ENKUR recruits FBXW7 to ubiquitinate and degrade MYH9 and further suppress MYH9-induced deubiquitination of β-catenin to block gastric cancer metastasis

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Funding information
Science and Technology Program of Guangzhou, Grant/Award Numbers: 202206010068, 201803010023; National Natural Science Foundation of China, Grant/Award Numbers: 81974460, 81572649; Discipline construction project of Guangdong Medical University, Grant/Award Number: 4SG2101I0; Natural Science Foundation of Hunan Province, Grant/Award Number: 2021JJ30402; Scientific Project of Guangzhou Medical University, Grant/Award Number: 2021Z0003.

Abstract
ENKUR was shown as a suppressor in some tumors. However, the biological role of ENKUR on gastric cancer (GC) and its related molecular mechanisms is not clear. Here, we first observed that ENKUR significantly inhibited cell migration, invasion, and metastasis in GC. The molecular basis showed β-catenin-mediated epithelial-mesenchymal transition (EMT) signaling was inactivated in ENKUR-overexpressing GC cells. In addition, ENKUR knockdown markedly restored cell migration and invasion. Subsequently, ENKUR bound to MYH9 and decreased its protein expression by recruiting E3 ubiquitin ligase FBXW7 to form an ubiquitinated degradation complex. The downregulated MYH9 protein weakened the recruitment of the deubiquitinase USP2 and thus promoted the degradation of β-catenin protein, which finally suppressed EMT signaling. Finally, the oncogenic transcription factor c-Jun bound to ENKUR promoter and reduced its expression in GC. In clinical samples, decreased ENKUR expression promoted the unfavorable prognosis of GC. Our data proved the vital role of ENKUR on suppressing cell migration, invasion, and metastasis and demonstrated its potential as a therapeutic target for GC.
INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies globally and the fourth leading cause of cancer-associated deaths. In the pathogenesis of GC, normal gastric mucosal cells exhibit gene mutations and thus harbor immortality and infiltration abilities. The higher metastasis property makes GC cells easily have direct infiltration, hematogenous metastasis, peritoneal implantation, and lymphatic metastasis, which limits the application and effect of surgical therapy. Despite advances in chemical therapy, targeted therapy, and immune therapy in recent years, the prognosis of patients with GC remains poor because of cancer metastasis.

ENKUR is highly expressed in the testis and vomeronasal organ and is identified as a transient receptor potential-canonical (TRPC) channel binding protein. Initially, ENKUR was found to participate in schizophrenia, the permeability of microvascular endothelial cells, human development. Subsequently, ENKUR has shown promising antitumor ability to decrease cell migration and invasion in lung adenocarcinoma and colorectal cancer. Our recent studies also showed that the ENKUR was an important tumor suppressor and mediated the small molecule chemical compound cinobufotalin to inhibit tumor proliferation, metastasis, and chemoresistance in nasopharyngeal carcinoma, hepatocellular carcinoma, and lung adenocarcinoma. However, the influence of the ENKUR on the biological function of GC and its related molecular mechanisms have not yet been studied.

MYH9 was reported to participate in some biological processes including cell adhesion, polarity formation as well as cell migration. Although MYH9 was originally identified as a tumor suppressive factor in squamous cell carcinoma of head and neck, recent studies support a role for MYH9 as an oncprotein in many tumors, including GC. Interestingly, in previous studies, we have demonstrated that MYH9 interacted with ENKUR and antagonized ENKUR to induce-cell growth and metastasis in hepatocellular carcinoma, lung adenocarcinoma, and nasopharyngeal carcinoma via suppressing p53 or elevating c-Myc. Nevertheless, there is no document to clarify the mechanism of ENKUR to modulate MYH9 in GC.

RESULTS

ENKUR functions as a tumor suppressor

Downregulated ENKUR level was confirmed in BGC-823 and AGS cells compared to GSE-1 cells by quantitative real-time PCR (qPCR) and western blot (WB) experiment. To explore the biological role of ENKUR, lentivirus carrying ENKUR cDNA was used to infect with GC cells. Subsequently, we found ENKUR overexpression reduced the ability of GC cells to migrate and invade. WB experiment showed that β-catenin and N-cadherin was decreased, while E-cadherin was increased in ENKUR overexpressing group.

In view of the suppressive role of ENKUR on the ability of GC cells to migrate and invade, we silenced ENKUR and performed the wound healing experiments and Boyden experiments. The interference efficiency of specific siRNAs against ENKUR was examined by WB experiments in AGS and BGC-823 cells overexpressing ENKUR. Suppression of ENKUR restored ability of GC cells to migrate and invade, while E-cadherin was increased, while E-cadherin was decreased in ENKUR-silenced group.
ENKUR suppresses cell migration and invasion in GC. Wound healing assays (A) and Boyden assays (B) were carried out in gastric cancer (GC) cells transfected with NC or ENKUR plasmids. (C) The protein expressions are shown of β-catenin, E-cadherin and N-cadherin in ENKUR-overexpressing GC cells transfected with NC or ENKUR plasmid. (D) A pulmonary metastasis model was used to assess the effect of ENKUR on GC metastasis. (E) Immunohistochemistry was utilized to detect the levels of E-cadherin and N-cadherin in pulmonary metastasis models.

(Figure S2C). These results proved that ENKUR inactive the EMT signals to block GC metastasis.

2.2 ENKUR interacts with MYH9

We employed the co-immunoprecipitation (Co-IP) and immunofluorescence assays to validate the protein that binding to ENKUR. After checking the results of Mass spectrometry analysis followed by Co-IP, MYH9 was taken as candidate protein that interacted with ENKUR with higher match scores in A549 cells with ENKUR overexpression. Therefore, we explored whether MYH9 binds to ENKUR to regulate cell metastasis in GC cells. The result of Co-IP showed MYH9 is the interacting protein of ENKUR, and immunofluorescence tests further confirmed that ENKUR and MYH9 colocalized in the cytoplasm (Figure 2A,B). Three domains were contained in the ENKUR: Enkurin domain, SH3 domain, and N-terminal region domain (Figure 2C). We observed that an Enkurin domain was a factor inhibiting cell migration and invasion rather than other domains (Figure S3A,B). To determine specific binding domains between ENKUR and MYH9, Co-IP was carried out in BGC-823 cells using domain plasmids. The data displayed that the combination of ENKUR and MYH9 occurred via the Enkurin domain rather than other two domains (Figure 2D). Furthermore, three domains of MYH9 including Myosin tail, Myosin head motor, and Myosin N were predicted based on InterPro (http://www.ebi.ac.uk/interpro/). We found that the Myosin tail domain binds to ENKUR (Figure 2F). These results indicated that ENKUR and MYH9 interact with each other via the Enkurin domain and Myosin tail domain, respectively.
FIGURE 2  The Enkurin domain mediates the interaction between ENKUR and MYH9. (A) Co-IP analysis was performed of the binding of ENKUR to MYH9 in ENKUR-overexpressing BGC-823 cells. (B) Immunofluorescence assays were used to visualize the colocalization of ENKUR and MYH9 in ENKUR-overexpressing BGC-823 cells (left). The fluorescence intensity was calculated to show the colocalization of ENKUR and MYH9 in the cytoplasm (right). (C) A schematic diagram of the Enkurin domain, SH3 domain, and N-terminal region domain in ENKUR protein structure is shown. (D) Co-IP analysis was performed to examine the interaction between the domains of ENKUR and MYH9 in BGC-823 cells with different ENKUR domain plasmids transfection. (E) A schematic diagram of the Myosin tail, Myosin head motor, and Myosin N in MYH9 is shown. (F) Co-IP analysis was performed to examine the interaction between domains and MYH9 in BGC-823 cells with different MYH9 domain plasmids transfection.

2.3  ENKUR recruits FBXW7 to ubiquitinate and degrade MYH9 protein

To clarify the underlying molecular mechanism of ENKUR to modulate MYH9, western blot and Co-IP assays were performed. Subsequently, ENKUR was found to shorten the half-life of MYH9 protein in BGC-823 cells incubated with cycloheximide (CHX) (Figure 3A). Co-IP assays showed that FBXW7, MYH9, and ubiquitin were the interacting proteins of ENKUR in BGC-823 cells (Figure 3B). Overexpressed ENKUR increased ubiquitinated expression of MYH9 protein in BGC-823 cells treated with MG132 (Figure 3C). Further, knockdown of FBXW7 elevated MYH9 protein in ENKUR-overexpressing GC cells (Figure 3D). In summary, our results indicated that ENKUR recruits FBXW7 to promote ubiquitination and degradation of MYH9 protein.

2.4  MYH9 attenuates ENKUR-induced EMT signaling inhibition by recruiting deubiquitinase USP2 to stabilize β-catenin protein

We detected the effect of MYH9 on the restoration of ENKUR-induced EMT signaling inhibition via western
FIGURE 3  ENKUR recruits FBXW7 to ubiquitinate and degrade MYH9. (A) The level of MYH9 was detected in ENKUR-overexpressing BGC-823 cells with or without MG132 incubation at 0, 4, 8, 12, and 16 h after cycloheximide (CHX) treatment. (B) Co-immunoprecipitation (Co-IP) assays were performed to detect the interacting proteins in BGC-823 cells with ENKUR overexpression. (C) The ubiquitinated MYH9 expression was determined in ENKUR-overexpressing BGC-823 cells after 6 h of incubation with MG132. (D) WB experiment was used to detect the level of MYH9 in FBXW7-silenced GC cells.

FiguRe 3  EnKur recruits FBxW7 to ubiquitinate and degrade MYH9. (A) The level of MYH9 was detected in ENKUR-overexpressing BGC-823 cells with or without MG132 incubation at 0, 4, 8, 12, and 16 h after cycloheximide (CHX) treatment. (B) Co-immunoprecipitation (Co-IP) assays were performed to detect the interacting proteins in BGC-823 cells with ENKUR overexpression. (C) The ubiquitinated MYH9 expression was determined in ENKUR-overexpressing BGC-823 cells after 6 h of incubation with MG132. (D) WB experiment was used to detect the level of MYH9 in FBXW7-silenced GC cells.

The results showed that MYH9 restored β-catenin and EMT-related protein expression patterns in ENKUR-overexpressing cells (Figure 4A). After introducing CHX into MYH9-overexpressing BGC-823 cells and control cells, we observed that MYH9 promoted the stabilization of β-catenin (Figure 4B). In addition, MYH9 was shown to recruit the deubiquitinase USP2, β-catenin, and ubiquitin to form a complex by Co-IP assays (Figure 4C). Furthermore, overexpressed MYH9 decreased β-catenin protein ubiquitination in BGC-823 cells incubated with MG132 (Figure 4D). Suppression of USP2 inhibited β-catenin in GC cells with MYH9 overexpression (Figure 4E). The above data demonstrated that MYH9 enlists deubiquitinase USP2 to stabilize β-catenin protein and thus attenuates ENKUR-induced EMT signaling inhibition.

2.5  C-Jun directly suppresses ENKUR transcription

To explore the transcription factor of ENKUR, qPCR and chromatin immunoprecipitation (ChIP) were performed in GC cells. Two c-Jun-binding sites (site-A: −250 to −238; site-B: −1077 to −1065) were predicted in ENKUR promoter region using the JASPAR (Figure 5A). To examine the action of c-Jun in modulating ENKUR, we transfected c-Jun plasmids into AGS and BGC-823 cells and found that overexpressing c-Jun decreased the expression of ENKUR (Figure 5B,C). ChIP with anti-c-Jun antibodies and gel electrophoresis confirmed that c-Jun bind to transcription regulatory sequences of ENKUR (Figure 5D and E). Luciferase reporter and electrophoretic mobility shift assay (EMSA) assays further proved the binding of c-Jun to transcription regulatory sequences of ENKUR (Figure 5F,G). These data suggest c-Jun directly suppresses ENKUR transcription.

2.6  Pathoclinical features of ENKUR and MYH9

To confirm the significance of ENKUR and MYH9 protein levels in pathogenesis of GC, immunohistochemistry was used to detect ENKUR and MYH9 protein in cancer and para-cancer tissues. Using immunohistochemistry, we confirmed ENKUR protein was reduced in GC tissues (Figure 6A and Table S1). As shown in Table 1, there is no significant correlation between ENKUR and clinicopathologic characteristics. Kaplan–Meier analysis showed that the prognosis of GC patients with high ENKUR protein levels was better than those with low ENKUR expression (Figure 6B). Stratum analysis showed a trend hinting higher ENKUR expression led to longer survival times in early and advanced stages (Figure 6C).

Compared with normal gastric tissues, MYH9 was shown to be upregulated in GC tissues (Figure 6D and
Figure 4 MYH9 attenuates ENKUR-induced EMT signaling inhibition by recruiting deubiquitinase USP2 to stabilize β-catenin protein. (A) The effect of MYH9 overexpression on β-catenin and EMT-related protein level changes is shown in ENKUR-overexpressing gastric cancer (GC) cells. (B) WB experiment was used to access the effect of MYH9 overexpression on β-catenin stability in BGC-823 cells with or without MG132 incubation at 0, 5, 10, 15, and 25 h after cycloheximide (CHX) treatment. (C) The interactions of MYH9, β-catenin, USP2, and Ubiquitin were detected in BGC-823 cells. (D) The ubiquitinated β-catenin expression was determined in control cells and MYH9-overexpressing BGC-823 cells after incubation with MG132 for 6 h. (E) The protein changes in MYH9-overexpressing GC cells transfected with si-USP2 were determined.

Table S2). As shown in Table 2, MYH9 protein levels were not significantly correlated with clinicopathologic characteristics. Survival analysis demonstrated that the prognosis of GC patients with elevated MYH9 expression is poor (Figure 6E). Further, MYH9 was negatively correlated with survival time in advanced stages, while this association was not found in early stages (Figure 6F).

Survival analysis combining ENKUR and MYH9 showed that high ENKUR and low MYH9 led to the longest survival time of GC patients, followed by those with low ENKUR + low MYH9, high ENKUR + high MYH9, and low ENKUR + high MYH9 (Figure 6G). Furthermore, Cox proportional hazards regression model analysis in GC patients showed that ENKUR and MYH9 served as independent prognostic factors (Table S3). In conclusion, abnormal protein levels of ENKUR and MYH9 were significantly involved in GC survival prognosis.

3 | DISCUSSION

Metastasis is one of the hardest obstacles for treating cancer.24,25 It is the main reason for cancer-related death.26–28 Here, we first found ENKUR was downregulated in GC cells, which suggested ENKUR as a candidate tumor suppressive factor. Subsequently, we proved downregulated ENKUR expression in GC cells contributes to cell migration and invasion. Mechanistic analysis identified β-catenin-mediated EMT as a negative downstream signal of ENKUR. In addition, immunohistochemistry was utilized to examine EMT-related protein in lung tissues of lung metastasis models. Decreased N-cadherin and elevated E-cadherin protein were observed in the group with ENKUR overexpression. The above results indicated that ENKUR significantly reduces the ability of cells to migrate and
invade as well as metastasis in GC by inactivating EMT signal. In previous studies, some genes or signals are significantly involved in GC EMT and metastasis such as Lgr5, DCLK1, MYH9, and miR-31/RhoA et al.\textsuperscript{29–33} In our studies, MYH9 had shown a powerful ability to promote pathogenesis of nasopharyngeal carcinoma (NPC) and hepatocellular carcinoma (HCC) via binding to GSK3β or HBV X protein (HBX).\textsuperscript{34,35} After checking the data of mass spectrometry from A549 cells with ENKUR overexpression, we interestingly found that MYH9, but not Lgr5, DCLK1, and RhoA, is a potential interacting protein with ENKUR in the development of GC. Co-IP and immunofluorescence assays further confirmed our hypothesis that ENKUR binds to MYH9. In the tertiary structure of a protein, domain is considered as an independent folding unit and has different functions.\textsuperscript{36,37} After constructing plasmids with different domains, we found that Enkurin was the key domain for ENKUR in playing an antimetastatic role and interacting with MYH9. In addition, the Myosin tail domain was also found to be the element interacting with ENKUR.

Ubiquitination is reported as a protein modification mode at posttranslational level, and ubiquitin-mediated MYH9 stability has been shown to participate in the development of cancer.\textsuperscript{38} However, there is no report on whether
ENKUR can reduce MYH9 protein expression through the ubiquitination degradation pathway. To explore the biological significance of the combination of ENKUR and MYH9, we detected the stability of MYH9 protein in GC cells with ENKUR overexpression and found that ENKUR shorten the half-life of MYH9 protein. FBXW7 is a significant E3 ubiquitination ligase inducing c-Myc in ubiquitination degradation in GC.39 Our previous studies have shown that the E3 ubiquitin ligase FBXW7 mediates the process by which CCDC65 promotes the ubiquitinated degradation of ENO1.40 Here, we unexpectedly found that ENKUR recruits FBXW7 to ubiquitinate and degrade MYH9 protein. These data demonstrated that ENKUR inhibits MYH9 expression under the regulation of protein modification.

Previous study has shown that β-catenin mediates MYH9 to promote GC metastasis22; thus, we speculated MYH9 reversed the antimetastatic role of ENKUR via increasing β-catenin expression level in GC. To confirm this speculation, we transfected MYH9 plasmids to ENKUR-overexpressing GC cells and found that β-catenin-mediated EMT signaling was restored. Yet, the molecular mechanism of MYH9-induced β-catenin expression has not been clarified. Subsequently, we found that MYH9...
TABLE 1  Correlation between the clinicopathologic characteristics and ENKUR expression in GC

| Factors                  | Low (n) | High (n) | p-Value |
|--------------------------|---------|----------|---------|
| Gender                   |         |          |         |
| Male                     | 123     | 66       | 57      |
| Female                   | 65      | 30       | 35      |
| Age (y)                  |         |          |         |
| ≤60                      | 68      | 34       | 34      |
| >60                      | 120     | 62       | 58      |
| T classification         |         |          |         |
| T1–T2                    | 21      | 7        | 14      |
| T3–T4                    | 167     | 89       | 78      |
| N classification         |         |          |         |
| N0–N1                    | 78      | 34       | 44      |
| N2–N3                    | 110     | 62       | 48      |
| M classification         |         |          |         |
| M0                       | 178     | 90       | 88      |
| M1                       | 10      | 6        | 4       |
| Pathological classification|   |          |         |
| 1–2                      | 30      | 11       | 19      |
| 3                        | 158     | 85       | 73      |
| AJCC stage               |         |          |         |
| I–II                     | 75      | 33       | 42      |
| III–IV                   | 113     | 63       | 50      |
| Tumor size (cm)          |         |          |         |
| ≤5                       | 94      | 42       | 52      |
| >5                       | 94      | 54       | 40      |
| Lymphovascular invasion  |         |          |         |
| No                       | 157     | 80       | 77      |
| Yes                      | 31      | 16       | 15      |

Abbreviation: AJCC, American Joint Committee on Cancer; GC, gastric cancer.

*χ² test was applied to access the correlation between the clinicopathologic characteristics and ENKUR expression.

TABLE 2  Correlation between the clinicopathologic characteristics and MYH9 expression in GC

| Factors                  | Low (n) | High (n) | p-Value |
|--------------------------|---------|----------|---------|
| Gender                   |         |          |         |
| Male                     | 123     | 36       | 87      |
| Female                   | 65      | 13       | 52      |
| Age (y)                  |         |          |         |
| ≤60                      | 68      | 18       | 50      |
| >60                      | 120     | 31       | 89      |
| T classification         |         |          |         |
| T1–T2                    | 21      | 6        | 15      |
| T3–T4                    | 167     | 43       | 124     |
| N classification         |         |          |         |
| N0–N1                    | 78      | 23       | 55      |
| N2–N3                    | 110     | 26       | 84      |
| M classification         |         |          |         |
| M0                       | 178     | 47       | 131     |
| M1                       | 10      | 2        | 8       |
| Pathological classification|   |          |         |
| 1–2                      | 30      | 7        | 23      |
| 3                        | 158     | 42       | 116     |
| AJCC stage               |         |          |         |
| I–II                     | 75      | 23       | 52      |
| III–IV                   | 113     | 26       | 87      |
| Tumor size (cm)          |         |          |         |
| ≤5                       | 94      | 27       | 67      |
| >5                       | 94      | 22       | 72      |
| Lymphovascular invasion  |         |          |         |
| No                       | 157     | 39       | 118     |
| Yes                      | 31      | 10       | 21      |

Abbreviation: AJCC, American Joint Committee on Cancer; GC, gastric cancer.

*χ² test was applied to access the correlation between the clinicopathologic characteristics and MYH9 expression.

increased β-catenin stability and thus speculated that MYH9 increases β-catenin protein stability possibly at the protein modification level. In contrast to ubiquitination, deubiquitination mediated by deubiquitinating enzymes is a process of removing ubiquitin from ubiquitinated proteins to increase protein stability. Recent study demonstrated that the deubiquitinase USP2 harbors oncogenic abilities by E2F4-facilitated autophagy and zinc homeostasis, and its elevation is correlated with poor outcome of GC patients. Interestingly, the deubiquitinase USP2 has also been reported to bind to the β-catenin protein and lead to a decreased degradation. Consistently, we discovered that MYH9 recruits USP2 and β-catenin, which contributed to a reduced ubiquitinated expression and ubiquitin-mediated degradation of β-catenin. These results indicated that ENKUR combined with MYH9 and degraded its protein subsequently reduced the recruitment of USP2, thus promoting the degradation of the β-catenin protein, which finally inhibited the β-catenin mediated-EMT signaling. The above-mentioned data demonstrated that ENKUR, as an antimetastatic factor, suppresses GC cell metastasis by modulating MYH9/USP2/β-catenin-mediated EMT signaling.
In previous studies, c-Jun was shown to be a transcription factor to promote the development of tumors.\textsuperscript{45–48} Interestingly, bioinformatics analysis predicted c-Jun binds to the transcription regulatory sequences of ENKUR. To confirm whether c-Jun is the transcription factor negatively modulating ENKUR expression, we transfected c-Jun plasmids to GC cells and observed the negative modulation of ENKUR by c-Jun. Subsequently, we verified that c-Jun binds to 2 c-Jun binding sites predicted in the transcription regulatory sequences of ENKUR by ChIP and gel electrophoresis. In the following study, luciferase reporter and EMSA assays further demonstrated that c-Jun binds to the ENKUR transcriptional regulatory region. These data demonstrated that c-Jun binds to ENKUR transcriptional regulatory region, which weakens its transcriptional expression in GC cells.

Finally, to confirm the suppressive role of ENKUR on GC pathogenesis, we performed immunohistochemistry and found that compared to normal tissues, ENKUR was shown to be decreased in clinical GC samples and was positively correlated with the prognosis of GC. In contrast to ENKUR, MYH9 was elevated in cancer tissues, and its upregulation led to a poor prognosis. In addition, high ENKUR and low MYH9 expression contributed to the best prognosis in GC patients. Furthermore, ENKUR as well as MYH9 were observed as independent prognostic factors in GC. These data demonstrated the important roles of ENKUR and MYH9 in GC carcinogenesis.

In summary, ENKUR was shown to be an antimetastatic factor by recruiting the E3 ubiquitin ligase FBXW7 to ubiquitin and degrade MYH9 protein. The decreased MYH9 protein reduced the recruitment of the deubiquitinase USP2 and thus increased the degradation of β-catenin protein, which finally suppressed EMT signaling. Our data proved the significantly antimetastatic effect of ENKUR on GC and revealed its detailed molecular basis, which hinted that ENKUR is a significantly therapeutic target for GC.

4 | MATERIALS AND METHODS

4.1 | Cell culture

GSE-1, AGS, and BGC-823 cells were obtained from Zhongqiaoxinzhou Co., Ltd. (Shanghai, China). RPMI-1640 and fetal bovine serum purchased from Huixiang Biotechnology Co., Ltd. (Guangzhou, China) and Haoyue Biotechnology Co., Ltd. (Guangzhou, China), respectively. RPMI-1640 with 10% fetal bovine serum was used to maintain these cell lines in a humidified chamber containing 5% CO\textsubscript{2} at 37°C.

4.2 | Cell transfection

C-Jun, MYH9 and its domain plasmids, ENKUR and its domain plasmids were respectively obtained from Vigene Biosciences (Shandong, China), BersinBio (Guangzhou, China), and GeneChem (Shanghai, China). SiRNAs for ENKUR, USP2, FBXW7 purchased from RiboBio Inc. (Guangzhou, China) were shown in Table S4. When GC cells were plated onto cell plates at 40%–50% confluence, Lipofectamine TM 2000 (Invitrogen Biotechnology, China) was used to transfect siRNAs or plasmids into cells. After 2–3 days, these treated cells were harvested and utilized for further experiments.

4.3 | qPCR

TRIZol (TaKaRa Bio, Inc., Shiga, Japan) was used to obtain total RNA of GC cell lines, and then the RNA was reverse-transcribed into cDNA. The primers are indicated in Table S5.

4.4 | Western blot

WB experiments were conducted as described previously.\textsuperscript{49} As shown in Table S6, primary antibodies include anti-ENKUR, c-Jun, β-catenin, FBXW7, ubiquitin, MYH9, USP2, E-cadherin, and N-cadherin. β-actin or GADPH was used as a loading control.

4.5 | Wound-healing assay

GC cells with transfection of plasmids or siRNAs were plated in six-well plates. Artificial wounds were scraped into cells with a 10-μl tip and subsequently washed. After incubating 0 h, 48, and 72 h in a humidified chamber, the distance moved into the wound was used to estimate cell shift ability.

4.6 | Boyden assay

The invasion ability was assessed using Boyden assay. First, 24 μg/ml Matrigel (BD Biosciences, NJ, USA) was applied to precoat transwell membranes (BD Biosciences, NJ, USA) for 30 min. Then, a total of 1 × 10\textsuperscript{5} cells suspended in 100-μl serum-free medium were added to the upper chamber. Subsequently, RPMI 1640 medium containing 10% fetal bovine serum was added to the lower chamber with a volume of 500 μl. The filter was collected 10 h later and subsequently stained with crystal violet.
solution for 3 min. The invasion capacity was accessed based on the average number of three random fields.

4.7 ChIP

Two c-Jun-binding sites in ENKUR transcriptional regulatory region were predicted by JASPAR (http://jaspar.genereg.net) and ALGGEN (http://alggen.lsi.upc.es) database. A ChIP assay (Thermo Scientific, Waltham, MA, USA) was used to examine whether c-Jun binds to ENKUR transcriptional regulatory region. The cross-linked DNA from AGS and BGC-823 cells was sonicated to 200–1000 base pairs, and then the immunoprecipitation process was conducted with an anti-c-Jun antibody. Finally, DNA fragments enriched in the ENKUR promoter region were examined by qPCR using specific primers.

4.8 Luciferase reporter assays

GC cells were transfected with c-Jun cDNA and luciferase reporter vectors that carrying wild or mutant binding site sequence of c-Jun to the ENKUR transcriptional regulatory region (GeneChem, Shanghai, China). After 48 h, Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) were used to test the luciferase activity of these treated cells.

4.9 Co-IP assay

The Co-IP assay (Thermo Scientific, Waltham, MA, USA) was used to identify interacting protein. Specific primary antibodies (10 μg) were added to 2-mg protein and incubated overnight at 4°C. The proteins bound to primary antibodies were eluted and subjected to denaturation and WB experiments. Anti-IgG served as a negative control.

4.10 Immunofluorescent staining

GC cells transfected with different plasmids were seeded onto coverslips. First, 3,5% paraformaldehyde was applied to fix cells for 30 min, and then 0.2% Triton X-100 was used to permeabilize. Further, primary antibodies were utilized to treat these cells overnight at 4°C. Finally, the cells were treated with Alexa Fluor 488 goat antirabbit IgG and Alexa Fluor 594 goat antimouse IgG (1:1,000, Proteintech Group Inc., Wuhan, China) for 1 h and stained with DAPI for 5 min. Confocal microscopy of Carl Zeiss LSM800 was used to capture images.

4.11 CHX chase assay

ENKUR and MYH9 cDNAs were transfected into GC cells. MG132 and CHX were respectively obtained from Sigma-Aldrich (MO, USA) and Abcam (Massachusetts, USA). These cells were treated with (20 μmol/l) for 6 h or not, and then CHX (50 μg/ml) was added. Finally, these treated cells were collected at different time points for western blots.

4.12 Immunohistochemistry

Paraffin-embedded tissue arrays with 172 adjacent nontumor tissues and 188 GC tissues were obtained from Shanghai Outdo Biotech. Co., Ltd. (Shanghai, China). After deparaffinization, antigen-unmasking solution was added to paraffin sections and heated at 100°C for 3 min. Serum and a peroxidase blocking reagent were used to eliminate the effects of nonspecific antigens and endogenous peroxidase activity. Primary antibodies were used to treat slides overnight at 4°C, and then 3,3-diaminobenzidine chromogen solution was utilized to visualize these slides. Sections were analyzed using a brightfield microscope. The immunohistochemical staining level was scored based on cell staining intensity (negative = 0, weakly positive = 1, moderate positive = 2, strongly positive = 3) and the percentage of positive staining areas (<25% = 1, 25% to <50% = 2, 50% to <75% = 3, ≥75% = 4). The staining index was analyzed by multiplying the staining intensity score by the positive area score. A staining index ≤4 or 6 was considered low expression for ENKUR or MYH9, respectively, and the rest was considered high expression.

4.13 EMSA

EMSA (BersinBio, Guangzhou, China) was conducted to detect whether c-Jun binds to ENKUR transcriptional regulatory region. The probe sequences are indicated in Table S7. After obtaining the nuclear extract from the GC cells, the nuclear extracts were added to the reaction mixture together with biotin-labeled probes. Unlabeled wild-type or mutant probes anti-c-Jun antibodies were used for competition assays and supershift assays, respectively. BioSens Gel Imaging System (BIOTOP) was utilized to analyze signals.

4.14 In vivo metastasis assays

A lung metastasis model was used for detecting the antimetastasis role of ENKUR. Four-week-old female
non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice were purchased from Gempharmatech Co., Ltd, (https://www.gempharmatech.us/en/about-china) and kept in specific pathogen free (SPF) room, and these mice were divided into negative control (NC) group and ENKUR group (n = 4 for each group). First, about 1.5 × 10⁶ GC cells stably overexpressing ENKUR or their control cells were resuspended with 100 μl of phosphate buffered saline (PBS), and then the cell suspension was injected into the tail vein of NOD-SCID mice. All mice were sacrificed after 2 weeks, and the lungs were extracted and embedded in paraffin. The mean number of tumors was detected in the pulmonary metastasis model, and statistical analysis was performed.

4.15 | Statistical analysis

All statistical analyses were carried out using SPSS ver. 22.0 (SPSS Inc. Chicago, IL, USA). Data were shown as the mean± s.d. from at least three independent experiments. The correlation of ENKUR expression with clinical features was analyzed using chi-square testing. Survival prognosis was performed based on the Kaplan–Meier method with a log-rank test. Student’s t-test and one-way ANOVA were utilized for comparisons between two groups and multiple groups, respectively. p < 0.05 was taken as statistically significant.

AUTHOR CONTRIBUTIONS

Weiyi Fang, Zhen Liu, Dayong Zheng, and Rong Li designed the research. Jiahao Liu, Zhan Liu, Huiling Yang, Weiwai Yan, Shiyi Fang, Shuting Deng, Yinghao Wen, Peng Shen, Yonghao Li, Rentao Hou, Xiong Liu, and Tao Huang performed the experiments and data analysis. Jiahao Liu wrote the manuscript. Weiyi Fang revised the manuscript. All authors have reviewed and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (grant numbers: 81974460 and 81572649), Science and Technology Program of Guangzhou (grant numbers: 202206010068 and 201803010023), Scientific Project of Traditional Chinese Medicine Bureau of Guangdong Province (grant number: 20181178), Discipline construction project of Guangdong Medical University (grant number: 4SG21011G), Natural Science Foundation of Hunan Province (grant number: 2021JJ30402), and Hunan Provincial Health and Family Planning Commission Project (grant number: 202103030035).

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all patients. This study was approved by the ethics committee of Shanghai Outdo Biotech Co., Ltd, China (ethical ID number: T18-1425). All animal experiments were approved by the animal ethics committee of Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Liu J, Liu Z, Yan W, et al. ENKUR recruits FBXW7 to ubiquitinate and degrade MYH9 and further suppress MYH9-induced deubiquitination of β-catenin to block gastric cancer metastasis. *MedComm.* 2022;3:e185. [https://doi.org/10.1002/mco2.185](https://doi.org/10.1002/mco2.185)