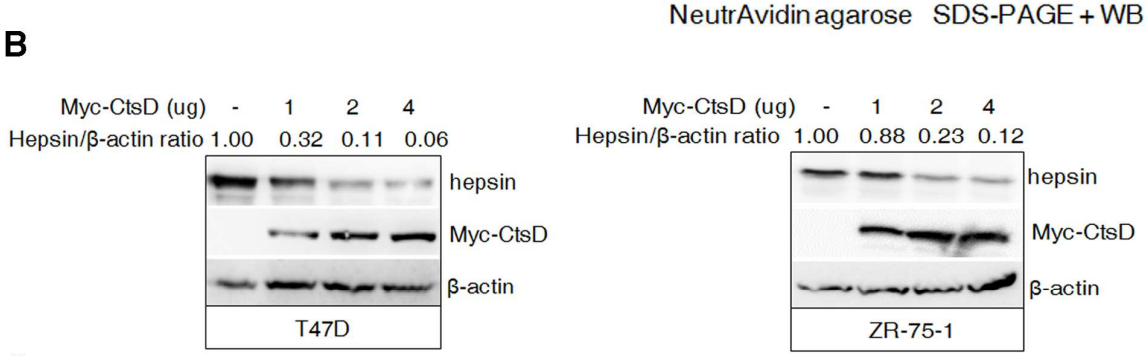
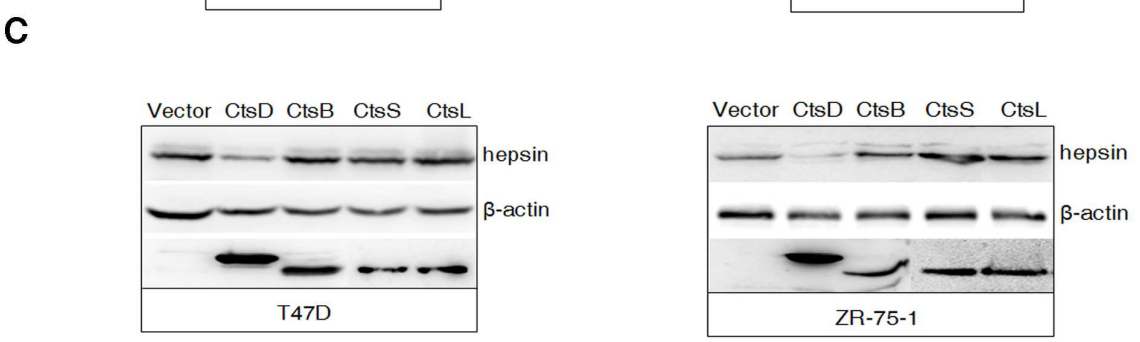
NeutrAvidin pull down
Breast cancer cells
Biotin
NeutrAvidin agarose
SDS-PAGE + WB


B
Myc-CtsD (ug) - 1 2 4
Hepsin/β-actin ratio 1.00 0.32 0.11 0.06
hepsin
Myc-CtsD
β-actin
T47D
Myc-CtsD (ug) - 1 2 4
Hepsin/β-actin ratio 1.00 0.88 0.23 0.12
hepsin
Myc-CtsD
β-actin
ZR-75-1


C
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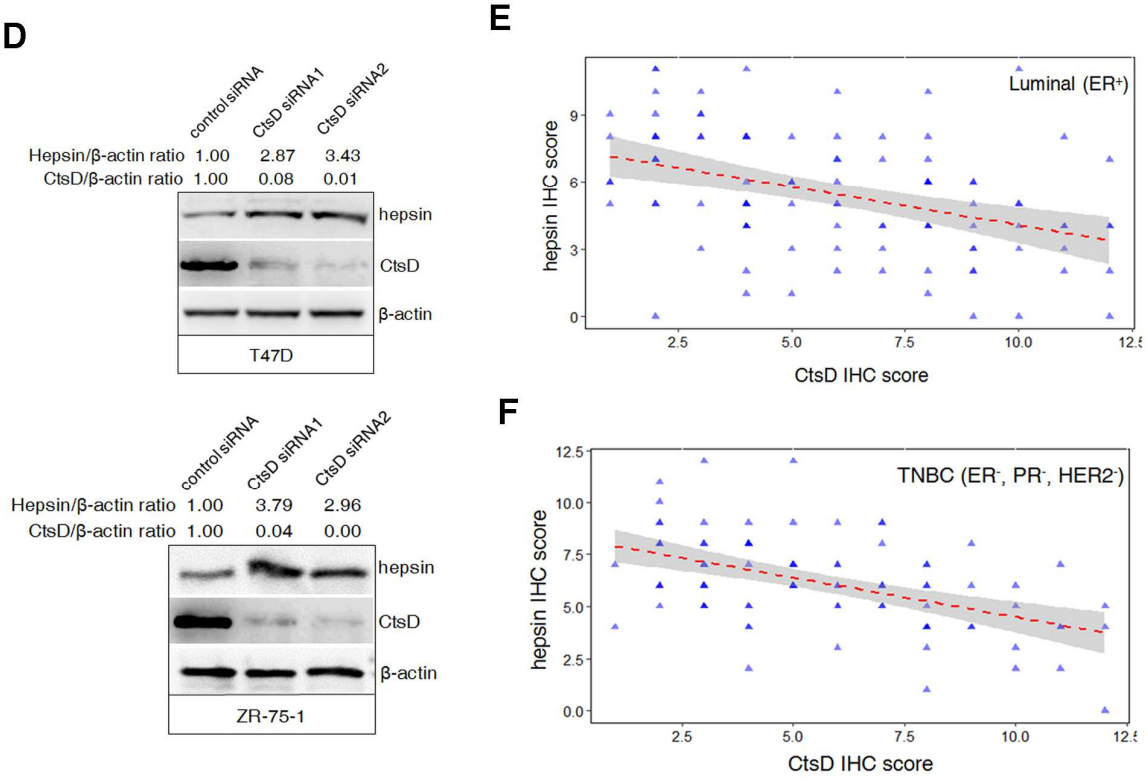
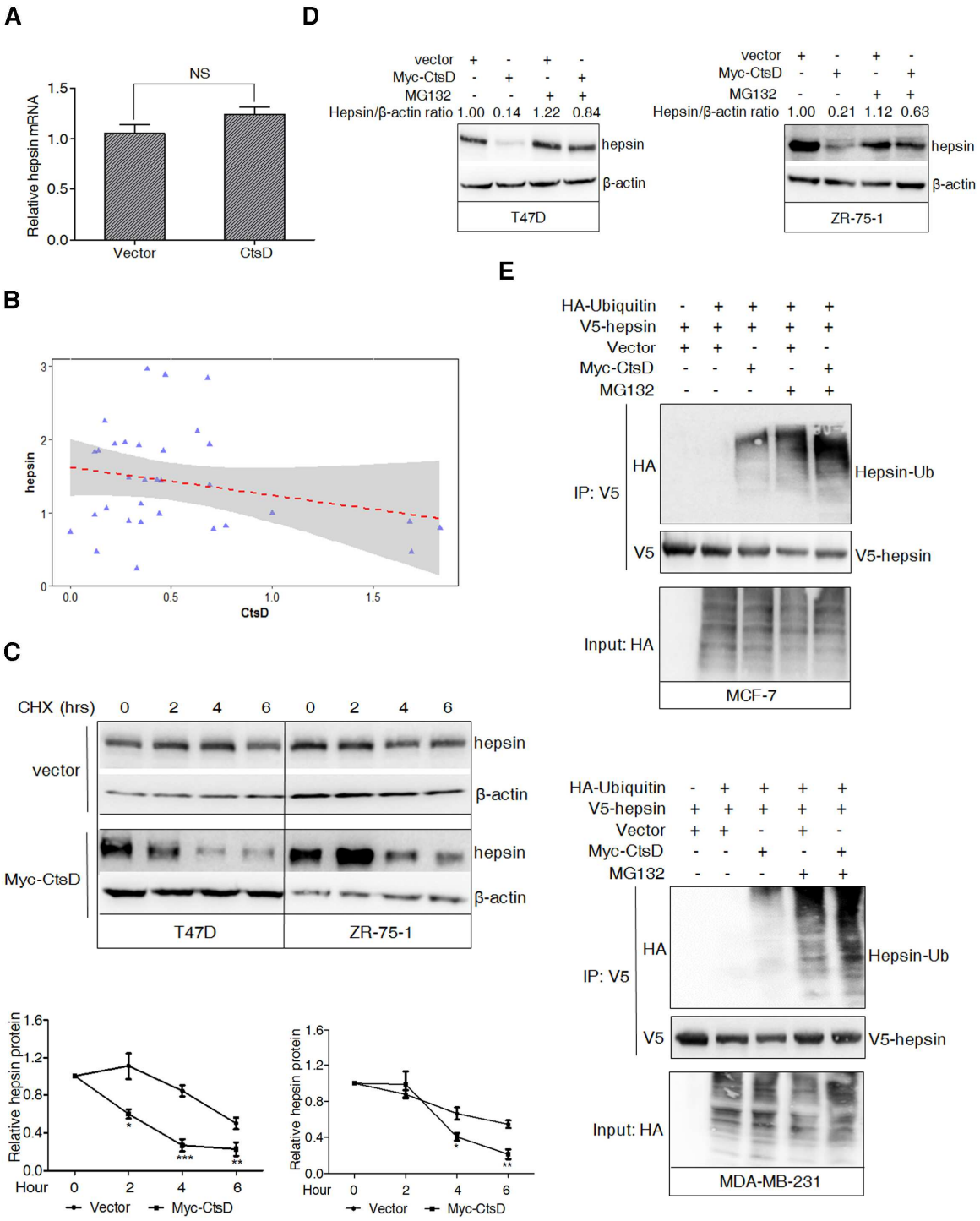
hepsin
β-actin
T47D
Vector CtsD CtsB CtsS CtsL
hepsin
β-actin
ZR-75-1


D
control siRNA
CtsD siRNA1
CtsD siRNA2
Hepsin/β-actin ratio 1.00 2.87 3.43
CtsD/β-actin ratio 1.00 0.08 0.01
hepsin
CtsD
β-actin
T47D
control siRNA
CtsD siRNA1
CtsD siRNA2
Hepsin/β-actin ratio 1.00 3.79 2.96
CtsD/β-actin ratio 1.00 0.04 0.00
hepsin
CtsD
β-actin
ZR-75-1
E
Luminal (ER+)
hepsin IHC score
CtsD IHC score
F
TNBC (ER-, PR-, HER2-)
hepsin IHC score
CtsD IHC score


A
NS
Relative hepsin mRNA
1.5
1.0
0.5
0.0
Vector
CtsD
B
hepsin
CtsD
C
CHX (hrs) 0 2 4 6 0 2 4 6
vector
Myc-CtsD
hepsin
β-actin
T47D
ZR-75-1
Relative hepsin protein
Hour
Vector
Myc-CtsD
D
vector
Myc-CtsD
MG132
Hepsin/β-actin ratio 1.00 0.14 1.22 0.84
Hepsin/β-actin ratio 1.00 0.21 1.12 0.63
hepsin
β-actin
T47D
ZR-75-1
E
HA-Ubiquitin
V5-hepsin
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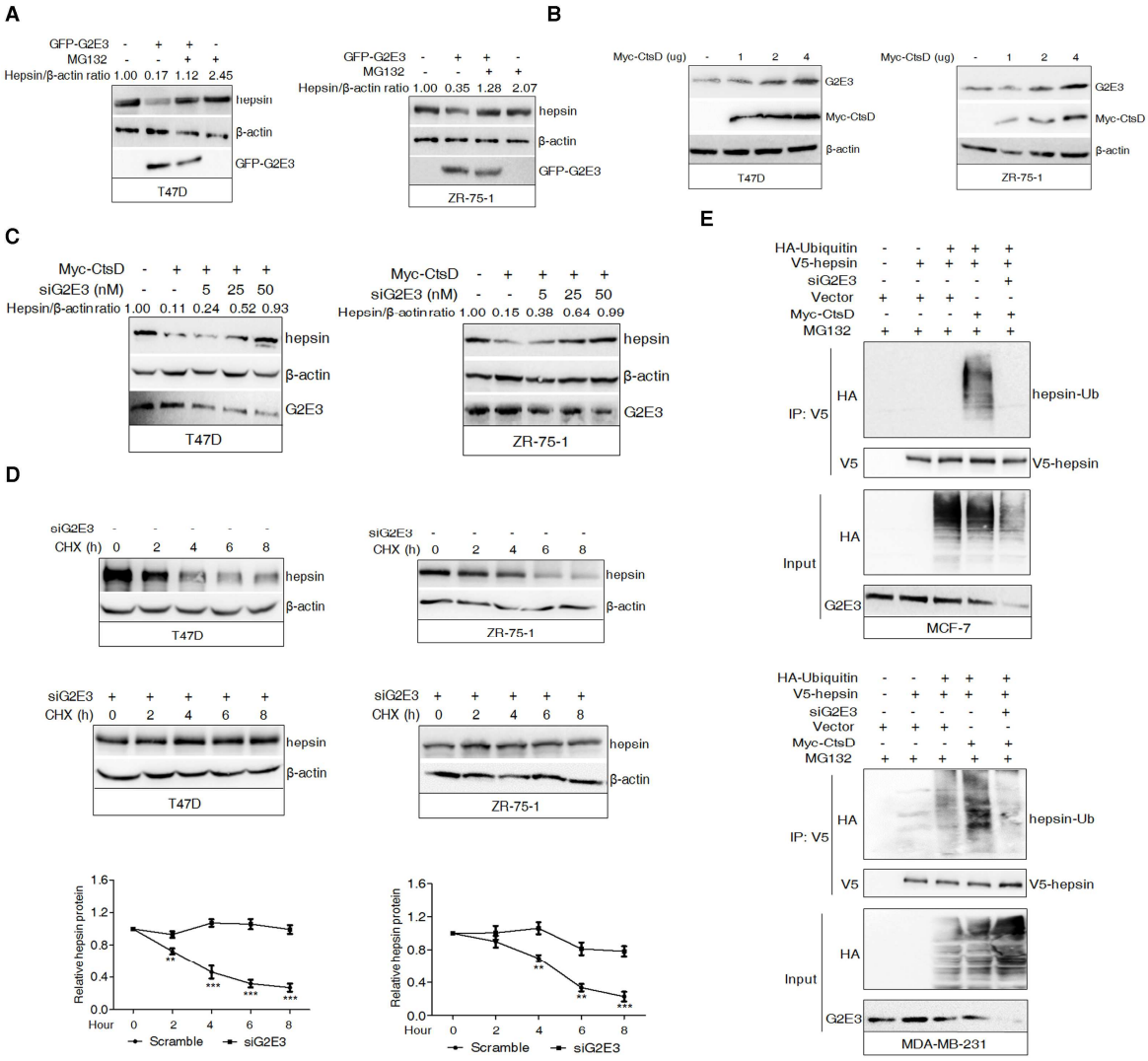

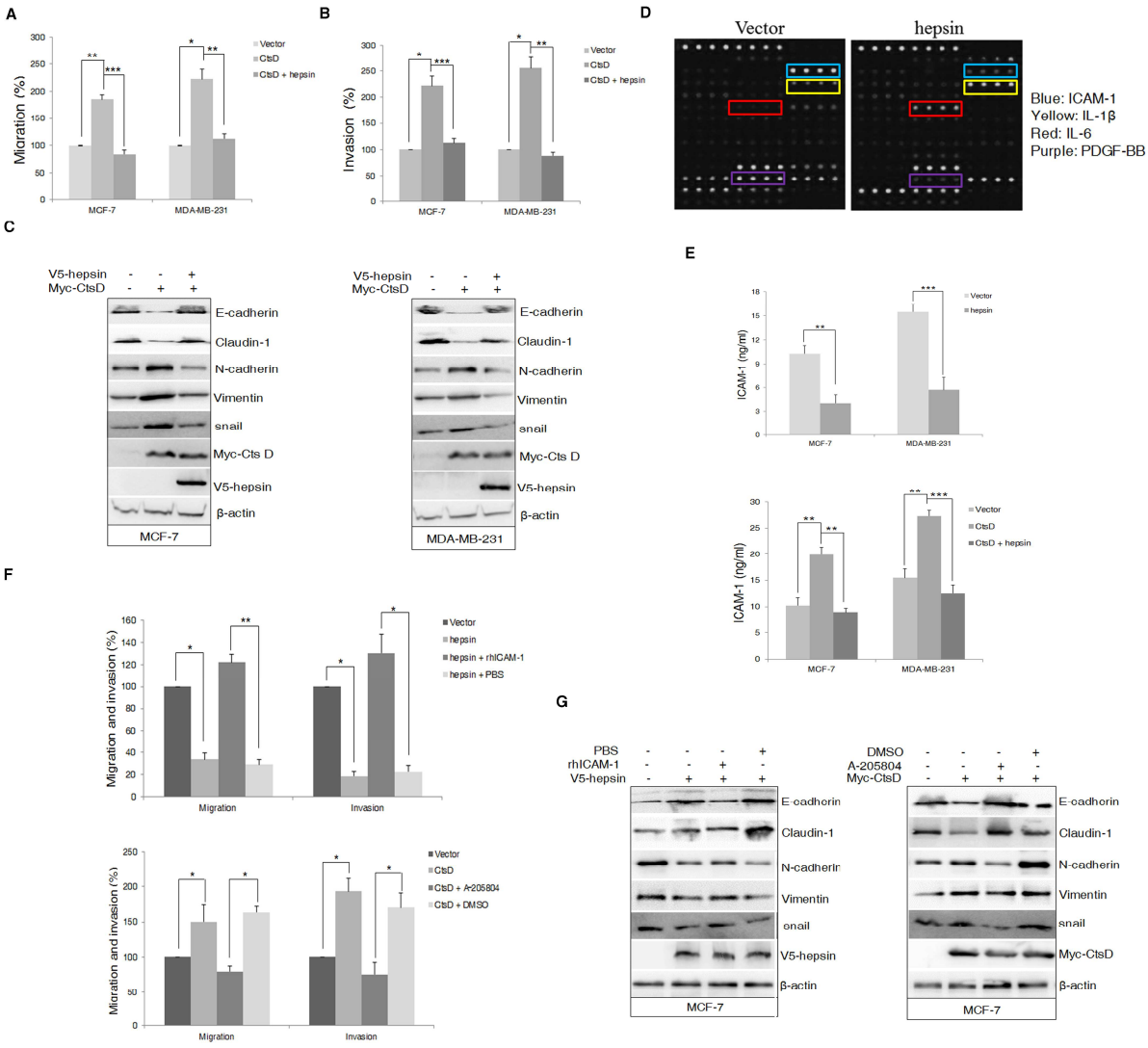

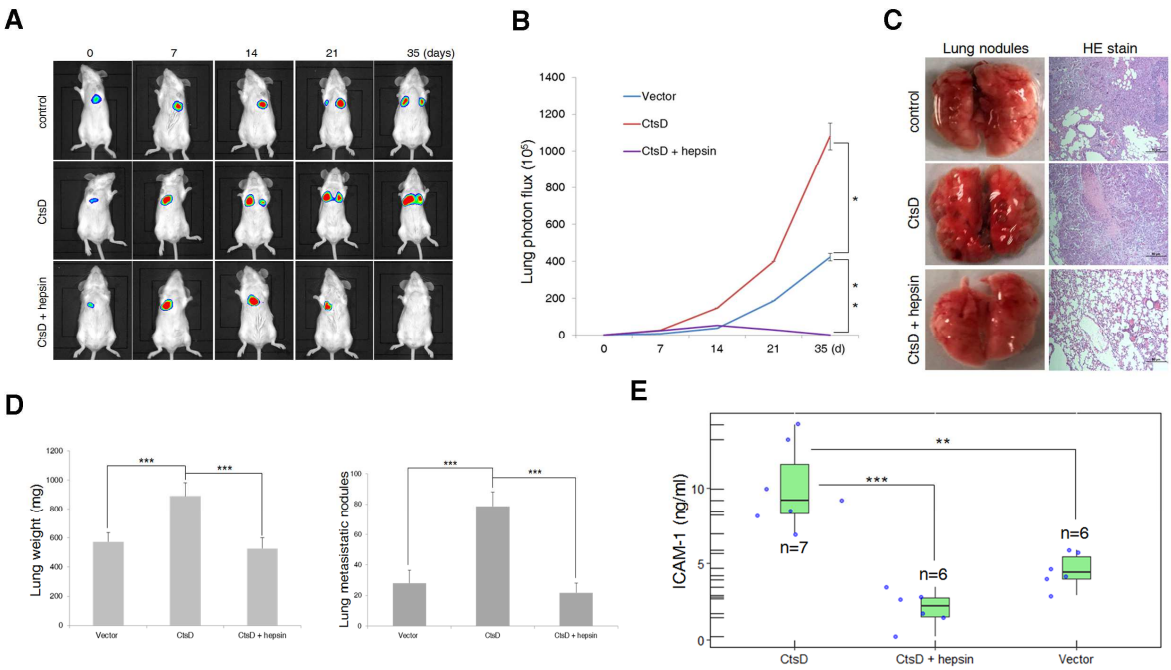

1. CtsD destabilized hepsin by promoting its ubiquitylated proteasomal degradation.
2. Hepsin expression was negatively correlated with CtsD in breast cancer TMA.
3. G2E3 targeted hepsin for degradation.
4. CtsD promoted BCC invasion and migration by enhancing the autocrine of ICAM-1.
5. Hepsin expression inhibited BCC invasion and migration induced by CtsD.