Flotillin-1 promotes EMT of gastric cancer *via* stabilizing Snail

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Gastric cancer is one of the most common malignancies worldwide and has been identified as the third leading cause of cancer-related mortality. Flotillin-1 is a lipid raft-associated scaffolding protein and plays an important role in the progression and development of several malignant carcinomas. Flotillin-1 is involved in epithelial-mesenchymal transition (EMT) process of several solid tumors to promote metastasis. However, the detailed characteristics and mechanisms of Flotillin-1 in gastric cancer have rarely been investigated. In this study, we found Flotillin-1 upregulated in gastric cancer, and the high expression of Flotillin-1 correlated with a worse prognosis. The migration and invasion ability of gastric cancer cells was upregulated by overexpressing Flotillin-1. Knockdown of Flotillin-1 inhibits gastric cancer cells metastasis. Flotillin-1 is a key regulator of EMT process and promotes gastric cancer cells metastasis through inducing EMT. Flotillin-1 may interact with a deubiquitinase to inhibit the ubiquitination of Snail in gastric cancer cells to promote EMT process. Our study provides a rationale and potential target for the treatment of gastric cancer.

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Abstract

Gastric cancer is one of the most common malignancies worldwide and has been identified as the third leading cause of cancer-related mortality. Flotillin-1 is a lipid raft-associated scaffolding protein and plays an important role in the progression and development of several malignant carcinomas. Flotillin-1 is involved in epithelial-mesenchymal transition (EMT) process of several solid tumors to promote metastasis. However, the detailed characteristics and mechanisms of Flotillin-1 in gastric cancer have rarely been investigated. In this study, we found Flotillin-1 upregulated in gastric cancer, and the high expression of Flotillin-1 correlated with a worse prognosis. The migration and invasion ability of gastric cancer cells was upregulated by overexpressing Flotillin-1. Knockdown of Flotillin-1 inhibits gastric cancer cells metastasis. Flotillin-1 is a key regulator of EMT process and promotes gastric cancer cells metastasis through inducing EMT. Flotillin-1 may interact with a deubiquitinase to inhibit the ubiquitination of Snail in gastric cancer cells to promote EMT process. Our study provides a rationale and potential target for the treatment of gastric cancer.

Keywords: Flotillin-1, metastasis, EMT, gastric cancer, Snail

Introduction

Gastric cancer is one of the most common malignancies worldwide and has been identified as the third leading cause of cancer-related mortality, with 1,089,103 newly diagnosed cases and 768,793 deaths in 2020 [1]. Early detection and curative resection resulted in improved outcomes for patients with early gastric cancer, but the prognosis for the advanced patients remains poor, the five-year survival rate is less than 40% [2]. The main cause of death in advanced gastric cancer patients is metastasis. In the past two decades, the proportion of patients with metastatic gastric cancer has risen to more than 40% [3-5]. Thus, it is necessary to study the molecular mechanism of gastric cancer metastasis to elucidate its pathogenesis.

Epithelial-mesenchymal transition (EMT) is an evolutionarily conserved developmental program that is associated with cancer development and confers metastasis properties to cancer cells by enhancing their mobility and invasiveness [6]. Cancer cells in primary tumors lose intercellular adhesion mediated by E-cadherin inhibition, break through the basement membrane with increased invasive properties, enter the bloodstream through intravasation, and exit the bloodstream to form micro-metastases [7, 8]. EMT is a cellular process in which epithelial cells lose their epithelial phenotypes and acquire mesenchymal-like characteristics. The initiation of metastasis requires invasion, which is achieved through EMT [9]. Several signaling pathways have been implicated to be involved in EMT, including TGF-β, Wnt/beta-catenin and Notch, mediating the loss of the epithelial marker E-cadherin and the gain of the expression of the mesenchymal marker N-cadherin and Vimentin [10-12].

Flotillin-1 is a lipid raft-associated scaffolding protein and it is implicated in a variety of cellular processes including axonal regeneration, cellular adhesion, endocytosis, phagocytosis and

signal transduction [13]. In addition, Flotillin-1 plays an important role in the progression and development of several malignant carcinomas [14]. Upregulation of Flotillin-1 predicts poor prognosis and promotes malignant progression, proliferation and metastasis of multiple tumors, including non-small cell lung, breast cancer, renal cell carcinoma, neuroblastoma, bladder cancer, prostate cancer and hepatocellular carcinoma [15-21]. In particular, studies shown that Flotillin-1 is involved in EMT process of several solid tumors to promote metastasis. In cervical cancer, Flotillin-1 facilitates cell metastasis through Wnt/beta-catenin and NF-κB pathway-regulated EMT [22]. Flotillin-1 promotes cell invasion and migration by inducing EMT and modulating the cell cycle in lung adenocarcinoma [23]. However, the detailed characteristics and mechanisms of Flotillin-1 in gastric cancer have rarely been investigated.

In this study, we determined the role of Flotillin-1 in the metastasis process of gastric cancer and molecular mechanisms of Flotillin-1 in the regulation of EMT.

Materials and Methods

Cell culture

The human gastric cancer cell line SGC-7901 was obtained from GeneChem (Shanghai, China), AGS was acquired from the Shanghai Institute for Biological Science. Both of the cell lines were cultured in RPMI/1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and incubated at 37℃ with 5% CO_2. HEK293T cell were purchased from American Type Culture Collection (ATCC) and grown in DMEM medium (Gibco, USA) plus with 10% FBS.

Plasmids and antibodies

Flotillin-1 and Snail were inserted into the pLVX vector. The Flotillin-1 shRNA sequences were cloned into the pSIH vector. The sequences of shRNAs are shown in Supplementary Table S1.

Antibodies used in this study: anti-Flotillin-1 (#18634, Cell Signaling Technology, USA), anti-Snail (#3879, Cell Signaling Technology, USA), anti-E-cadherin (#3195, Cell Signaling Technology, USA), anti-N-cadherin (#13116, Cell Signaling Technology, USA), anti-Vimentin (#5741, Cell Signaling Technology, USA), anti-β-actin (A5441, Sigma-Aldrich, USA), anti-Flag (#14793, Cell Signaling Technology, USA), anti-HA (sc-53516, Santa Cruz Biotechnology, USA), Goat anti-Rabbit IgG (AS014, ABclonal, China), Goat anti-mouse IgG (AS003, ABclonal, China).

Generation of stable transfected cells

The stable transfected cells including Flotillin-1-overexpressing and -knockdown were generated by lentivirus. The lentivirus was generated using packaging vectors PSPAX2, pMD2G and Flotillin-1-overexpressing plasmid or Flotillin-1-shRNA plasmid. SGC-7901 and AGS cells were infected with indicated lentivirus and performed the stable transfected cell screening using puromycin. The third generation of stable transfected cells were used for subsequent experiments.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent and cDNA was prepared from total RNA using the Quantscript RT Kit following manufacturer's instruction. qRT-PCR assay was performed using SYBR Green Mix following the manufacturer's instruction. The primers used in qRT-PCR were shown in Supplementary Table 2.

Immunoblotting assay

Cells were lysed by RIPA lysis buffer plus a cocktail of protease inhibitor. The protein concentration was detected using Bicinchoninic acid (BCA) assay. Total proteins (10μg for each well) were separated by SDS-PAGE gel and transferred to Polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% fat-free milk for 1 hour and incubated with primary antibodies overnight. Next day, the membrane was incubated with conjugated secondary antibody. The protein signal was visualized using ECL detection reagents.

Migration and invasion assay

Cell migration and invasion assay were performed using 24-well Transwell plate with or without coated Matrigel. Cells (5×10^4 for each well) were plated into Boyden chambers with serum-free media. Media containing 10% FBS were added into the bottom chambers, and the cells were cultured for 24 hours at 37℃ with 5% CO_2. Finally, the cells were fixed by methanol and stained with crystal violet.

Wound healing assay

Cells (2×10^5 for each well) were plated into 6-well plates and scratched using a sterile pipette tip. The cells were washed with phosphate-buffered saline (PBS) and incubated in media containing 2% FBS. Images were obtained at 0, 24, 48, 72 hours.

Immunoprecipitation assay

Cells were treated with 20 μg/ml MG132 for 6 hours and lysed with RIPA lysis buffer plus a cocktail of protease inhibitor. For Flag-tagged proteins, cell lysates were incubated with anti-Flag M2 magnetic beads overnight. Finally, the immunoprecipitates were subjected to immunoblotting.

106 ***Ubiquitination assay***

107 For ubiquitination assay, the cells were treated with MG132 for 6 hours and lysed using RIPA
108 lysis buffer plus a cocktail of protease inhibitor. The cell lysates were incubated with anti-Flag M2
109 magnetic beads overnight. The ubiquitin level of Flag-Snail was detected with anti-HA antibody.

110 ***Protein degradation analysis***

111 For Snail protein degradation analysis, cells were treated with cycloheximide (CHX,
112 50μg/ml) for 1, 2 hours. The protein level of Snail was detected using immunoblotting assay.

113 ***Statistical analysis***

114 Survival analysis was analyzed using a two-sided log-rank test, others were analyzed using
115 unpaired *t*-test. Except for special instructions, all results were shown as mean±SEM of three
116 independent experiments. All statistical analyses were performed with Graphpad Prism. A $p < 0.05$
117 was considered significant.

118

119 **Results**

120 **Increased Flotillin-1 predicts a poor prognosis of gastric cancer patients**

121 To identify the effect of Flotillin-1 expression level on the clinical prognosis of gastric cancer,
122 we analyzed the data from The Cancer Genome Atlas (TCGA). A Flotillin-1 amplification occurs
123 in several solid tumors including gastric cancer (Figure 1A). Compared to normal tissues, the
124 expression level of Flotillin-1 was significantly higher in gastric cancer samples (Figure 1B).
125 Gastric cancer patients with high Flotillin-1 expression level correlated with a worse survival
126 overall survival, advanced TNM stage and distant metastasis (Figure 1C-E). Together, those results

shown that Flotillin-1 can participate in the development of gastric cancer.

Overexpression of Flotillin-1 promotes gastric cancer metastasis

To identify the function of Flotillin-1 in gastric cancer, we overexpressed Flotillin-1 in two gastric cancer cell lines SGC-7901 and AGS. The overexpression efficiency was confirmed by qRT-PCR and immunoblotting (Figure 2A-D). To examine the role of Flotillin-1 in gastric cancer metastasis, we performed Transwell and wound healing assays to detect the abilities of migration and invasion. Overexpression of Flotillin-1 not only promoted the migration and invasion of SGC-7901 cells, but also increased the wound healing ability of gastric cancer cells (Figure 2E-F). In addition, we obtained the same results in AGS cells (Figure 2G-H). Taken together, the results show that overexpression of Flotillin-1 promotes gastric cancer cell migration and invasion and suggested that Flotillin-1 might have a role in cancer metastasis.

Knockdown of Flotillin-1 inhibits gastric cancer metastasis

To further study the role of Flotillin-1 in gastric cancer metastasis, we knocked down Flotillin-1 in SGC-7901 and AGS cells using shRNA. The knockdown efficiency was confirmed by qRT-PCR and immunoblotting (Figure 3A-D). We performed Transwell and wound healing assays to detect the cell migration and invasion abilities of Flotillin-1-knockdown and control gastric cancer cells. As expected, knockdown of Flotillin-1 inhibited the migration, invasion and wound healing abilities of SGC-7901 and AGS cells (Figure 3E-H). Thus, the results show that knockdown of Flotillin-1 inhibits gastric cancer cell migration and invasion and suggested that Flotillin-1 might have a role in cancer metastasis.

Flotillin-1 promotes gastric cancer metastasis through inducing EMT

148 The initiation of metastasis requires invasion, which is achieved through EMT. To identify
149 whether Flotillin-1 promotes gastric cancer metastasis through EMT process. We detected the
150 expression of several EMT markers including epithelial marker E-cadherin and mesenchymal
151 markers N-cadherin, Vimentin and Snail. Overexpression of Flotillin-1 downregulated the protein
152 and mRNA levels of E-cadherin, and upregulated the N-cadherin, Vimentin and Snail levels
153 (Figure 4A-D). In contrast, knockdown of Flotillin-1 increased the expression of E-cadherin and
154 inhibited the expression of N-cadherin, Vimentin and Snail (Figure 4E-H). Moreover, we found
155 that Flotillin-1 regulated the protein level of Snail, but has no effect on the mRNA level. Taken
156 together, those results determined that Flotillin-1 promotes the EMT process in gastric cancer cells.

157 **Flotillin-1 promotes EMT of gastric cancer via stabilizing Snail**

158 Snail, as an EMT-transcription factor, has been confirmed to play an integral role throughout
159 EMT of all types. In addition, Snail is a labile protein and degraded by ubiquitin-proteasome
160 system, and Flotillin-1 regulates the protein expression of Snail but no effect on mRNA level.
161 Thus, we speculated that Flotillin-1 promoted the EMT process through increasing the protein
162 stability of Snail. To identify the hypothesis, we performed immunoprecipitation and
163 ubiquitination assays. As expected, Flotillin-1 interacted with Snail (Figure 5A) and decreased the
164 ubiquitination level of Snail (Figure 5B). In addition, Flotillin-1 inhibited the degradation of Snail
165 in gastric cancer cells (Figure 5C-D). Taken together, the results shown that Flotillin-1 decreased
166 the ubiquitination level of Snail and inhibited its degradation.

167 **Discussion**

168 Flotillin-1, a lipid raft protein, is involved in cell migration and invasion, cell signaling,

proliferation, differentiation and endocytosis [13, 24-26]. More studies have shown that Flotillin-1 plays an important role in the development of malignant tumors and may act as a prognosis factor in solid carcinomas [27, 28]. The upregulated expression of Flotillin-1 was associated with tumor cell progression and poor prognosis in hepatocellular carcinoma [21]. Knockdown of Flotillin-1 impairs cell proliferation and tumorigenicity in breast cancer through upregulation of FOXO3a [16]. MiR-214-3p inhibited cell proliferation and metastasis in hepatocellular carcinoma by downregulating Flotillin-1 [29]. In tongue squamous cell cancer, the expression of Flotillin-1 was correlated with pathological stage, depth of invasiveness, lymph node metastasis, recurrence and shorter survival [27]. In this study, we found Flotillin-1 upregulated in gastric cancer, and the high expression of Flotillin-1 correlated with a worse prognosis. In addition, the migration and invasion ability of gastric cancer cells was upregulated by overexpressing Flotillin-1. Knockdown of Flotillin-1 inhibited the cell metastasis in gastric cancer. Therefore, our results demonstrate Flotillin-1 participates in the development of gastric cancer, especially in promoting metastasis.

EMT is a process by which epithelial cells lose their apical-basal polarity and intercellular adhesion properties and transform into invasive mesenchymal cells, all of which provide conditions for the invasion and metastasis of cancer cells [30-33]. There are numerous inducers of EMT, and the determinant inducers corresponding to different sites of tumors will be the key to the targeted therapy. In this study, we identified that overexpression of Flotillin-1 decreased the epithelial marker E-cadherin and upregulated mesenchymal markers such as N-cadherin, Vimentin and Snail in gastric cancer cells. E-cadherin was upregulated, and N-cadherin, Vimentin and Snail were decreased in Flotillin-1-knockdown gastric cancer cells. Therefore, our study demonstrates

that Flotillin-1 can participate in EMT process and promotes gastric cancer metastasis through inducing EMT.

Snail is a zinc-finger transcription factor that regulates EMT during mesoderm and neural crest development [34]. In breast cancer, Snail expression has been shown to be upregulated in recurrent tumors [35]. In addition, it is associated with reduced metastasis and recurrence-free survival. Similar to zinc finger E-box-binding homeobox 1 (ZEB1), Snail represses cadherin 1 (CDH1) transcription by binding to the E-box in the CDH1 promoter [36]. It also regulates CDH1 expression in concert with histone methyltransferases (HMT) and DNA methyltransferases (DNMTs) [37]. Snail interacts directly with the E-cadherin promoter, recruiting histone deacetylase 1 (HDAC1), HDAC2 and co-repressor Sin3A to the CDH1 promoter and silencing their expression through deacetylation of histones H3 and H4 [38]. Snail comprehensively affects the expression profile of epithelial cells, besides E-cadherin, it also downregulates claudin, occludin, mucin, vimentin, fibronectin and matrix metalloproteinases [39]. Thus, the role of Snail is mainly to downregulate epithelial markers and upregulate mesenchymal markers, and the increased expression of matrix metalloproteinases it regulates makes the cells migratory. In addition, Snail is a labile protein and degraded by ubiquitin-proteasome system. In our study, we demonstrate the interaction between Flotillin-1 and Snail. The protein expression of Snail is upregulated by overexpressing Flotillin-1, and overexpression of Flotillin-1 delays the degradation of Snail in gastric cancer cells. The ubiquitination level of Snail is downregulated in gastric cancer cells overexpressing Flotillin-1. Therefore, Flotillin-1 may interact with a deubiquitinase to inhibit the ubiquitination of Snail in gastric cancer to promote EMT process. This is a direction of our

211 further study.

212 In summary, our study demonstrate Flotillin-1 can participate in the development of gastric
213 cancer, and promotes gastric cancer metastasis. Increased Flotillin-1 predicts a poor prognosis of
214 gastric cancer patients. Flotillin-1 promotes gastric cancer metastasis through inducing EMT, and
215 promotes EMT of gastric cancer via stabilizing Snail. Our study provides a rationale and potential
216 target for the treatment of gastric cancer.

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218

219 **Conflicts of interest**

220 The authors declare that there is no conflict of interest.

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224

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Figure legends

Fig. 1 Increased Flotillin-1 predicts a poor prognosis of gastric cancer patients

(A) A histogram shows the amplification frequency of Flotillin-1 in 23 tumor types. The data were taken from TCGA PanCancer Altas studies. (B) The expression of Flotillin-1 in gastric cancer tissues and normal samples. T represents tumor tissues (n=408), N represents normal tissues (n=36). TPM represents transcripts per million. (C) Kaplan-Meier plot showing the overall survival of 875 gastric cancer patients stratified by Flotillin-1 expression level. Low-Flotillin-1 and high-Flotillin-1 were distinguished using the median of the expression of Flotillin-1. (D-E)

Comparison between the proportion of gastric cancer patients with different Flotillin-1 expression level in tumor size stage (T stage) (D) and distant metastasis (E). RSEM represents RNA-seq by expectation maximization. *p<0.05, **p<0.01, p value was calculated with t test, except survival analysis was analyzed using a two-sided log-rank test.

Fig. 2 Overexpression of Flotillin-1 promotes gastric cancer metastasis

(A-B) The mRNA (A) and protein (B) levels of Flotillin-1 in SGC-7901 cells overexpressing Flotillin-1. (C-D) The mRNA (C) and protein (D) levels of Flotillin-1 in AGS cells overexpressing Flotillin-1. (E) The migration and invasion abilities of SGC-7901 cells overexpressing Flotillin-1 were detected by Transwell assay. Scale bars, 500μm. (F) The migration ability of SGC-7901 cells overexpressing Flotillin-1 was examined by wound healing assay. Scale bars, 500μm. (G) The migration and invasion abilities of AGS cells overexpressing Flotillin-1 were detected by Transwell assay. Scale bars, 500μm. (H) The migration ability of SGC-7901 cells overexpressing Flotillin-1 was examined by wound healing assay. Scale bars, 500μm. **p<0.01, ***p<0.001, ****p<0.0001, p value was calculated with t test, compared to control group. All results were shown as mean±SEM of three independent experiments.

Fig. 3 Knockdown of Flotillin-1 inhibits gastric cancer metastasis

(A-B) The mRNA (A) and protein (B) levels of Flotillin-1 in Flotillin-1-knockdown (using shRNA targeting Flotillin-1 including shFlotillin-1-1 and shFlotillin-1-2) SGC-7901 cells. (C-D) The mRNA (left) and protein (right) levels of Flotillin-1 in Flotillin-1-knockdown AGS cells. (E) The migration and invasion abilities of Flotillin-1-knockdown SGC-7901 cells were detected by Transwell assay. Scale bars, 500μm. (F) The migration ability of Flotillin-1-knockdown SGC-

7901 cells was examined by wound healing assay. Scale bars, 500μm. (G) The migration and invasion abilities of Flotillin-1-knockdown AGS cells were detected by Transwell assay. Scale bars, 500μm. (H) The migration ability of Flotillin-1-knockdown AGS cells was examined by wound healing assay. Scale bars, 500μm. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, p value was calculated with t test, compared to control group. All results were shown as mean±SEM of three independent experiments.

Fig. 4 Flotillin-1 promotes gastric cancer metastasis through inducing EMT

(A-B) The protein expression levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1 overexpressing SGC-7901 (A) and AGS (B) cells. (C-D) The mRNA levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1 overexpressing SGC-7901 (C) and AGS (D) cells. (E-F) The protein expression levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1-knockdown SGC-7901 (E) and AGS (F) cells. (G-H) The mRNA levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1-knockdown SGC-7901 (G) and AGS (H) cells. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, p value was calculated with t test, compared to control group. All results were shown as mean±SEM of three independent experiments.

Fig. 5 Flotillin-1 promotes EMT of gastric cancer via stabilizing Snail

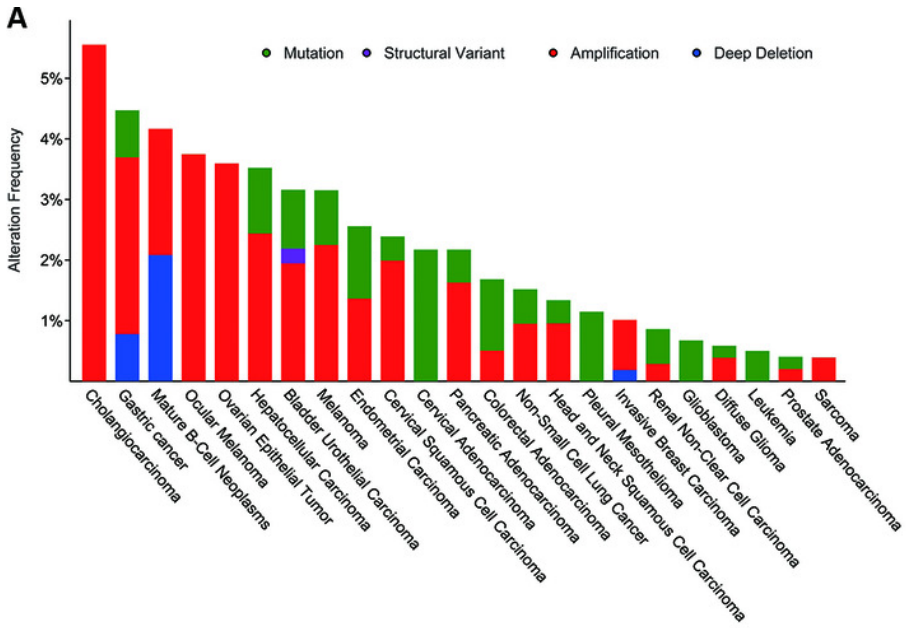
(A) Snail and Flag-Flotillin-1 were co-transfected into HEK293T cells. The cells were treated with MG132 for six hours and subjected into immunoprecipitation assay with anti-Flag antibody to pulldown Flag-Flotillin-1 immunoprecipitates. (B) HA-ubiquitin, Flag-Snail and Flotillin-1 were co-transfected into HEK293T cells. After MG132 treatment, the cells were subjected into ubiquitination assay with anti-Flag antibody. The ubiquitination level of Snail was detected using

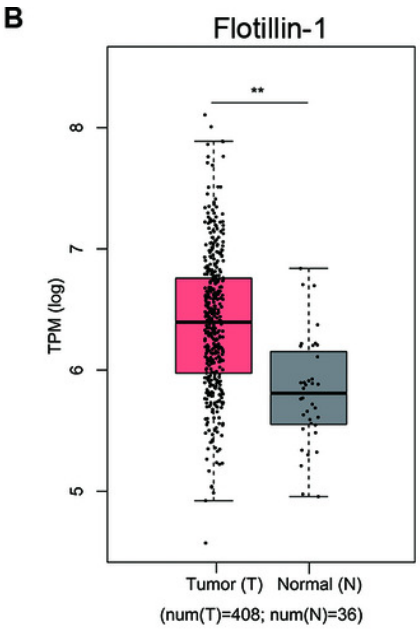
anti-HA antibody. (C) Flotillin-1-overexpressing and control SGC-7901 cells were treated with CHX as indicated. The degradation of Snail was detected by immunoblotting. (D) Flotillin-1-overexpressing and control AGS cells were treated with CHX as indicated. The degradation of Snail was detected by immunoblotting. *$p<0.05$, p value was calculated with t test, compared to control group at 2 h point. All results were shown as mean±SEM of three independent experiments.

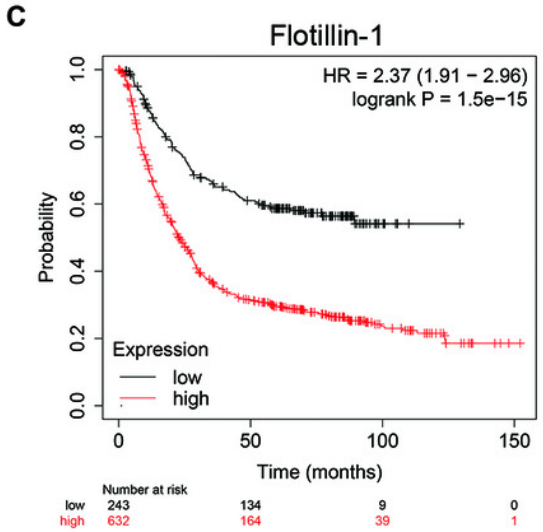
Figure 1

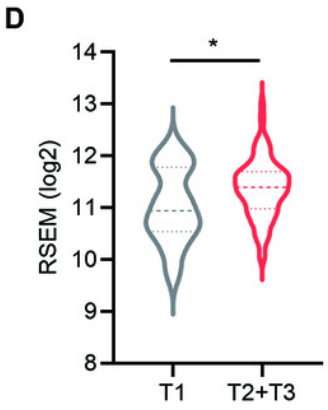
Increased Flotillin-1 predicts a poor prognosis of gastric cancer patients

(A) A histogram shows the amplification frequency of Flotillin in 23 tumor types. The data were taken from TCGA PanCancer Altas studies. (B) The expression of Flotillin-1 in gastric cancer tissues and normal samples. T represents tumor tissues (n=408), N represents normal tissues (n=36). (C) Kaplan-Meier plot showing the overall survival of 875 gastric cancer patients stratified by Flotillin-1 expression level. (D-E) comparison between the proportion of gastric cancer patients with different Flotillin-1 expression level in T stage (D) and distant metastasis (E). *$p<0.05$, **$p<0.01$, p value was calculated with t test, except survival analysis was analyzed using a two-sided log-rank test.


A
Mutation
Structural Variant
Amplification
Deep Deletion
Alteration Frequency
5%
4%
3%
2%
1%
Cholangiocarcinoma
Gastric cancer
Mature B-Cell Neoplasms
Ocular Melanoma
Ovarian Epithelial Tumor
Hepatocellular Carcinoma
Bladder Urothelial Carcinoma
Melanoma
Endometrial Carcinoma
Cervical Squamous Cell Carcinoma
Cervical Adenocarcinoma
Pancreatic Adenocarcinoma
Colorectal Adenocarcinoma
Non-Small Cell Lung Cancer
Head and Neck Squamous Cell Carcinoma
Pleural Mesothelioma
Invasive Breast Carcinoma
Renal Non-Clear Cell Carcinoma
Glioblastoma
Diffuse Glioma
Leukemia
Prostate Adenocarcinoma
Sarcoma


B
Flotillin-1
**
TPM (log)
8
7
6
5
Tumor (T)
Normal (N)
(num(T)=408; num(N)=36)


C
Flotillin-1
HR = 2.37 (1.91 − 2.96)
logrank P = 1.5e−15
Probability
1.0
0.8
0.6
0.4
0.2
0.0
Expression
low
high
0
50
100
150
Time (months)
Number at risk
low 243 134 9 0
high 632 164 39 1


D
*
RSEM (log2)
14
13
12
11
10
9
8
T1
T2+T3

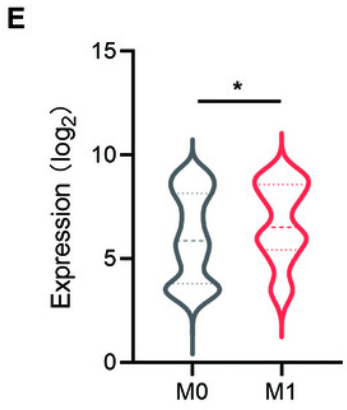

E
*
Expression (log2)
15
10
5
0
M0
M1

Figure 2

Overexpression of Flotillin-1 promotes gastric cancer metastasis

(A-B) The mRNA (A) and protein (B) levels of Flotillin-1 in SGC-7901 cells overexpressing Flotillin-1. (C-D) The mRNA (C) and protein (D) levels of Flotillin-1 in AGS cells overexpressing Flotillin-1. (E) The migration and invasion abilities of SGC-7901 cells overexpressing Flotillin-1 were detected by Transwell assay. Scale bars, 500μm. (F) The migration ability of SGC-7901 cells overexpressing Flotillin-1 was examined by wound healing assay. Scale bars, 500μm. (G) The migration and invasion abilities of AGS cells overexpressing Flotillin-1 were detected by Transwell assay. Scale bars, 500μm. (H) The migration ability of SGC-7901 cells overexpressing Flotillin-1 was examined by wound healing assay. Scale bars, 500μm. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, p value was calculated with t test, compared to control group. All results were shown as mean±SEM of three independent experiments.

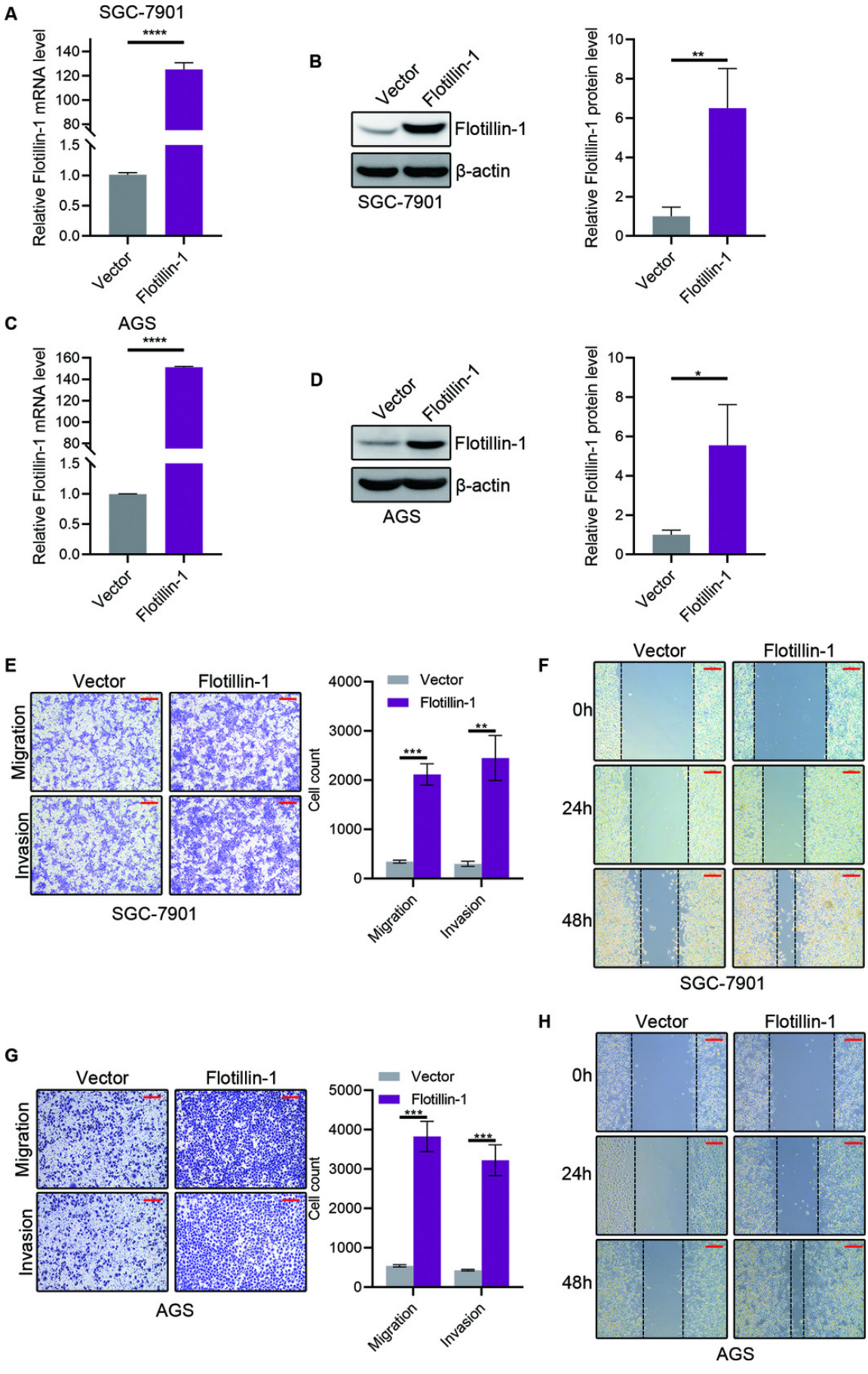

A
SGC-7901
****
Relative Flotillin-1 mRNA level
140
120
100
80
1.5
1.0
0.5
0.0
Vector
Flotillin-1
B
Vector
Flotillin-1
Flotillin-1
β-actin
SGC-7901
Relative Flotillin-1 protein level
**
10
8
6
4
2
0
Vector
Flotillin-1
C
AGS
****
Relative Flotillin-1 mRNA level
160
140
120
100
80
1.5
1.0
0.5
0.0
Vector
Flotillin-1
D
Vector
Flotillin-1
Flotillin-1
β-actin
AGS
Relative Flotillin-1 protein level
*
10
8
6
4
2
0
Vector
Flotillin-1
E
Vector
Flotillin-1
Migration
Invasion
SGC-7901
Vector
Flotillin-1
Cell count
4000
3000
2000
1000
0
***
**
Migration
Invasion
F
Vector
Flotillin-1
0h
24h
48h
SGC-7901
G
Vector
Flotillin-1
Migration
Invasion
AGS
Vector
Flotillin-1
Cell count
5000
4000
3000
2000
1000
0
***
***
Migration
Invasion
H
Vector
Flotillin-1
0h
24h
48h
AGS

Figure 3

Knockdown of Flotillin-1 inhibits gastric cancer metastasis

(A-B) The mRNA (A) and protein (B) levels of Flotillin-1 in Flotillin-1-knockdown (using shRNA targeting Flotillin-1 including shFlotillin-1-1 and shFlotillin-1-2) SGC-7901 cells. (C-D) The mRNA (left) and protein (right) levels of Flotillin-1 in Flotillin-1-knockdown AGS cells. (E) The migration and invasion abilities of Flotillin-1-knockdown SGC-7901 cells were detected by Transwell assay. Scale bars, 500μm. (F) The migration ability of Flotillin-1-knockdown SGC-7901 cells was examined by wound healing assay. Scale bars, 500μm. (G) The migration and invasion abilities of Flotillin-1-knockdown AGS cells were detected by Transwell assay. Scale bars, 500μm. (H) The migration ability of Flotillin-1-knockdown AGS cells was examined by wound healing assay. Scale bars, 500μm. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$, p value was calculated with t test, compared to control group. All results were shown as mean±SEM of three independent experiments.

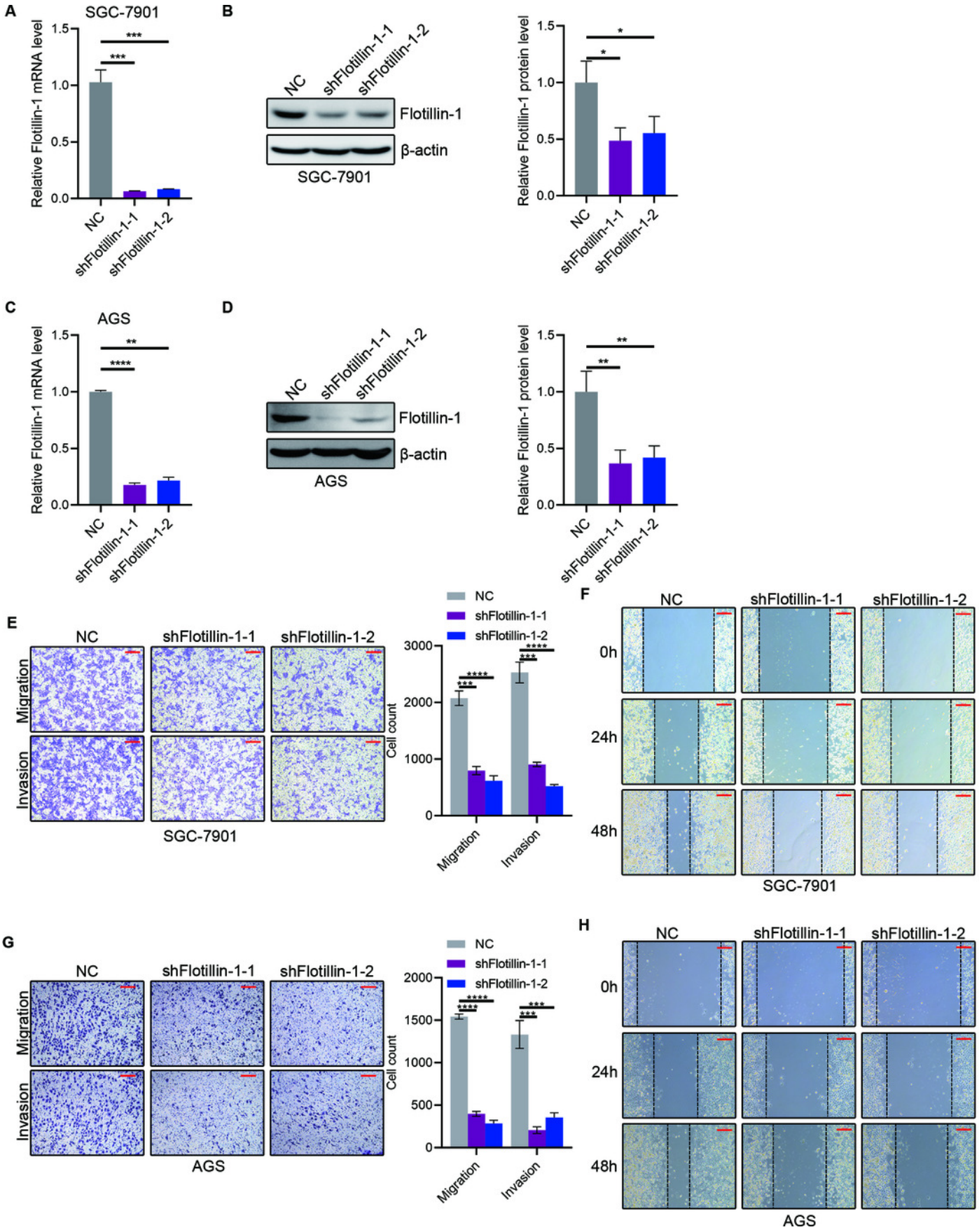

A
SGC-7901
Relative Flotillin-1 mRNA level
1.5
1.0
0.5
0.0
***
***
NC
shFlotillin-1-1
shFlotillin-1-2
B
NC
shFlotillin-1-1
shFlotillin-1-2
Flotillin-1
β-actin
SGC-7901
Relative Flotillin-1 protein level
*
*
C
AGS
**
****
D
AGS
**
**
E
NC
shFlotillin-1-1
shFlotillin-1-2
Migration
Invasion
SGC-7901
Cell count
3000
2000
1000
0
F
0h
24h
48h
SGC-7901
G
AGS
1500
500
H
AGS

Figure 4

Flotillin-1 promotes gastric cancer metastasis through inducing EMT

(A-B) The protein expression levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1 overexpressing SGC-7901 (A) and AGS (B) cells. (C-D) The mRNA levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1 overexpressing SGC-7901 (C) and AGS (D) cells. (E-F) The protein expression levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1-knockdown SGC-7901 (E) and AGS (F) cells. (G-H) The mRNA levels of E-cadherin, N-cadherin, Vimentin and Snail in Flotillin-1-knockdown SGC-7901 (G) and AGS (H) cells. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, p value was calculated with t test, compared to control group. All results were shown as mean±SEM of three independent experiments.

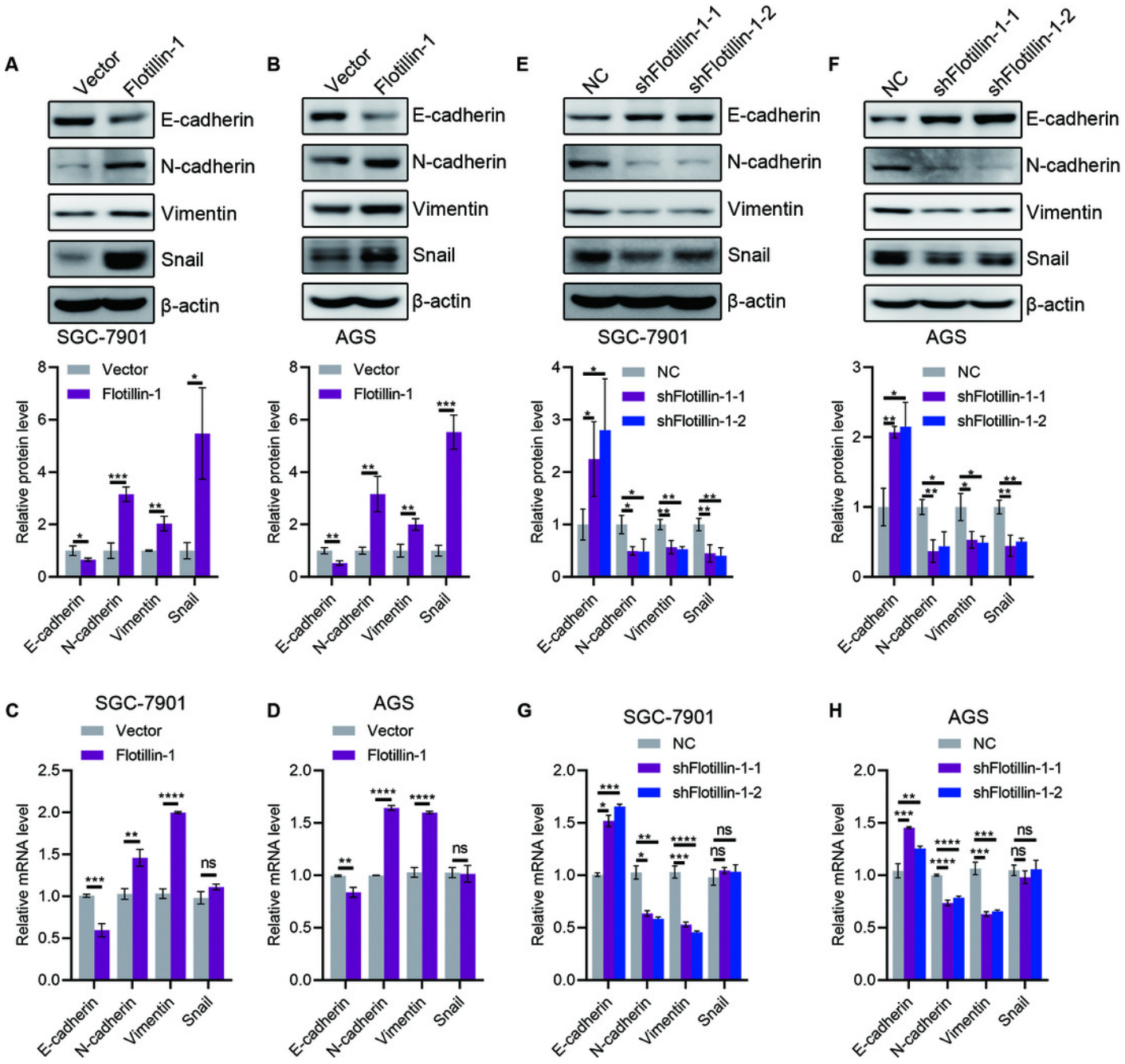
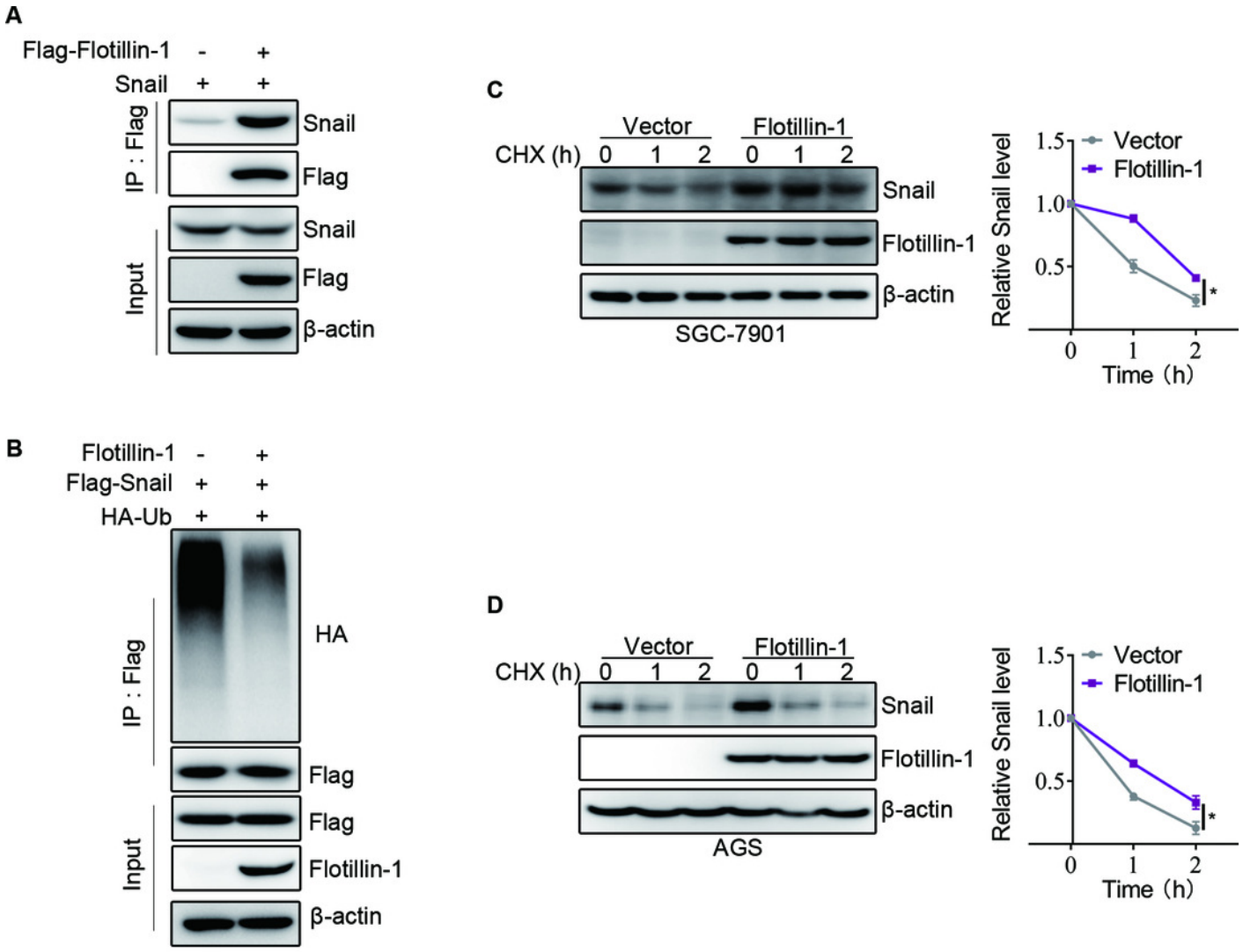

A
Vector
Flotillin-1
E-cadherin
N-cadherin
Vimentin
Snail
β-actin
SGC-7901
B
AGS
E
NC
shFlotillin-1-1
shFlotillin-1-2
SGC-7901
F
AGS
Relative protein level
C
SGC-7901
Relative mRNA level
D
AGS
G
SGC-7901
H
AGS
ns

Figure 5

Flotillin-1 promotes EMT of gastric cancer via stabilizing Snail

(A) Snail and Flag-Flotillin-1 were co-transfected into HEK293T cells. The cells were treated with MG132 for six hours and subjected into immunoprecipitation assay with anti-Flag antibody to pulldown Flag-Flotillin-1 immunoprecipitates. (B) HA-ubiquitin, Flag-Snail and Flotillin-1 were co-transfected into HEK293T cells. After MG132 treatment, the cells were subjected into ubiquitination assay with anti-Flag antibody. The ubiquitination level of Snail was detected using anti-HA antibody. (C) Flotillin-1-knockdown and control SGC-7901 cells were treated with CHX as indicated. The degradation of Snail was detected by immunoblotting. (D) Flotillin-1-knockdown and control AGS cells were treated with CHX as indicated. The degradation of Snail was detected by immunoblotting. *$p<0.05$, p value was calculated with t test, compared to control group at 2 h point.


A
Flag-Flotillin-1 - +
Snail + +
IP : Flag
Snail
Flag
Input
Snail
Flag
β-actin
B
Flotillin-1 - +
Flag-Snail + +
HA-Ub + +
IP : Flag
HA
Flag
Input
Flag
Flotillin-1
β-actin
C
Vector
Flotillin-1
CHX (h) 0 1 2 0 1 2
Snail
Flotillin-1
β-actin
SGC-7901
Relative Snail level
Vector
Flotillin-1
1.5
1.0
0.5
0 1 2
Time (h)
*
D
Vector
Flotillin-1
CHX (h) 0 1 2 0 1 2
Snail
Flotillin-1
β-actin
AGS
Relative Snail level
Vector
Flotillin-1
1.5
1.0
0.5
0 1 2
Time (h)
*