


## ORIGINAL ARTICLE

# Capn4 promotes esophageal squamous cell carcinoma metastasis by regulating ZEB1 through the Wnt/ $\beta$ -catenin signaling pathway

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**Keywords**

Capn4; esophageal squamous cell carcinoma (ESCC); metastasis; ZEB1.

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**Abstract**

**Background:** Capn4 and ZEB1 play important roles in the metastasis of several types of cancer. However, the roles and relationship of Capn4 and ZEB1 in esophageal squamous cell carcinoma (ESCC) remain unclear.

**Methods:** ESCC tumor tissues and corresponding normal esophageal epithelial tissues were obtained from 86 patients undergoing resection surgery at the Department of General Surgery, First Affiliated Hospital of Chinese PLA General Hospital from 2012 to 2017. Cell migration and invasion were examined via quantitative real-time PCR and Western blot assay.

**Results:** Our results indicate that both Capn4 and ZEB1 are significantly upregulated in ESCC tissues compared to corresponding adjacent tissues, and a positive correlation between expression and associated malignant characteristics was found. Silencing of Capn4 expression markedly inhibited ESCC invasion and metastasis in vitro and in vivo, and was accompanied by decreased ZEB1 expression. Furthermore, the anti-metastasis role of Capn4 silencing was reversed by ZEB1 overexpression, whereas knockdown of ZEB1 decreased ESCC metastasis driven by the upregulation of Capn4. Mechanistically, Capn4 regulated ZEB1 expression via activation of the Wnt/ $\beta$ -catenin signaling pathway in ESCC cells.

**Conclusion:** Overall, our results show that enhanced Capn4 expression activates the Wnt/ $\beta$ -catenin signaling pathway, resulting in increased ZEB1 expression and the promotion of ESCC cell metastasis.

**Introduction**

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive carcinomas of the gastrointestinal tract worldwide.<sup>1,2</sup> Despite improvements in detection and therapy, the five-year survival of this malignancy remains below 20%.<sup>3</sup> High invasive potential and distant metastasis are considered major obstacles to satisfactory treatment outcomes.<sup>4</sup> Therefore, a better understanding of the molecular mechanisms of invasion and metastasis of ESCC may be helpful for developing new treatment strategies for this deadly disease.

Capn4, also known as CAPNS1, acts as a binding partner to form a heterodimer with the 80 kDa large catalytic subunit and plays an essential role in maintaining calpain activity.<sup>5,6</sup> Recent studies have shown that Capn4

expression is associated with oncogenesis in solid tumors, such as ovarian,<sup>7</sup> breast,<sup>8</sup> and colorectal cancers.<sup>9</sup> Emerging evidence has linked the biologic function of Capn4 to tumor metastasis.<sup>10–12</sup> For instance, Capn4 silencing results in markedly decreased migration and invasion of nasopharyngeal carcinoma cells.<sup>11</sup> Dai *et al.* found that Capn4 promotes hepatocellular carcinoma metastasis by activating the focal adhesion kinase-steroid receptor coactivator signaling pathway.<sup>12</sup> However, the role of Capn4 expression and its specific mechanism of action in the process of ESCC metastasis are still unclear.

Metastasis of epithelium-derived cancers, including ESCC, is a multistep process regulated by various factors.<sup>13</sup> ZEB1, which belongs to the human ZEB family transcription factors that bind to E-box promoter elements, is one of the most common prometastatic factors.<sup>14</sup> ZEB1

expression has frequently been observed at the migration front of a variety of epithelial tumors and reported as an activator in many cancers with metastatic capability.<sup>15,16</sup> In particular, previous studies have shown that high ZEB1 expression in ESCC is closely correlated with advanced tumor stage and positive lymph node metastasis.<sup>17,18</sup> However, the regulatory mechanism of ZEB1 expression in ESCC remains largely unknown.

In the present study, we found that Capn4 and ZEB1 expression were aberrantly upregulated in ESCC tissues and show a significantly positive correlation. Functional analysis showed that Capn4 facilitates ESCC invasion in vitro and metastasis in vivo by increasing ZEB1 expression. Further investigation indicated that Capn4 regulated ZEB1 expression by activating the Wnt/ $\beta$ -catenin signaling pathway. Thus, Capn4 may become a new therapeutic target or clinical biomarker for metastatic ESCC.

## Methods

### Patients and samples

Esophageal squamous cell carcinoma tumor tissues and corresponding normal esophageal epithelial tissues were obtained from 86 patients undergoing resection surgery at the Department of General Surgery, First Affiliated Hospital of Chinese PLA General Hospital from 2012 to 2017. All specimens from resection surgery were frozen and stored at  $-80^{\circ}\text{C}$  until required. Informed consent was obtained from each patient, and the Ethics Committee of First Affiliated Hospital of Chinese PLA General Hospital approved the study protocol.

### Cell lines and culture conditions

Human ESCC cell lines (ECA109, TE7, TE10, and EC9760) and human normal esophageal epithelial cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin (Sigma-Aldrich, St Louis, MO, USA) and maintained in a humidified incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ .

### RNA extraction and quantitative real-time PCR assay

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed using the PrimeScript RT Reagent Kit (Invitrogen, USA) and quantitative real-time PCR (qRT-PCR) was performed

using SYBR Premix Ex Taq (TaKaRa, Dalian, China), following the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used for qRT-PCR assay were as follows: Capn4 primers, forward: 5'-CAATGTCCGCTTCGGCTCTA-3'; reverse: 5'-GGAGTTCTGGGAGTCGGTTC-3'. ZEB1 primers, forward: 5'-AGCGAGGTAAAGTTGCGTCT-3'; reverse: 5'-AGGTTTTCTGGGCCATACCG-3'. GAPDH primers, forward: 5'-CTGGGCTACACTGAGCACC-3', reverse: 5'-AAGTGGTCGTTGAGGGCAATG-3'.

The comparative  $2^{-\Delta\Delta\text{Ct}}$  method was applied to develop qRT-PCR to analyze the Capn4 or ZEB1 expression pattern in ESCC. A value of  $\Delta\text{Ct} = -1$  was established as the cutoff line between high or low Capn4 or ZEB1 expression in qRT-PCR.

### Western blot assay

Western blot analysis for specific protein expression was performed using standard methods. The antibodies used were as follows: anti-Capn4 (1:1000), anti- $\beta$ -catenin (Abcam, Cambridge, MA, USA); anti-ZEB1 (1:1000, Cell Signaling Technology, Danvers, MA, USA); and anti-GAPDH (1:1000, Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA). The signals were detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

### Constructs and plasmids

The RNA duplexes for short hairpin RNA (shRNA)-mediated Capn4, ZEB1, and  $\beta$ -catenin silencing were synthesized by Genepharma (Shanghai, China). Capn4, ZEBQ, and  $\beta$ -catenin plasmids were purchased from Genepharma. Transfections of the shRNA and overexpression vector in ESCC cells were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) following the manufacturer's recommended protocol.

### Cell migration and invasion assay

The migration ability of ESCC cells was tested in a Transwell Boyden Chamber (8 mm pore size; BD Biosciences, San Jose, CA, USA), as previously described.<sup>19</sup> For the cell invasion assay, the polycarbonate membranes of the upper compartment of the chambers were precoated with a matrix gel.

### In vivo tumor metastatic assay

Esophageal squamous cell carcinoma cells overexpressing or silencing Capn4 were transplanted into SCID beige mice via tail vein injection. The mice were sacrificed and dissected 12 weeks later to examine lung tissue. Lung metastatic nodules were counted for statistical analysis.

The Ethics Committee for Animal Experiments of the First Affiliated Hospital of Chinese PLA General Hospital approved the animal experiments, which were performed in accordance with the “Guide for the Care and Use of Laboratory Animals.”

**Statistical analysis**

All data are expressed in terms of means ± standard error of the mean. Significant differences were analyzed using the Student’s *t*-test and two-tailed distribution. Spearman’s correlation analyses were used to identify the correlation between Capn4 and ZEB1. *P* < 0.05 was considered statistically significant.

**Results**

**Capn4 and ZEB1 expression are both upregulated in esophageal squamous cell carcinoma (ESCC) tissues and are closely related to progression**

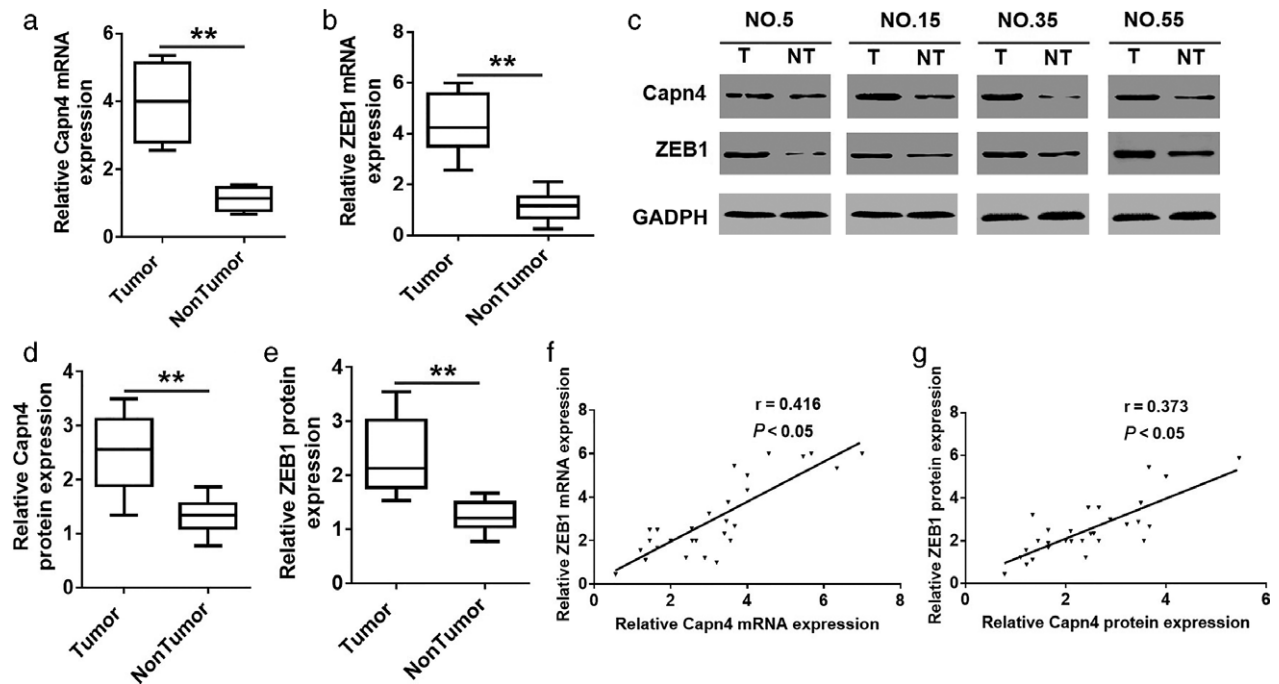
To explore the expression and significance of Capn4 and ZEB1 in ESCC tissues, we initially examined their expression in 86 ESCC tissues and corresponding normal esophageal epithelial tissues. As shown in Figure 1a,b, the average fold

change of Capn4 and ZEB1 messenger RNA (mRNA) expression in tumor tissues was significantly higher than that in paired normal tissues. The qRT-PCR results revealed that Capn4 was overexpressed in 65.1% (56/86) of ESCC specimens detected, and ZEB1 was upregulated in 61.6% (53/86) of ESCC tissues. Consistently, Western blot results showed that the Capn4 and ZEB1 protein levels were significantly elevated in the ESCC tissues (Fig 1c–e). Scatter plots showed that Capn4 and ZEB1 mRNA and protein expression levels were positively correlated in ESCC tissues (Fig 1f,g).

We then analyzed the correlation between high Capn4 and ZEB1 expression and ESCC clinicopathologic parameters. The results showed that both Capn4 and ZEB1 overexpression were closely correlated with advanced tumor stage, positive lymph node metastasis, and greater tumor depth (Table 1). These results suggested a positive correlation between Capn4 and ZEB1 expression in ESCC tumors, and positive or enhanced Capn4 expression was an indicator for metastasis.

**Silencing Capn4 reduces ZEB1 expression and suppresses ESCC invasion and metastasis**

To explore whether Capn4 regulated ZEB1 expression in ESCC cells, we initially examined the expression level of



**Figure 1** Capn4 overexpression is correlated with ZEB1 expression in human esophageal squamous cell carcinoma (ESCC). (a,b) Quantitative real-time PCR analysis of Capn4 and ZEB1 messenger RNA (mRNA) expression in 86 ESCC and paired non-tumor tissues (\*\**P* < 0.01). (c–e) Determination and quantification of Capn4 and ZEB1 protein levels in ESCC and in paired non-tumor tissues by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (f,g) Scatter plots show a positive correlation between Capn4 and ZEB1 at the mRNA and protein level in 30 ESCC samples (*r* = 0.416, \**P* < 0.05 and *r* = 0.373, \**P* < 0.05, \*\**P* < 0.01).

**Table 1** Correlation between Capn4 or ZEB1 and clinicopathologic characteristics in esophageal squamous cell carcinoma patients

Pathologic characteristics	N	Capn4		P	ZEB1		P
		Low	High		Low	High	
Age (years)				NS			NS
< 50	36	12	24		14	22	
≥ 50	50	18	32		19	31	
Gender				NS			NS
Female	26	8	18		10	16	
Male	60	22	38		23	37	
Histological grade				P = 0.036			P = 0.031
G1	22	13	9		14	8	
G2	46	15	31		16	30	
G3	18	2	16		3	15	
Tumor location				NS			NS
Upper	19	6	13		7	12	
Middle	40	15	25		16	24	
Lower	27	9	18		8	17	
Tumor stage				P = 0.009			P = 0.0029
I	26	17	9		18	8	
II	30	11	19		10	20	
III	20	2	18		4	16	
IV	10	0	10		1	9	
Tumor depth				P = 0.001			P = 0.001
T1–2	22	14	8		16	6	
T3–4	64	16	48		17	47	
Lymph node metastasis				P = 0.003			P = 0.001
Negative	50	24	26		27	23	
Positive	36	6	30		6	30	

NS, no statistical significance.

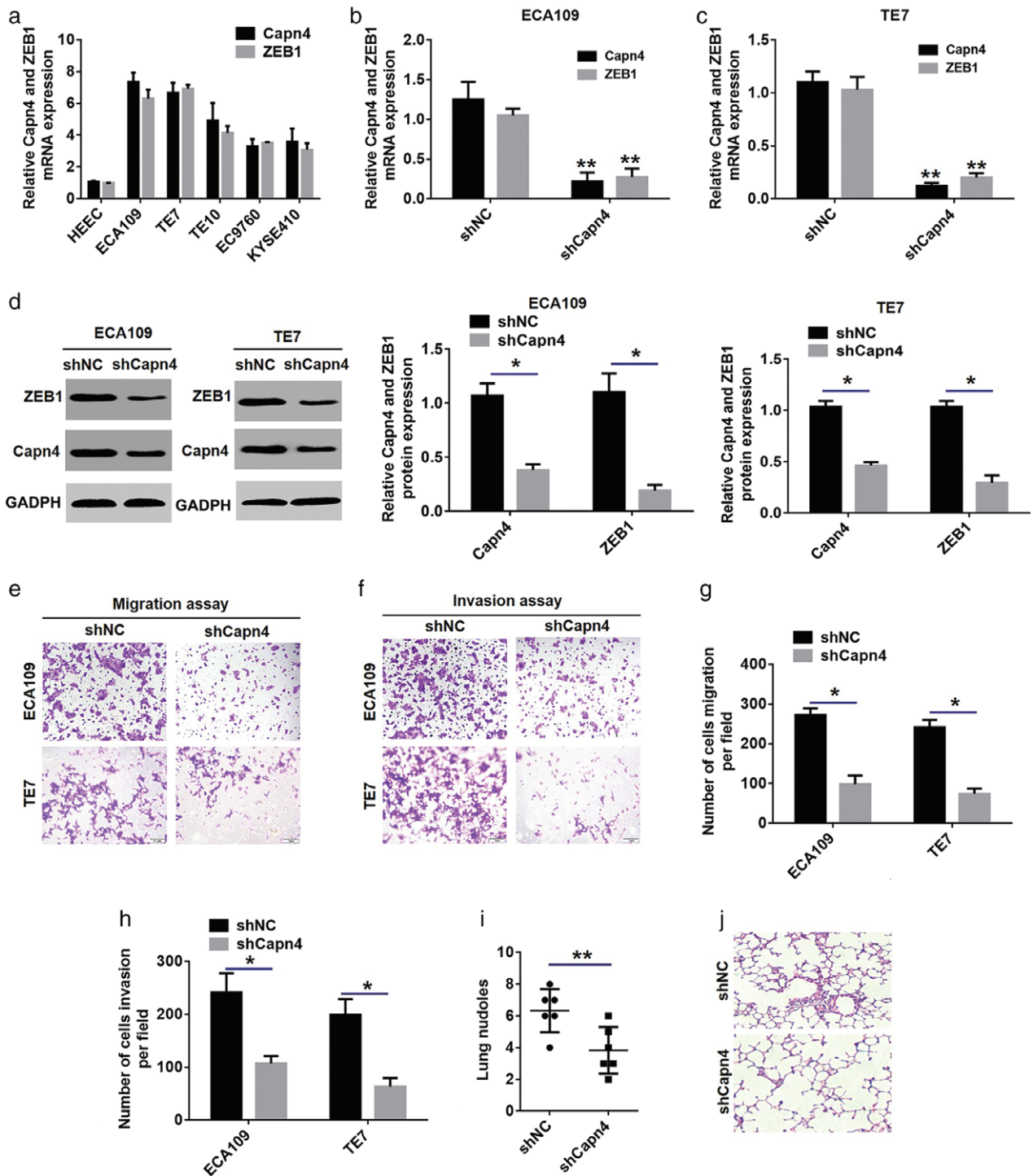
Capn4 and ZEB1 in four human ESCC cell lines (ECA109, TE7, TE10, and EC9760) and human normal esophageal epithelial cells. The results indicated that Capn4 and ZEB1 expression in ESCC cells was higher than in normal esophageal epithelial cells (Fig 2a). We then stably transfected a Capn4-specific shRNA (shCapn4) into ECA109 and TE7 cells. qRT-PCR and Western blotting results showed that knockdown of Capn4 reduced ZEB1 mRNA and protein levels in the ECA109 and TE7 cells (Fig 2b–d). Furthermore, we examined the effects of Capn4 on ESCC cell metastasis by Transwell assay. As shown in Figure 2e–h, Capn4 knockdown significantly suppressed ESCC cell metastasis. Our results showed that the simultaneous silencing of Capn4 and ZEB1 dramatically suppressed ESCC cell migration and invasion (Fig S1). We further examined the effects of Capn4 on ESCC metastasis by in vivo tumor metastatic assay. As shown in Figure 2i, there were fewer lung metastatic nodules in the shCapn4 group than in the control group. Histological staining was performed to further confirm the presence of lung metastasis (Fig 2j). The results indicated that Capn4 silencing could reduce ZEB1 expression and thus inhibit ESCC invasion and metastasis.

### ZEB1 is required for Capn4-mediated metastasis of ESCC cells

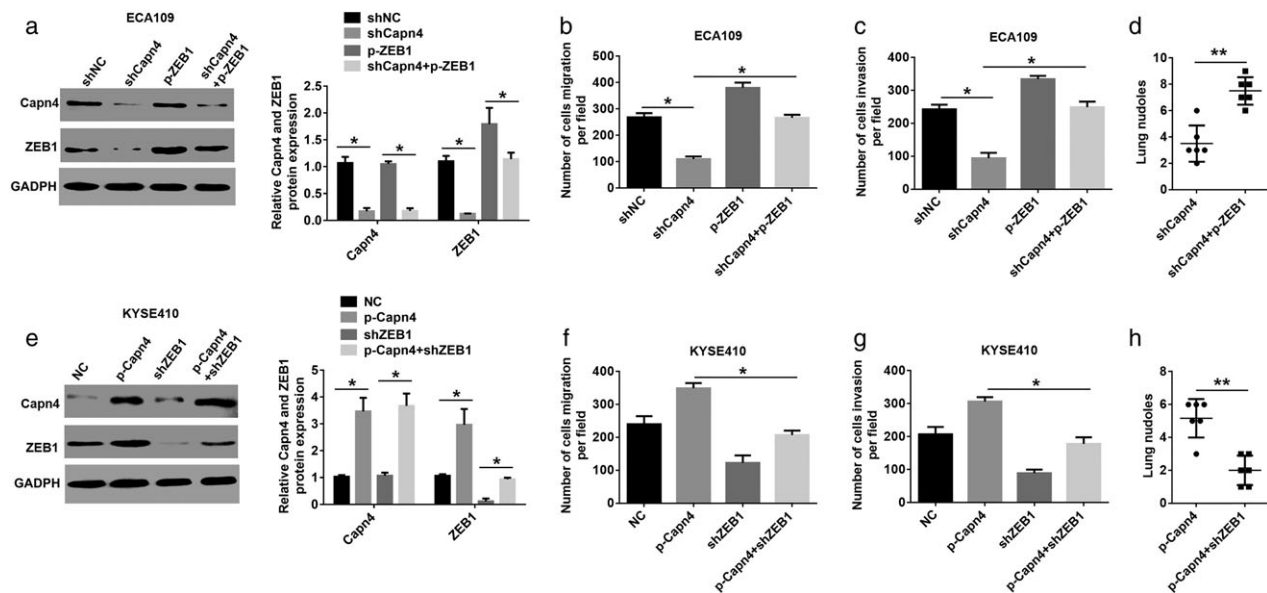
To further confirm that Capn4 mediates ESCC metastasis by regulating ZEB1, we increased the expression of ZEB1 in Capn4 silenced ESCC cells. Capn4 knockdown decreased ZEB1 expression, whereas ZEB1 upregulation attenuated the loss of ZEB1 expression in Capn4 silenced ECA109 cells. ZEB1 overexpression reversed the effects of decreased migration and invasion induced by Capn4 downregulation (Fig 3a–c). Additionally, the in vivo tumor metastatic assay showed that ZEB1 upregulation increased lung metastasis in ESCC cells, which had been inhibited by Capn4-knockdown (Fig. 3d). Conversely, ZEB1 downregulation inhibited ZEB1 expression and significantly reduced cell metastasis in Capn4-overexpressing ESCC cells (Fig 3e–h). Collectively, these results confirm that ZEB1 is crucial for Capn4-mediated ESCC metastasis.

### Capn4 regulates ZEB1 expression through the Wnt/ $\beta$ -catenin signaling pathway in ESCC cells

Previous studies have reported that Capn4 promotes the invasion and metastasis of many cancers by activating the



**Figure 2** Stable knockdown of Capn4 reduced ZEB1 expression and inhibited esophageal squamous cell carcinoma (ESCC) invasion and metastasis in vitro and in vivo. **(a)** Quantitative real-time PCR (qRT-PCR) analysis of Capn4 and ZEB1 expression in human esophageal epithelial and ESCC cell lines. **(b,c)** qRT-PCR analyses were used to detect Capn4 and ZEB1 messenger RNA (mRNA) expression in ECA109 and TE7 cells stably transfected with the short hairpin negative control (shNC) vector or shCapn4 plasmid (\*\* $P < 0.01$ ). **(d)** Determination (left) and quantification (right) of Capn4 and ZEB1 protein levels in ECA109 and TE7 cells stably transfected with the shNC vector or the shCapn4 plasmid. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control (\* $P < 0.05$ ). **(e,g)** Transwell migration assays of ECA109 and TE7 cells with stable Capn4 knockdown (\* $P < 0.05$ ). **(f,h)** Transwell invasion assays of ECA109 and TE7 cells transfected with shCapn4 plasmid (\* $P < 0.05$ ). **(i)** Statistical analysis of lung metastatic nodules. ( $n = 6$ /group; \*\* $P < 0.01$ ). **(j)** Hematoxylin and eosin staining of the paraffin embedded sections of lung metastatic nodules.



**Figure 3** Capn4 promotes esophageal squamous cell carcinoma (ESCC) invasion and metastasis by upregulating ZEB1 expression. **(a)** Determination (left) and quantification (right) of Capn4 and ZEB1 protein levels in the indicated cells by Western blotting. ZEB1 upregulation attenuated the loss of ZEB1 expression in ECA109-short hairpin Capn4 (shCapn4) cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control ( $*P < 0.05$ ). **(b,c)** Transwell assays showed that the upregulation of ZEB1 significantly rescued the cell migration and invasion in ECA109-shCapn4 cells ( $*P < 0.05$ ). **(d)** Statistical analysis of lung metastatic nodules. ( $n = 6$ /group;  $**P < 0.01$ ). **(e)** Western blotting was used to detect Capn4 and ZEB1 expression. **(f,g)** Transwell assays showed that ZEB1 inhibition reduced Capn4-enhanced cell migration and invasion ( $*P < 0.05$ ). **(h)** Statistical analysis of lung metastatic nodules ( $n = 6$ /group;  $**P < 0.01$ ).

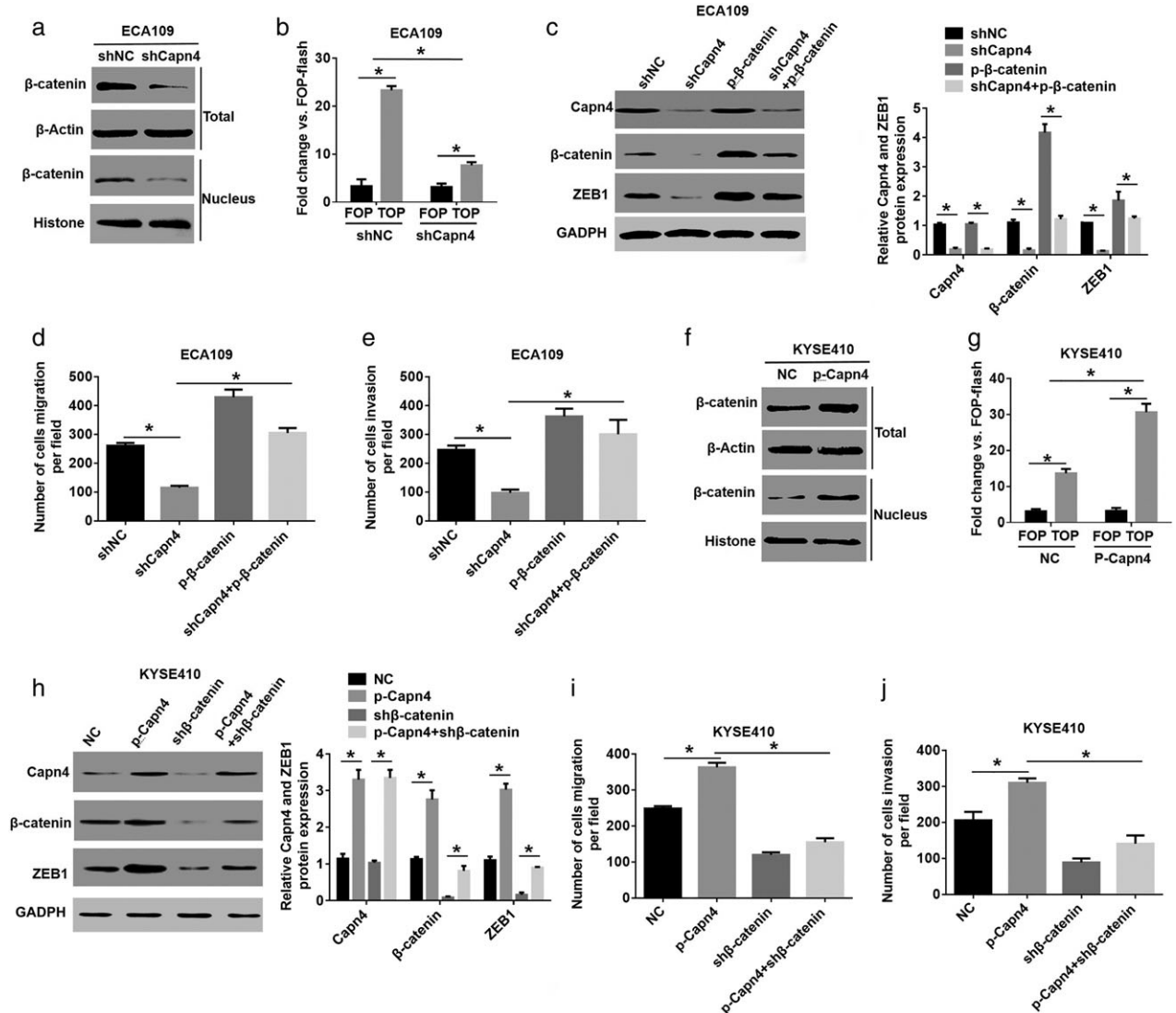
Wnt/ $\beta$ -catenin signaling pathway.<sup>20–22</sup> Many studies have also shown that ZEB1 is a downstream target of the Wnt/ $\beta$ -catenin signaling pathway.<sup>23,24</sup> Thus, we speculated that Capn4 regulates ZEB1 expression by activating the Wnt/ $\beta$ -catenin signaling pathway in ESCC cells.

To test this hypothesis, we first observed whether Capn4 regulated the Wnt/ $\beta$ -catenin signaling pathway in ESCC cells. The results showed that as Capn4 expression decreased, total and nuclear  $\beta$ -catenin expression reduced in ECA109 cells (Fig 4a). Meanwhile, in a TOP-Flash reporter luciferase assay, silencing of Capn4 in ECA109 cells decreased the transcriptional activity of TCF4 compared to the control groups (Fig 4b). In addition, we found that upregulation of  $\beta$ -catenin reversed the effects of decreased ZEB1 expression and cell migration and invasion induced by Capn4 knockdown (Fig. 4c–e). In contrast, upregulation of Capn4 significantly increased total and nuclear  $\beta$ -catenin expression levels, and the transcriptional activity of TCF4 was also increased (Fig 4f,g). Knockdown of  $\beta$ -catenin significantly suppressed the increase in ZEB1 expression and reduced cell metastasis in Capn4-overexpressing ESCC cells (Fig 4h–j). Overall, these data showed that Capn4 promotes ESCC metastasis via the Wnt/ $\beta$ -catenin/ZEB1 axis.

## Discussion

Esophageal squamous cell carcinoma is one of the most common malignancies, with low five-year overall survival. Because metastasis causes 90% of all cancer-related deaths, including ESCC,<sup>25</sup> a deep understanding of the mechanisms of metastasis is required. Capn4 is a potent oncogene and plays a crucial role in the metastasis of several tumors. However, no information is currently available on the specific role or molecular mechanism of Capn4 in ESCC. Herein, we prove that both Capn4 and ZEB1 overexpression are closely associated with the invasive pathologic features of ESCC. We also found that Capn4 silencing could reduce ZEB1 expression and decrease ESCC invasion and metastasis in vitro and in vivo. Furthermore, our results show that Capn4 positively regulates ZEB1 expression by activating the Wnt/ $\beta$ -catenin signaling pathway in ESCC. Our results indicate that Capn4 plays a critical role in ESCC metastasis, and could be a potential therapeutic target.

Metastasis is an essential biological characteristic of many cancers,<sup>26</sup> and is associated with cancer progression.<sup>27</sup> ZEB1 is important factor in the metastatic process, which drives epithelial-to-mesenchymal transition.<sup>28</sup> Increasing evidence indicates that ZEB1 is deeply involved



**Figure 4** Capn4 regulates ZEB1 expression through the Wnt/β-catenin pathway in esophageal squamous cell carcinoma (ESCC). (a) The total and nuclear protein β-catenin levels were assessed by Western blotting in ECA109 cells transfected with short hairpin Capn4 (shCapn4). (b) The relative luciferase activity levels in cells transfected with TOP-flash and FOP-flash vectors in ECA109 cells transfected with short hairpin negative control (shNC) and cells transfected with shRNA against Capn4 are shown ( $*P < 0.05$ ). (c) Determination (left) and quantification (right) of Capn4, ZEB1, and β-catenin protein levels in the indicated cells by Western blotting. (d,e) Upregulation of β-catenin reversed cell migration and invasion in ECA109-shCapn4 cells ( $*P < 0.05$ ). (f) Western blotting was used to assess the total and nuclear protein β-catenin levels in KYSE410 cells transfected with p-Capn4. (g) The relative luciferase activity levels in cells transfected with TOP-flash and FOP-flash vectors in ECA109 cells transfected with the Capn4 plasmid ( $*P < 0.05$ ). (h) Determination (left) and quantification (right) of Capn4, ZEB1, and β-catenin protein levels in the indicated cells by Western blotting. (i,j) Downregulation of β-catenin reduced Capn4-enhanced migration and invasion in KYSE410 cells ( $*P < 0.05$ ). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control ( $*P < 0.05$ ).

in cancer invasion and metastasis.<sup>15,16</sup> Thus, elucidation of ZEB1 expression mechanisms will improve understanding of the process of ESCC metastasis. Our data confirm that downregulation of Capn4 expression decreases ZEB1 expression and inhibits ESCC metastasis. Furthermore, ZEB1 overexpression reverses the effects of decreased cell invasion and migration induced by Capn4 knockdown,

whereas ZEB1 silencing significantly decreases Capn4-enhanced cell proliferation. These results show that Capn4 positively regulates ZEB1 expression and thus influences ESCC metastasis, suggesting a new regulatory mechanism of ZEB1.

Capn4 is reported to contribute to the invasion and metastasis of many cancers by activating the Wnt/β-catenin

signaling pathway. Additionally,  $\beta$ -catenin and TCF4 can bind the promoter region of ZEB1 to promote its expression.<sup>29,30</sup> Therefore, we predicted that Capn4 regulates the Wnt signaling pathway target gene ZEB1 to promote ESCC metastasis. First, we validated that Capn4 could activate the Wnt/ $\beta$ -catenin signaling pathway in ESCC cells. Second, Capn4 knockdown led to the recovery of ZEB1 protein levels in cells overexpressing  $\beta$ -catenin. Third, Capn4 upregulation decreased ZEB1 in cells silencing  $\beta$ -catenin. Therefore, Capn4 increases Wnt/ $\beta$ -catenin signaling pathway activity, which induces increased ZEB1 expression in ESCC cells.

In summary, our results provide the first evidence that Capn4 functions as an oncogene in ESCC. Moreover, we not only clarify the function of Capn4 in ESCC metastasis, but also provide novel insight into the mechanism of Capn4, which modulates the Wnt/ $\beta$ -catenin/ZEB1 axis. These results highlight the significant power of Capn4 expression, which could serve as a therapeutic target in ESCC in the future.

## Disclosure

No authors report any conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** Simultaneous silencing Capn4 and ZEB1 significantly inhibited the migration and invasion abilities of esophageal squamous cell carcinoma (ESCC) cells. (a,c) Determination and (b,d) quantification of Capn4 and ZEB1 protein levels in ECA109 and TE7 cells stably transfected with the shNC vector, shCapn4 or the shCapn4 + shZEB1 plasmid. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control (\* $P < 0.05$ ). (e,f) Transwell migration assays of ECA109 and TE7 cells following simultaneous knockdown of Capn4 and ZEB1 (\* $P < 0.05$ ).