ORIGINAL ARTICLE

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# HOTAIR induces the ubiquitination of Runx3 by interacting with Mex3b and enhances the invasion of gastric cancer cells

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## Abstract

**Background** Long non-coding RNAs (LncRNAs) exert their functions mainly by binding to their corresponding proteins. Runt-related transcription factor 3 (Runx3) is an important transcription factor that functions as a tumor suppressor in gastric cancer. Whether there is an interplay between LncRNAs and Runx3 remains unclear.

**Methods** RPISeq was applied to screen the LncRNAs that potentially bind to Runx3. The interaction between LncRNA HOX antisense intergenic RNA (HOTAIR) and Runx3 was validated by RNA Immunoprecipitation and RNA pull-down assays. The role of Mex3b in the ubiquitination of Runx3 induced by HOTAIR was assessed by immunoprecipitation. Pearson's correlation between HOTAIR mRNA expression and Runx3 protein expression was analyzed. Cell migration and invasion were explored by transwell assays.

**Results** We found that HOTAIR was bound to Runx3 protein and identified the fragment of HOTAIR spanning 1951–2100 bp as the specific binding site. In addition, mex-3 RNA binding family member B (Mex3b) was an E3 ligase involved in HOTAIR-induced ubiquitous degradation of Runx3. Silencing the expression of HOTAIR or Mex3b attenuated the degradation of Runx3. In human gastric cancer tissues, HOTAIR was negatively associated with the expression level of Runx3 protein (Pearson coefficient − 0.501, $p = 0.025$). Inhibition of HOTAIR significantly suppressed gastric cancer cell migration and invasion through upregulating claudin1, which could be reversed by co-deficiency of Runx3.

**Conclusions** These results uncovered the novel interaction between HOTAIR and Runx3, and provided potential therapeutic targets on the metastasis of gastric cancer.

**Keywords** HOTAIR · Runx3 · Gastric cancer · Ubiquitination

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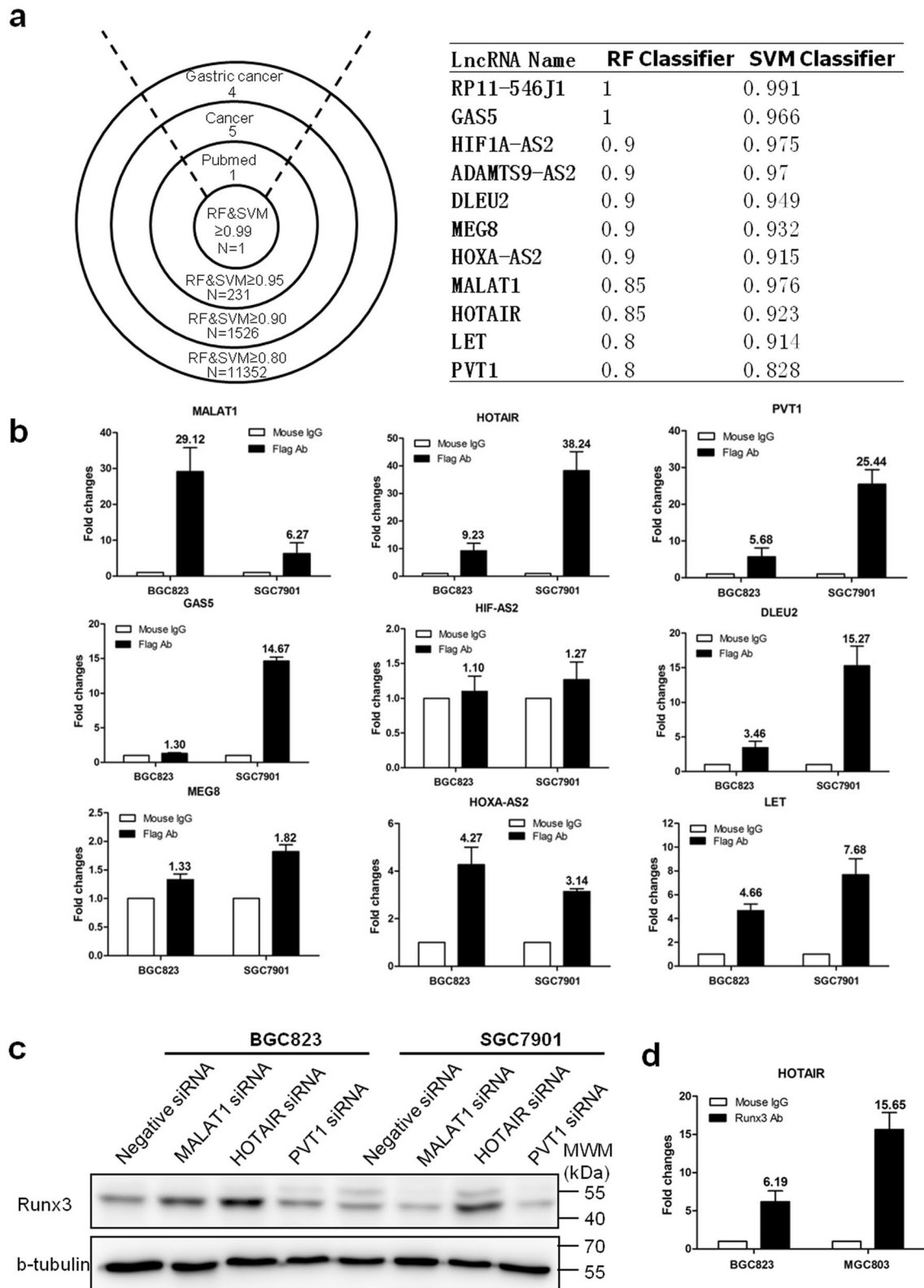
# Background

Runt-related transcription factor 3 (Runx3) is a transcription factor with 3 different splice variants. Variants 1 and 3 share the same amino sequence, while variant 2 has a shorter amino sequence. Runx3 inhibits the metastasis of malignant

**a**

| LncRNA Name | RF Classifier | SVM Classifier |
|---|---|---|
| RP11-546J1 | 1 | 0.991 |
| GAS5 | 1 | 0.966 |
| HIF1A-AS2 | 0.9 | 0.975 |
| ADAMTS9-AS2 | 0.9 | 0.97 |
| DLEU2 | 0.9 | 0.949 |
| MEG8 | 0.9 | 0.932 |
| HOXA-AS2 | 0.9 | 0.915 |
| MALAT1 | 0.85 | 0.976 |
| HOTAIR | 0.85 | 0.923 |
| LET | 0.8 | 0.914 |
| PVT1 | 0.8 | 0.828 |

**b**

**c**

**d**

tumors, and a list of invasiveness-related genes has been validated as downstream targets [1, 2]. The expression of Runx3 was significantly downregulated in gastric cancer [3].

Both transcription mechanism such as methylation [4] and post-transcription regulation including ubiquitination change [5] were responsible for the low expression of Runx3.

Long non-coding RNAs (LncRNAs) are non-protein-coding transcripts (> 200 nt) that have gained widespread attention as a crucial regulator of cancer development [6]. Previous studies have provided evidence that LncRNAs might regulate gene expression and post-transcription modification by multiple mechanisms [7]. Binding to their corresponding proteins, especially transcription factors, is considered as one of the most common mechanisms [8]. However, whether LncRNAs could bind to Runx3 and affect its expression remains unclear.

In this study, HOTAIR (HOX antisense intergenic RNA), an LncRNA localizing in the boundaries of Hox gene cluster, was screened out to be a potential upstream LncRNA regulating Runx3. HOTAIR is an oncogene responsible for malignant behavior in various types of cancer [9], including the invasiveness of gastric cancer [10]. Here, we identified the interaction between HOTAIR and Runx3, and validated that HOTAIR induced the invasiveness of gastric cancer through Runx3.

## Methods

### Patient specimens

This study was approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University. All patients signed the informed consent before surgery. Twenty gastric cancer tissues and paired adjacent noncancerous tissues were obtained. Total RNA and protein were extracted for further analysis.

### Cell culture

Seven gastric cancer cell lines (AGS, MKN28, MKN45, BGC823, HGC27, MGC803 and SGC7901) were purchased from Riken Gene Bank (Tsukuba, Japan) and American Type Culture Collection (Manassas, USA), and GES-1, a noncancerous gastric cell line, was obtained from Beijing Institute for Cancer Research (Beijing, China). RPMI 1640

medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Huzhou, China) was used to culture those cells in a condition of 5% $CO_2$, 37 °C and 95% humidity.

### Construction of mammalian plasmids

Full-length Runx3 CDS, HOTAIR CDS sense/antisense, HOT-A, B, C, D and D1–D4 were amplified by reverse transcription-polymerase chain reaction (RT-PCR). Then those fragments were cloned into the mammalian expression plasmids of either GV141 (XhoI/KpnI, Genechem, Shanghai, China; having an element of 3 × Flag) or GV219 (XhoI/EcoRI, Genechem; having an element of T7 RNA polymerase promoter). The primers consisting enzyme cleavage sites are listed in Online Resource 1 in the electronic supplementary material.

### Cell transfection

BGC823 and SGC7901 cells were transfected with GV141–Runx3 or GV141 empty vector using Fugene HD (Promega). For siRNA-mediated gene knockdown, gastric cancer cells were transfected with negative control siRNA, HOTAIR siRNA or Runx3 siRNA (Qiagen, Hilden, Germany) using Lipofectamine RNAiMAX Reagent (Invitrogen). All 3 variants of Runx3 have the same 3′-UTR sequence, and could be inhibited by one siRNA.

### Screening of potential LncRNAs that bind to Runx3

We input the amino sequence of Runx3 (NM_004350) and the sequence of LncRNAs in Fasta format from LNCiPedia (V3.1) into RPISeq (http://pridb.gdcb.iastate.edu/RPISeq/, a free online software predicting RNA–protein interactions). Two classifiers of random forest (RF) and support vector machine (SVM) for each LncRNA were calculated. Potential Runx3-binding LncRNAs were selected using the following rules: (1) RF ≥ 0.99 and SVM ≥ 0.99; (2) RF ≥ 0.95, SVM ≥ 0.95 and was mentioned in the references from PubMed; (3) RF ≥ 0.90, SVM ≥ 0.90 and was associated with tumor as reported in PubMed; (4) RF ≥ 0.80, SVM ≥ 0.80 and was associated with gastric cancer as reported in PubMed.

### Western blot analysis

Whole cell lysates were prepared using RIPA lysis buffer (Cell Signaling Technology Inc., Danvers, USA) supplemented with 1 nM PMSF (Cell Signaling Technology Inc.), while tissue lysates were prepared with PARIS Kit (Thermo Fisher, Boston, USA). Lysates were loaded on SDS-PAGE gel and transferred to PVDF membranes (Millipore,

Bedford, USA). Runx3 (#9647 for Figs. 1c, 4b, #13089 for the rest of the western blot analysis, 1:1000, Cell Signaling Technology Inc.), Dzip3 (ab155782, 1:1000, Abcam, Cambridge, USA), Mex3b (ab129938, 1:1000, Abcam), b-tubulin (CW0098M, 1:1000, Cwbiotech, Beijing, China) antibodies. The blots were visualized by chemiluminescence using the Amersham Imager 600 System (GE Healthcare Life Sciences, Pittsburgh, USA).

## Quantitative real-time PCR (qPCR)

Total RNAs were either isolated from tumor cells with TRIzol reagent (Invitrogen, Carlsbad, USA) or from tissue samples with PARIS Kit (Thermo Fisher). cDNA was synthesized using RT reagent kit with gDNA Eraser (TaKaRa, Otsu, Japan). SYBR Green Master Mix Kit (Takara) was used to perform qPCR on a LightCycler 480 Instrument (Roche, Penzberg, Germany). GAPDH was used as an internal control for protein-coding genes and U6 was used for LncRNAs. Primers used for PCR reactions are listed in Online Resource 2 in the electronic supplementary material.

## RNA immunoprecipitation (RIP)

RIP was performed according to the instructions of Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, 3 × Flag–Runx3 overexpressed BGC823 and SGC7901 cells were suspended in RIP lysis buffer and mixed with Flag M2 antibody (F1804, Sigma, Darmstadt, Germany)-coated magnetic beads. Then, the precipitated RNA was purified and converted into cDNA using the reverse transcription kit (Taraka). Quantitative PCR (qPCR) was applied to measure the enrichments of 11 LncRNAs, using U1 RNA as a negative control. The RNAs precipitated by normal mouse IgG antibody and input RNAs were applied as negative and positive control, respectively. For endogenous RIP assay, Runx3 antibody was used to precipitate RNAs in wild BGC823 and MGC803 cells.

## RNA pull-down

Plasmids containing LncRNA HOTAIR or its short fragments were linearized in 3′ end with a restriction enzyme EcoRI. Biotinylated RNA was synthesized in vitro by T7 RNA polymerase using those linearized plasmids as templates, and then purified on G-50 Sephadex RNA Columns (Roche). After validating the size by agarose gel electrophoresis, proper RNA secondary structure was folded from biotin-labeled RNAs following incubation at different temperatures. Meanwhile, nuclear lysates were harvested from gastric cancer cells with addition of prewashed streptavidin–agarose beads. A mixture of folded biotinylated RNAs and nuclear lysates were then incubated with tRNA and prewashed streptavidin–agarose beads in sequence. The pellets were washed and then boiled with loading buffer. The pulled-down Runx3 protein was measured by western blotting.

## Co-immunoprecipitation

Cell lysates were extracted from whole cells with IP lysis buffer, mixed with protein G agarose beads and then incubated with Runx3 or Mex3b antibody overnight. Rabbit or mouse IgG was used as negative control. Prewashed protein G agarose beads were then added and shaken gently for 3 h to capture the antigen–antibody complex. The beads were pelleted and washed 3 times with IP lysis buffer before boiling in SDS loading buffer. The pellets were further subjected to immunoblotting with appropriate antibodies.

## Transwell assay

Cell migration was assessed by modified Boyden transwell chambers (Corning Inc., Corning, USA), and cell invasion was measured in chambers coated with Matrigel (BD Biosciences, Franklin Lakes, USA). In brief, 36 h after being transfected with siRNA targeting HOTAIR or Runx3, gastric cancer cells were trypsinized, resuspended in culture medium containing 1% FBS and then seeded in the upper chamber with culture medium containing 15% FBS added in the lower chamber. After 24 h of incubation in a $CO_2$ incubator, non-migratory cells in the upper chamber were carefully removed with swab cotton. Those cells migrating through the transwell membrane were stained with 1.0 µg/ml DAPI staining solution (Roche) and then counted microscopically (× 400). The cells invading through Matrigel and transwell membrane were incubated with crystal violet (Beyotime, Shanghai, China) and then counted microscopically (× 200).

## Results

### Screening HOTAIR as a potential LncRNA binding to Runx3

To search for the LncRNAs that potentially bind to Runx3, a RNA–protein binding prediction software RPISeq was applied to perform *in silico* analysis. Based on RF/SVM classifiers and searching results in PubMed, eleven candidate LncRNAs with the potential to bind to Runx3 were screened (Fig. 1a). To validate these binding LncRNAs, RNAs in gastric cancer cells (BGC823 and SGC7901) were precipitated by RIP assay after transfecting with Runx3 plasmids (see Online Resource 3 in the electronic supplementary material). Q-PCR results showed that HOTAIR, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and Pvt1

oncogene (PVT1) were significantly amplified (over five-fold) in both BGC823 and SGC7901 cells (Fig. 1b). Interestingly, silencing HOTAIR remarkably induced the expression level of Runx3 protein in gastric cancer cells, while knockdown of MALAT1 or PVT1 had no significant effect on the expression of Runx3 (Fig. 1c and Online Resource 4 in the electronic supplementary material). These results suggested that HOTAIR interacts with Runx3 in gastric cancer cells.

To validate the interaction between endogenous Runx3 and HOTAIR, RNAs in wild BGC823 and MGC803 cells which have a relatively high endogenous expression of Runx3 (Online Resource 5 in the electronic supplementary material) were precipitated by RIP assay using the Runx3 antibody. Q-PCR results showed that HOTAIR was significantly amplified in both cell lines (Fig. 1d).

## Identification of specific HOTAIR binding sites with Runx3

To identify the specific binding sites of HOTAIR with Runx3, full-length HOTAIR sense and antisense biotinylated transcripts were synthesized in vitro (Fig. 2a) and the molecular sizes were confirmed by RNA electrophoresis (Online Resource 6 in the electronic supplementary material). Biotin-labeled RNAs were mixed with cell lysates in the presence of tRNA and streptavidin–agarose beads. Pulled-down Runx3 protein was recruited by the sense transcript of HOTAIR rather than the antisense one by immunoblotting assay (Fig. 2b). 5′
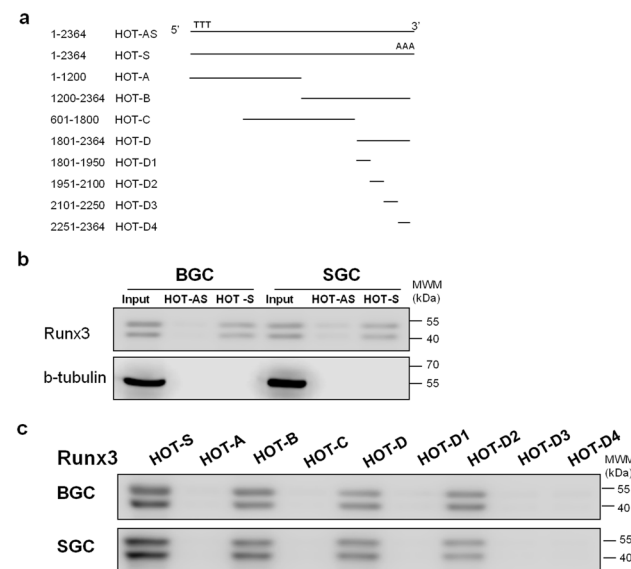


**Fig. 2** Identification of specific HOTAIR binding sites with Runx3. **a** Deletion mapping of Runx3-binding domain in HOTAIR. Runx3 immunoblot of 5% input and proteins pulled down by HOTAIR sense probe in BGC823 and SGC7901 cells. **b** HOTAIR antisense probe was used as a negative control. **c** Western blotting of Runx3 showed its interaction with different HOTAIR fragments

deletions of HOTAIR transcript were further designed to testify the specific binding sequence with Runx3 (Fig. 2a and Online Resource 6 in the electronic supplementary material). The 2.4 kb full sense transcript was divided into HOT-A, HOT-B (each of about 1.2 kb) and HOT-C (1.2 kb, crossing A and B). The remaining sequence of HOT-B (about 0.6 kb) was named as HOT-D, which was divided into D1–D4 fragments (each of about 0.15 kb). Serial RNA pull-down assay revealed that the fragment D2 (1951–2100 bp) was a specific Runx3 binding site by HOTAIR (Fig. 2c).

## HOTAIR induces the degradation of Runx3 dependent on Mex3b

Consistent with the above result that silencing of HOTAIR reversed the expression of Runx3 protein, overexpression of HOTAIR could suppress the protein level of Runx3 in gastric cancer cells (Fig. 3a). However, Q-PCR analysis observed that Runx3 mRNA level was not affected by forced expression with HOTAIR (Fig. 3b). MG132, a ubiquitin–proteasome inhibitor, was able to recover the Runx3 protein in cells transfected with HOTAIR (Fig. 3a), indicating that HOTAIR might regulate the degradation of Runx3 through a ubiquitin–proteasome pathway. To further clarify the degradation mechanisms, we investigated the interaction between Runx3 and two key E3 ubiquitin ligases with RNA-binding domains, DAZ interacting zinc finger protein 3 (Dzip3) and mex-3 RNA binding family member B (Mex3b). Co-immunoprecipitation assays revealed that Mex3b indeed interacted with endogenous Runx3 (Fig. 3c). In addition, we found that the interplay between Runx3 and Mex3b was enhanced when co-transfected with HOTAIR plasmids in both BGC823 and SGC7901 cells (Fig. 3d).

To further explore the role of HOTAIR and Mex3b in the ubiquitination of Runx3, we depleted either HOTAIR or Mex3b in both BGC823 and SGC7901 cells, and observed that the ubiquitination level of Runx3 was significantly reduced (Fig. 3e). In addition, the effect of HOTAIR and Mex3b on the stability of Runx3 was evaluated by protein synthesis assay by adding cycloheximide (CHX), a general inhibitor of protein biosynthesis. The degradation half-life time of Runx3 after the treatment of CHX was 2–4 h. In contrast, Runx3 degradation half-life time was extended to 4–8 h by knockdown of either HOTAIR or Mex3b (Fig. 3f). Collectively, our result suggested that HOTAIR induced the degradation of Runx3 by enhancing its interaction with Mex3b.

## HOTAIR is negatively associated with Runx3 in gastric cancer tissues

To further investigate the relationship between HOTAIR and Runx3 in gastric cancer, we measured the expression
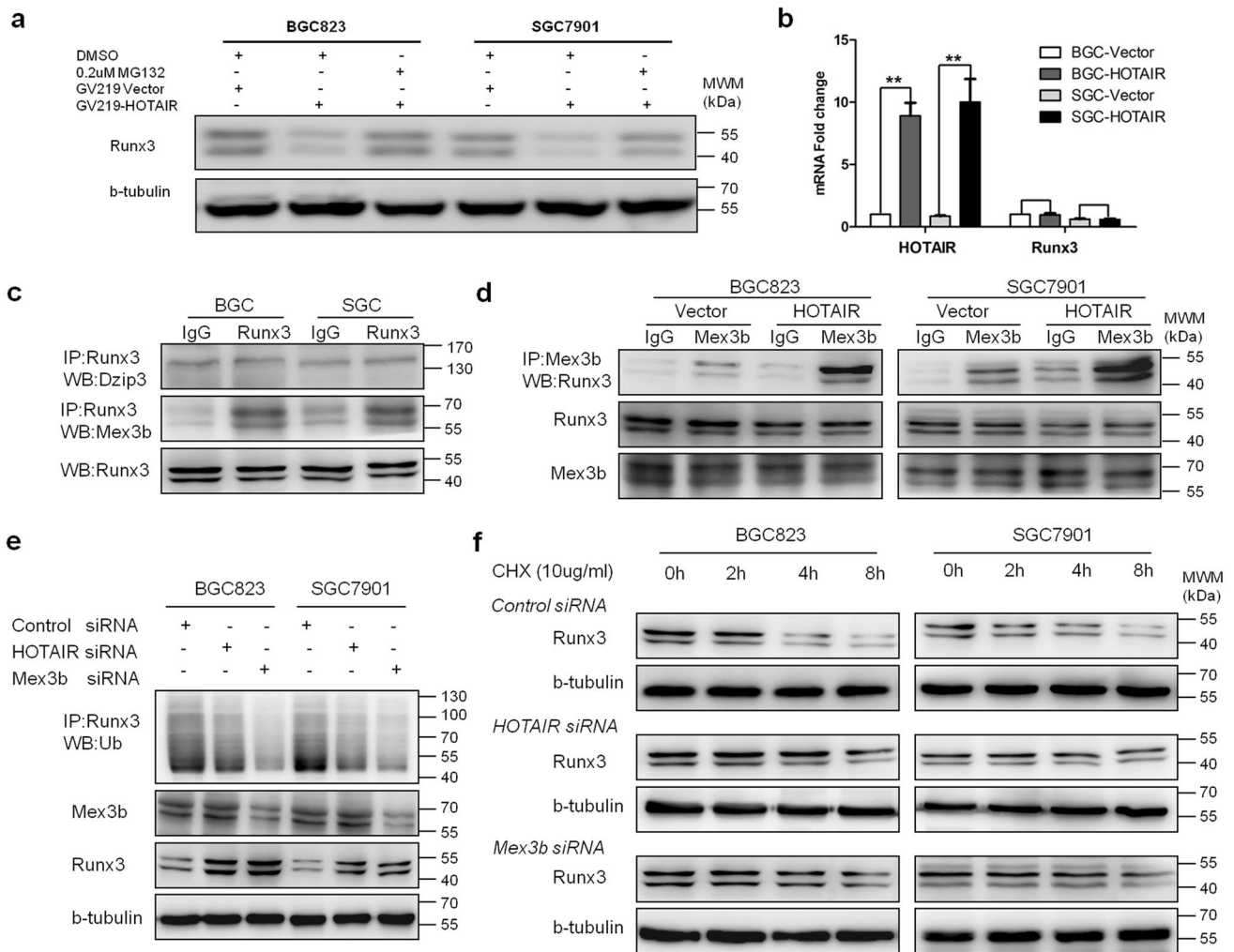
**Fig. 3** HOTAIR destabilizes Runx3 through enhancing its ubiquitination. **a** Runx3 protein was determined in HOTAIR-overexpressing gastric cancer cells after MG132 treatment. **b** The simultaneous change of Runx3 mRNA expression was assessed by qRT-PCR following the inhibition of HOTAIR expression. **c** The interaction between Runx3 and its potential E3 ligases (Dzip3 and Mex3b) was tested by immunoprecipitation. **d** Western analysis showed that the interaction between Runx3 and Mex3b was enhanced in HOTAIR-overexpressing cells. **e** Knockdown of either HOTAIR or Mex3b significantly decreased the ubiquitination of Runx3. **f** Gastric cancer cells were transfected with negative control (top), HOTAIR (medium) or Mex3b (bottom) siRNAs for 48 h. Then, the cells were treated with CHX, and the cell lysates were collected at a series of indicated times. Western blotting was applied to assess the Runx3 protein stability ($t1/2$)

levels of HOTAIR mRNA and Runx3 protein in 20 pairs of gastric cancer and adjacent noncancerous tissues (detailed pathologic characteristics and the relative expression levels of HOTAIR and Runx3 in each specimen are listed in Online Resource 7 in the electronic supplementary material). Consistent with previous reported studies [3, 11], HOTAIR was significantly upregulated in gastric cancer when compared with adjacent noncancerous tissues, while lower expression of Runx3 was detected in gastric cancer tissues (Fig. 4a, b). The relative expression of HOTAIR was negatively correlated with Runx3 protein (Pearson coefficient = − 0.501, $p = 0.025$, Fig. 4c).

## HOTAIR promotes gastric cancer cell invasiveness through Runx3

In transwell assays, knockdown of HOTAIR impaired the migration and invasion of gastric cancer cells (BGC823 and SGC7901). However, this effect was significantly reversed with dual silencing of Runx3 in the above cells (Fig. 5a–d), suggesting that the effect of HOTAIR on the invasiveness of gastric cancer was dependent on Runx3.

Both HOTAIR and Runx3 were shown to regulate matrix metalloproteinase (MMP), E-cadherin and other invasion-related genes. However, only Runx3 was reported to be
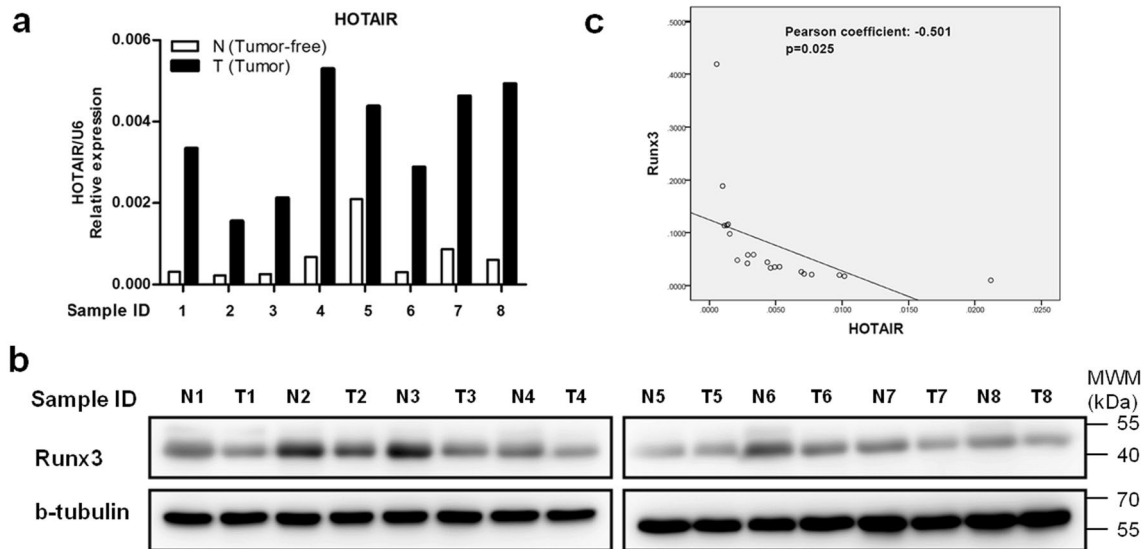
**Fig. 4** HOTAIR is negatively associated with Runx3 in gastric cancer tissues. **a** qRT-PCR measured the expression difference of HOTAIR in gastric cancer and adjacent noncancerous tissues, and representative 8-pair results are presented. **b** Representative results of Runx3 protein expression in gastric cancer and adjacent tissues are shown. **c** HOTAIR mRNA expression in gastric cancer tissues was negatively correlated with Runx3 protein expression

an upstream regulator of claudin1, which is a member of the tight junction protein family and plays a pivotal role in the process of epithelial–mesenchymal transition (EMT). Results revealed that gastric cancer cells with HOTAIR deficiency had an increased expression of claudin1 mRNA and protein, while co-deficiency of Runx3 would partially reverse this phenotype (Fig. 5e, f).

## Discussion

The expression of Runx3 was regulated by several mechanisms including promoter methylation, histone acetylation, miRNAs, etc. Previous studies have reported that the promoter of Runx3 was hypermethylated in gastric cancer [4]. Interestingly, *Helicobacter pylori* infection and lipopolysaccharide also increase the frequency of Runx3 promoter hypermethylation [12]. miRNA was another regulator responsible for the epigenetic modification of Runx3 [13]. In addition, various post-transcriptional and post-translational regulations are responsible for the expression of Runx3. For example, transforming growth factor-β (TGF-β) could stimulate p300-dependent Runx3 acetylation, thus affecting its degradation and transactivation [14]. In this study, we first reported that LncRNA HOTAIR induced the degradation of Runx3, supporting that HOTAIR is another post-translational regulator of Runx3.

As one of the important members of protein degradation enzymes, E3 ubiquitin ligases could induce the transfer of ubiquitin from E2 conjugating enzyme to the protein

molecules for degradation [15]. Recent reports demonstrated that Mex3b and Dzip3 were two certain E3 ligases possessing RNA binding domains, and both were associated with HOTAIR [16]. Mex3b is detectable both in the nuclear and cytoplasm, while Dzip3 is only distributed in cytoplasmic vesicles [17]. As a transcription factor, Runx3 is mainly localized in the nucleus. In this study, we revealed that Mex3b, and not Dzip3, functioned as an E3 ligase binding to Runx3. Either silencing HOTAIR or Mex3b impaired the ubiquitination of Runx3 and enhanced protein stability. Downregulation of Runx3 induced by HOTAIR was partially reversed by siRNA targeting Mex3b, indicating that HOTAIR accelerates the degradation of Runx3 though enhancing its interaction with Mex3b.

In this study, the RNA pull-down assay confirmed that the domain of HOTAIR to which Runx3 binds is located at 1951–2100 bp along the segment spanning nucleotides of HOTAIR. Runx3 contains the fewest number of exons in the Runx family, and the former 3 exons constitute the Runt domain [18]. The binding domain of Runx3 could be identified by RIP with truncated plasmids and site mutations. In addition, ubiquitinated proteins are often conjugated to the Ile44 hydrophobic patch of ubiquitin via specific lysine residues [19]. The interacting domains with Mex3b along the amino sequence of Runx3 is undetermined.

HOTAIR is involved in tumor metastasis through different pathways in several previous studies [20, 21]. Wang et al. [22] found that knockdown of HOTAIR decreased the secretion of MMP2 and MMP9, thus impairing the metastasis of osteosarcoma. In another study, HOTAIR depletion
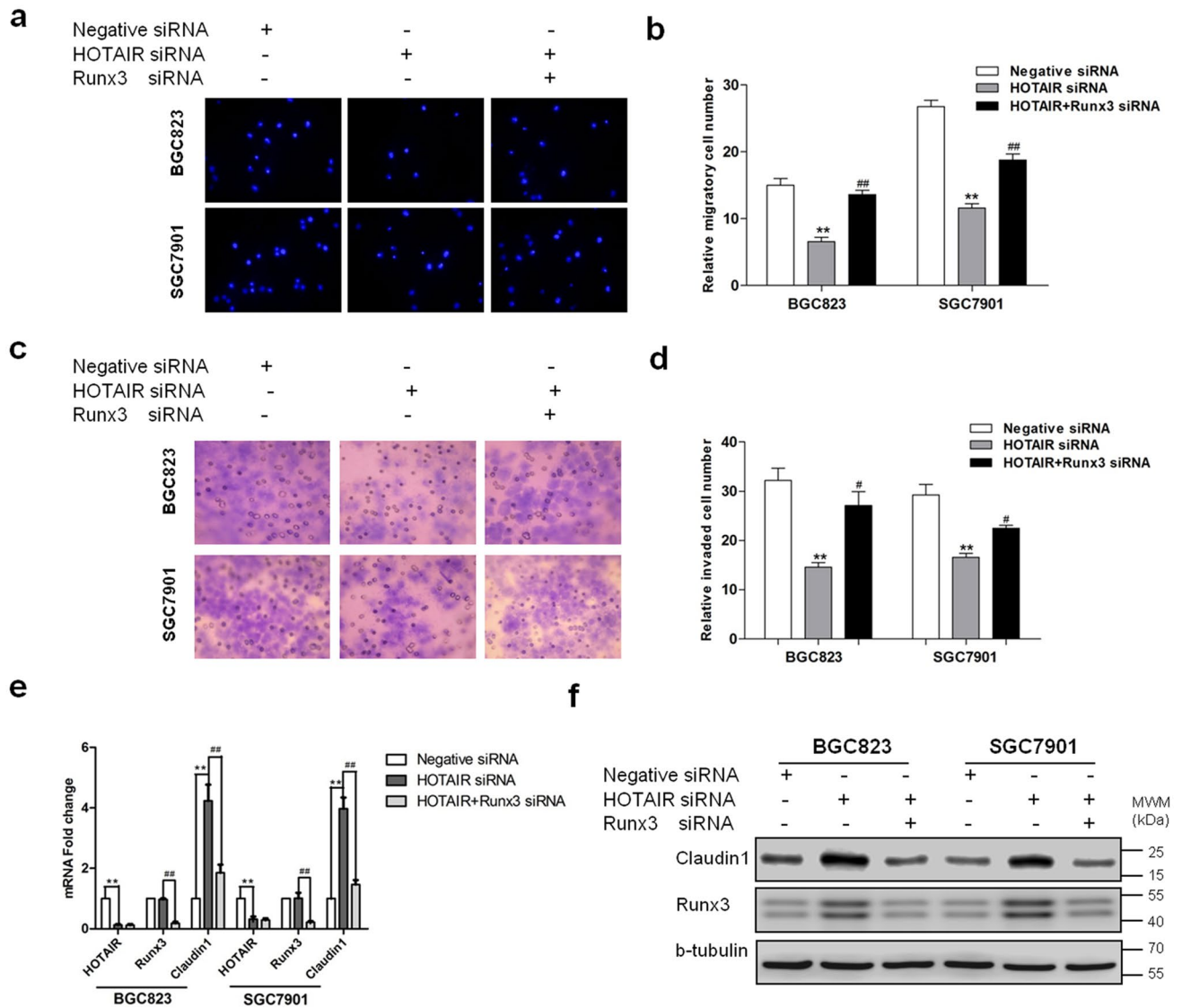
**Fig. 5** HOTAIR promotes the invasiveness of gastric cancer depending on Runx3. **a, b** BGC823 and SGC7901 cells were treated with the indicated siRNA or scramble siRNA, and subjected to the following analyses. Cell migration was assessed by modified Boyden transwell chambers assays. After incubation for 16 h, cells that migrated to the bottom of the membrane were stained with DAPI. The mean number of visible migratory cells was counted in five random high power fields. **c, d** Invaded cells at the bottom of the membrane were stained with crystal violet. Invaded cells were washed with 10% acetic acid and detected on a microplate reader (560 nm). **e, f** Q-PCR and western blotting revealed that knockdown of HOTAIR significantly increased the mRNA and protein levels of claudin1, and co-transfection of Runx3 siRNA partially reversed this phenotype. These experiments were performed at least for three times. $*p < 0.05$ vs. negative siRNA, $**p < 0.01$ vs. negative siRNA, $\#p < 0.05$ vs. HOTAIR siRNA, $\#\#p < 0.01$ vs. HOTAIR siRNA

inhibited the invasion and metastasis of colon cancer stem cells with decreased vimentin and increased E-cadherin [23]. Acquiring mesenchymal characteristics confers a pro-migratory advantage to tumor cells, with altered expressions of E-cadherin, ß-catenin and other EMT-related genes [24]. Claudin1 is a member of the tight junction-related proteins and could block the EMT process. Runx3 promoted the transcriptional expression of claudin1 both in vivo and in vitro [25], while no previous literature reported the effect of HOTAIR on the expression of claudin1. In this study,

knockdown of Runx3 significantly attenuated the decrease of migrated cells and concomitant increase of claudin1 expression induced by siRNA targeting HOTAIR, suggesting the role of HOTAIR–Runx3–claudin1 in the invasiveness of gastric cancer.

In conclusion, we demonstrated that HOTAIR interacted with Runx3 and induced its degradation dependent on the E3 ubiquitin ligase Mex3b, which provided a novel regulatory mechanism responsible for the silencing the expression of Runx3 in gastric cancer. HOTAIR–Runx3 interaction

might work as a new therapeutic target for metastatic gastric cancer.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research involving human participants and/or animals and informed consent** All samples were obtained with patients' informed consent. The Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University, approved this study. This study does not involve animals.

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