EIF3H promotes aggressiveness of esophageal squamous cell carcinoma by modulating Snail stability

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Abstract

Background: Overexpression of eukaryotic translation initiation factor 3H (EIF3H) predicts cancer progression and poor prognosis, but the mechanism underlying EIF3H as an oncogene remains unclear in esophageal squamous cell carcinoma (ESCC).

Methods: TCGA database and the immunohistochemistry (IHC) staining of ESCC samples were used and determined the upregulation of EIF3H in ESCC. CCK8 assay, colony formation assay and transwell assay were performed to examine the ability of cell proliferation and mobility in KYSE150 and KYSE510 cell lines with EIF3H overexpression or knockdown. Xenograft and tail-vein lung metastatic mouse models of KYSE150 cells with or without EIF3H knockdown were also used to confirm the function of EIF3H on tumor growth and metastasis in vivo. A potential substrate of EIF3H was screened by co-immunoprecipitation assay (co-IP) combined with mass spectrometry in HEK293T cells. Their interaction and co-localization were confirmed using reciprocal co-IP and immunofluorescence staining assay. The function of EIF3H on Snail ubiquitination and stability was demonstrated by the cycloheximide (CHX) pulse-chase assay and ubiquitination assay. The correlation of EIF3H and Snail in clinical ESCC samples was verified by IHC.

Results: We found that EIF3H is significantly upregulated in esophageal cancer and ectopic expression of EIF3H in ESCC cell lines promotes cell proliferation, colony formation, migration and invasion. Conversely, genetic inhibition of EIF3H represses ESCC tumor growth and metastasis in vitro and in vivo. Moreover, we identified EIF3H as a novel deubiquitinating enzyme of Snail. We demonstrated that EIF3H interacts with and stabilizes Snail through deubiquitination. Therefore, EIF3H could promote Snail-mediated EMT process in ESCC. In clinical ESCC samples, there is also a positive correlation between EIF3H and Snail expression.

Conclusions: Our study reveals a critical EIF3H-Snail signaling axis in tumor aggressiveness in ESCC and provides EIF3H as a promising biomarker for ESCC treatment.

Keywords: EIF3H, Snail, Deubiquitination, Esophageal squamous cell carcinoma

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Background
Esophageal cancer is the 7th most common cancer and the 6th most common cause of death from cancer worldwide, with an approximate 572,000 new cases and 509,000 deaths in 2018 [1]. Esophageal squamous cell carcinoma (ESCC) accounts for about 90% cases of esophageal cancer worldwide [2], and more than 50% of ESCC occur in China [3]. Esophageal cancer has a very poor five-year survival rate below 30% [4], mainly because of limited clinical approaches for early diagnosis, tumor metastasis before diagnosis and tumor recurrence [5–7]. However, molecular mechanisms underlying these processes are still not clear. Thus, a compelling study is required for the better understanding of the molecular basis of ESCC, for shaping new diagnostic approaches and developing new therapeutic modalities to improve the prognosis of ESCC patients.

The eukaryotic translation initiation factor 3 (EIF3) is one of the largest complexes in mammalian cells. It contains 13 putative subunits named from EIF3A to EIF3M [8]. EIF3H is one of non-conserved subunits of EIF3 [9]. Expression of the EIF3H gene was shown to be significantly upregulated in many normal tissues, including non-small cell lung cancer [10], breast cancer [11], hepatocellular carcinomas [12], colorectal cancer [13], prostate cancer [14] and osteocarcinoma [15]. A siRNA screen identifies EIF3H as a driver gene within the 8q23.3 amplicons contributing to cell growth, survival and transformation in breast cancer [11]. In lung adenocarcinoma, EIF3H functions as an oncogene by inducing EMT signaling pathway, which could be inhibited by PDCD4 [16]. Moreover, amplification of the EIF3H is associated with advanced stage and poor prognosis in prostate cancer [17]. Besides, the METTL3-EIF3H interface is required for enhanced translation and oncogenic transformation [18]. These observations indicate that EIF3H might have great contribution to establishing and maintaining the aggressive state of cancer. In consistence with previous studies, we also found EIF3H is overexpressed in ESCC tissues. In order to get a comprehensive understanding about the significance of EIF3H and the mechanism of its function in ESCC, we performed a liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis and identified that EIF3H could interact with Snail and correlate positively with Snail expression. Furthermore, we demonstrated Snail, as the novel identified substrate of EIF3H, could be deubiquitinated and stabilized by EIF3H.

Snail is a well-known transcription factor capable of promoting epithelial-mesenchymal transition (EMT) and tumor metastasis [19], inducing cancer cell stemness and differentiation [20], contributing to cancer cell proliferation [21] and survival [22, 23], impacting on metabolism [24], suppressing immune surveillance [25] and inducing drug resistance [26]. Snail is a highly labile protein which is degraded through the ubiquitin-proteasome pathway at post-translational levels [27]. Multiple E3 ubiquitin ligases, including β-TrCP [28], FBXO11 [29], FBXL14 [30], FBXL5 [31] and SPSB3 [32], are involved in Snail ubiquitination and degradation. Protein expression is meticulously regulated by the balance between ubiquitination and deubiquitination [33], so deubiquitinating enzymes (DUBs) may play a crucial role in regulating Snail protein in the opposite direction to ubiquitination. Approximately 100 DUBs have been identified so far [34], nevertheless, a few DUBs such as DUB3 [35], OTUB1 [36], USP47 [37], PSMD14 [38], USP27X [39], USP26 [40] and USP1 [41] have been demonstrated to promote deubiquitination and stabilization of Snail. Most of these DUBs belong to cysteine proteases. The molecular mechanism underlying the post-translational regulation of Snail by other types of DUBs remains not fully understood.

Here, we identify EIF3H as a potential deubiquitinating enzyme, which is essential to deubiquitinate and stabilize Snail. Furthermore, we demonstrate that elevated expression of EIF3H in ESCC evokes enhanced malignant phenotypes of ESCC cells. Overall, our study demonstrates the importance of EIF3H in regulating ESCC tumor aggressiveness, and provides a potential therapeutic targets of ESCC.

Methods
Cell lines and cell culture conditions
Human esophageal squamous cell carcinoma cell lines (KYSE30, KYSE140, KYSE150, KYSE180, KYSE410, KYSE450, and KYSE510) were maintained in RPMI/1640 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone). Human esophageal epithelial cell line HET-1A and HEK293T cell line purchased from the American Type Culture Collection, were maintained in DMEM medium (Gibco) supplemented with 10% fetal bovine serum. Cells were cultured in an atmosphere of 5% CO2 at 37 °C.

Antibodies and reagents
Antibodies against EIF3H (#ab251743, Abcam), Snail (#3879, CST; #14–9859-82, Thermo Scientific; #53519, Abcam), Ubiquitin (#3936, CST), Vimentin (#5741, CST), N-cadherin (#13116, CST), E-cadherin (#3195, CST), EIF3A (#3411, CST), EIF3I (#ab40745, Abcam), Ubiquitin (#3936, CST), Vimentin (#5741, CST), N-cadherin (#13116, CST), E-cadherin (#3195, CST), EIF3A (#3411, CST), EIF3I (#ab40745, Abcam) were used. Normal rabbit IgG (#2729, CST), anti-c-MYC agarose affinity gel (A7470, Sigma Aldrich) and anti-Flag M2 affinity gel (A2220, Sigma Aldrich) were used for immunoprecipitation assay. MG132 (C2211, Sigma Aldrich) was dissolved in dimethyl sulfoxide. Lipofectamine 2000 (Invitrogen) was used for transfection. CCK-8 kit was purchased from Dojindo Labs.
Plasmids and primers
Full length of human EIF3H cDNA (NM_003756.2; CCDS6319.1) was subcloned into the pLVX-IRES-puro vector (Clontech) with or without tags. Plasmids of wild-type and deletion mutants for Snail were constructed as described [38]. One short hairpin RNAs targeting Snail (shSnail: 5′-CCACTCAGATGTCAAAGAATATA-3′) and two targeting EIF3H (shEIF3H#1: 5′-CAACTCTTGGAAATAATATA-3′; shEIF3H#2: 5′-CTGTGTGCAGATTAAAACATGAA-3′) were constructed into the pSiH-H1 vector (System Biosciences). The following RT-qPCR primers were used in this study: EIF3H-F: 5′-AAAGTGGTCTAGAAGATA-3′; S n a i l - F : 5′-GCGAGTCAGCTGTTGCAGA-3′; S n a i l - R : 5′-CCAGCAAGATGAGCATTGGCAG-3′; EIF3H-R: 5′-ATATTCTCCTTCGGAAGCCTAACT-3′; Snail-F: 5′-CTGGAAGCCTAACTACAGCGA-3′; Snail-R: 5′-AGATGACATTTGCGACGAG-3′. All sequences were verified by DNA sequencing.

Cell proliferation and colony formation assays
CCK-8 assay and colony formation assay were used to evaluate cells proliferation. Briefly, cells were seeded in 96-well culture plates (Corning Life Science). At 24, 48, 72, 96 and 120 h post seeding, 10 μl CCK-8 reagent was added to the wells and incubated for 1 h. The absorbance was determined at 450 nm using a SpectraMax 190 Microplate Reader (Molecular Devices). The colony formation assay needs 1000–2000 indicated cells seeded in six-well plates and after 14 days, these cells were fixed with 1.5% crystal violet/ methanol solution and counted. For the softagar colony formation assay, 2000 established HET1A cells were mixed in 0.36% agarose culture medium as the upper layer. The bottom layer is composed of 0.75% agarose and DMEM culture medium. After an incubation for 2 weeks, the colony were stained by crystal violet and photographed.

In vitro transwell assays
Invasion assays were performed in 24-well Milli cell chamber coated with 30 μl of Matrigel (Corning Incorporated). 3–5 × 10^5 indicated cell lines were seeded on the coated filters in 100 μl of serum-free medium, the bottom chamber was filled with 600 μl complete culture medium. After 24 h incubation at 37 °C in 5% CO_2, the invasive ESCC cells were stained with crystal violet. The migration assays were conducted in a similar method without coating with Matrigel.

Immunofluorescence staining
Cells were cultivated on the μ slide VI (Ibidi) tunnels for the experiment. The cells were fixed for 15 min with 4% formaldehyde, permeabilized for 15 min with 0.5% Triton X-100 and blocked for 1 h with 5% BSA. Cells were then incubated with anti-EIF3H and anti-Snail primary antibodies overnight, and with secondary antibodies conjugated with Alexa Fluor in dark place for 1 h. The sample was then incubated with 0.5 μg/ml DAPI for 5 min. Localization of EIF3H and Snail were visualized by confocal microscopy.

Co-immunoprecipitation (co-IP) assay
For Co-immunoprecipitation assay, 10 μM MG132 were used for incubation for 4 h before harvesting protein extraction. Whole cell lysates were mixed with anti-Flag M2 affinity gel at 4 °C for 12 h. Next, after washing the beads with RIPA buffer for five times, the immunoprecipitated protein complexes were collected for immunoblotting. Endogenous co-IP assay was performed as described above, except that total protein extraction was incubated with appropriate antibodies at 4 °C for 12 h, and then conjugated with protein A/G agarose beads (#9863, CST).

Mass spectrometry
Vector or EIF3H-Flag plasmid was transfected and cells were cultured for 24 h. 10 μM MG132 was added into these cells for an additional 4 h. The co-IP assay and mass spectrometry procedures were performed as previously described [38]. The LC-MS/MS analysis was performed by Qinglianbio Co. Ltd. (Beijing, China).

In vitro ubiquitination assay
Snail-FLAG together with HA-Ub and EIF3H-MYC plasmids were transfected into HEK293T, respectively. Ubiquitinated Snail and EIF3H protein were immunoprecipitated by anti-FLAG or anti-MYC affinity gel with an incubation of 10 μM MG132 for 4 h. Purified Snail and EIF3H were obtained after elution with FLAG or MYC peptides and dialysis. They were incubated in a deubiquitination reaction buffer at 30 °C for 2 h and analyzed by immunoblotting as previously described [35].

Immunohistochemistry (IHC) analysis
To investigate the presence of EIF3H, Snail and Ki67 proteins in tumor tissues of mice, the IHC assay was performed as previously described [38]. The following antibodies were used in this assay: Ki67 with 1:200 dilution; EIF3H with 1:2000 dilution; Snail with 1:50 dilution; E-cadherin with 1: 200 dilution; N-cadherin with 1: 100 dilution. The human ESCC tissues and paired adjacent tissues used in this study were obtained from Nanjing First Hospital. These individuals did not receive any radiotherapy, chemotherapy or immunotherapy before surgery and were informed consent.

Animal experiments
Eighteen 6 weeks old male BALB/c-nude mice were randomly divided into three groups, and subcutaneously injected with 6 × 10^5 cancer cells into the right flank.
region of each mouse. After visible tumors had developed, tumor volume was measured and calculated as length \times width^2 / 2 every 2 days. Eighteen 6-week-old male SCID/Beige mice were randomly divided into three groups and injected with the KYSE150 cells (6 \times 10^5 cells each mouse) into the tail vein. One month after injection, the mice lungs were harvested, stained and weighed. These mice were purchased from Vital River (Beijing). The Institutional Animal Care and Use Committee of Chinese Academy of Medical Sciences Cancer Hospital approved these experiment procedures.

**Statistical analysis**

Comparisons between two groups were performed using two-tailed Student’s t-test. The correlation between two groups was determined by the Pearson correlation analysis. Results from in vitro experiments shown here are representative of three independent experiments. All quantitative data are presented as mean ± s.e.m. GraphPad Prism Version 6.01 was used for statistical analysis. A P-value < 0.05 was considered statistically significant.

**Results**

**EIF3H is overexpressed in ESCC**

We first analyzed the expression of EIF3H in esophageal cancer of TCGA data in UALCAN [42] (n = 195). Box plots showed that EIF3H is significantly upregulated in tumor tissues (Fig. 1a). Next, we performed the immunoblotting analysis on a panel of human esophageal epithelial cell line and ESCC cell lines. The results indicated that EIF3H is overexpressed in tumor cell lines (Fig. 1b). Using immunohistochemistry staining, we found that EIF3H expression was much higher in the ESCC tissues compared with adjacent normal esophageal epithelial tissues (Fig. 1c and d). Taken together, these results demonstrate that EIF3H is overexpressed in ESCC.

**EIF3H promotes ESCC tumorigenesis and growth in vitro and in vivo**

To elucidate functional significance of EIF3H in tumor progression, we used EIF3H overexpression or knockdown KYSE150 and KYSE510 cells to detect the impact of EIF3H on cell growth. The efficacy of EIF3H overexpression or knockdown in these cells was detected by immunoblotting and RT-qPCR (Fig. 2a-d). We observed that EIF3H overexpression in both KYSE150 and KYSE510 cells increased cell proliferation in vitro (Fig. 2e and f), whereas depletion of EIF3H significantly reduced cell proliferation (Fig. 2g and h). Additionally, to detect the function of EIF3H in tumorigenesis, we established HET1A EIF3H-overexpression cell line (Fig. S1A-B). The proliferation, colony formation and malignant transformation ability were significantly improved with EIF3H overexpression (Fig. S1C-F). Moreover, in in vivo xenograft model, tumor growth and the percentage of Ki67 positivity were markedly reduced in mice injected with EIF3H knockdown KYSE150 cells compared with the controls (Fig. 2i-j). Together, these data indicate an
Fig. 2 (See legend on next page.)
essential role of EIF3H in cell proliferation of ESCC in the process of tumorigenesis and progression.

EIF3H facilitates metastatic phenotypes of ESCC cells
To further investigate the function of EIF3H on ESCC cell migration and invasion, we performed the transwell assay using ESCC cells with EIF3H overexpression or knockdown. We found that EIF3H overexpression significantly increased the abilities of migration and invasion in KYSE150 and KYSE510 cells (Fig. 3a and b). In contrast, EIF3H knockdown substantially weakened these abilities (Fig. 3c and d). To find out whether EIF3H enhances cancer metastasis ability in vivo, we then performed lung metastatic model assay. Mice injected with EIF3H knockdown cells lacked metastatic nodules in the lung tissues and the lung weight was significantly lighter than the shControl group (Fig. 3e). Taken together, these results indicate that EIF3H knockdown inhibits cell mobility and tumor metastasis in ESCC.

EIF3H interacts with Snail
To find out the substrate of EIF3H, we examined the proteins interacting with EIF3H using co-IP and LC-MS/MS assay (Fig. 4a). A total of 963 proteins were recognized, and among them, Snail attracted our attention because it is an EMT-associated transcription factor and ranks high on the list (Table S1). To confirm the interaction between EIF3H and Snail, we then performed a co-IP assay with co-expressed EIF3H-Flag and Snail in HEK293T cells. After the immunoprecipitation of EIF3H, we detected an associated Snail (Fig. 4b). Then, the reciprocal co-IP assay was performed to further identify their interaction (Fig. 4c). Similar results were observed. Next, immunoprecipitation of endogenous Snail from KYSE510 cells also confirmed the interaction between endogenous Snail and EIF3H (Fig. 4d). Additionally in vivo co-IP assay was performed in HEK293T and KYSE510 EIF3H-knockdown cells to test the specificity of the interaction between Snail and EIF3H (Fig. S2A-B). In the MS analysis, multiple EIF3 subunits were identified to interact with EIF3H. However, as shown in Fig. S2A-B, we only observed the immunoprecipitated EIF3H by anti-Snail antibodies but could not detect the interaction between Snail and another two EIF3 subunits EIF3A and EIF3I. In previous study, most of the EIF3H are present exclusively in the cytoplasm [43].

EIF3H stabilizes Snail through deubiquitination
The interaction between EIF3H and Snail suggests the ability of EIF3H to regulate Snail expression. To verify this hypothesis, we co-expressed Snail with vector or EIF3H in HEK293T cells and examined the expression of Snail and EIF3H. Results showed that EIF3H obviously upregulated the Snail protein, but did not affect the mRNA level of Snail (Fig. 5a). Subsequently, we performed a cycloheximide (CHX) pulse-chase assay to detect the function of EIF3H on Snail stability by blocking protein synthesis. EIF3H overexpression could strikingly stabilize Snail protein levels, while Snail protein degraded obviously in cells transfected with vector (Fig. 5b). We further validated whether EIF3H modulates Snail stability through deubiquitination. When co-expressed Snail-Flag with EIF3H in HEK293T cells, we found that Snail ubiquitination could be abolished by EIF3H overexpression (Fig. 5c). To further confirm the function of EIF3H on Snail stability in ESCC, we stably overexpressed or knocked down EIF3H in two ESCC cell lines, KYSE150 and KYSE510. We detected the mRNA and protein level of Snail in these cells, and revealed that the protein expression of Snail was regulated by EIF3H.
Fig. 3 (See legend on next page.)
but the mRNA expression has no significant changes (Fig. 2a-d). Therefore, EIF3H regulates Snail expression at the posttranslational level. Then we demonstrated that EIF3H overexpression prolonged Snail half-life in the CHX pulse-chase assay (Fig. 5d and e), while endogenous Snail became unstable and degraded acceleratedly through EIF3H knockdown (Fig. 5f and g). To further extend these findings, endogenous ubiquitination assay was performed. We observed overexpression of EIF3H deubiquitinated endogenous Snail (Fig. 5h), but

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**Fig. 3** Effects of EIF3H on ESCC cell motility and tumor metastasis in vitro and in vivo, EIF3H was stably overexpressed (a and b) or knocked down (c and d) in KYSE150 and KYSE510 cells, in which migration and invasion assays were performed. The migratory and invasive cells were counted. Data are obtained from three independent experiments. Scale bars, 500 μm. e EIF3H knockdown suppressed lung metastasis in vivo in mice. KYSE150 cells with or without knockdown of EIF3H were injected into the tail vein of SCID/Beige mice (n = 6) and the mice lung was harvested, weighed and stained 1 month later. HE staining was performed to analyze the nodules inside the lung. The lung weight was measured after the experiment. Representative images, representative HE staining (left panel) and quantitative results of lung weight (right panel) are shown. Scale bars, 1 mm. *P < 0.05; **P < 0.01; ***P < 0.001

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**Fig. 4** EIF3H interacts with Snail. a Vector or EIF3H-Flag plasmid was transfected in HEK293T cells for 24 h. After a 4 h treatment of 10 μM MG132, cell lysate was immunoprecipitated with anti-FLAG M2 beads, and the proteins interact with EIF3H were analyzed by mass spectrometry. Silver staining image was shown in the left panel. Representative proteins detected in mass spectrometry were shown in the right panel. b and c EIF3H-Flag was co-expressed with the Snail plasmid in HEK293T cells (b). Snail-Flag was co-transfected with the EIF3H plasmid in HEK293T cells (c). The co-IP assay was performed as described in (a). d Endogenous Snail was captured by anti-Snail antibody from KYSE510 cells, and the endogenous EIF3H and Snail were examined by immunoblotting. e Endogenous EIF3H and Snail in KYSE510 cells was detected by immunofluorescence staining. Scale bars, 30 μm. f The Flag-tagged empty vector, wild-type Snail or mutants plasmids were co-expressed with EIF3H plasmid. Extracts were subjected to the co-IP assay.
Fig. 5 (See legend on next page.)
Having established that EIF3H tightly regulates Snail in ESCC cells, we next explored our findings in the lung tissue in the tail-vein injection model in which EIF3H knockdown KYSE150 cells compared with the controls was used (Fig. 7a). The results demonstrated that knockdown of EIF3H suppressed the expression of Snail and Snail-mediated EMT switch in vivo. The percentage of Ki67 positive tumor cells in lung tissues of shControl group is also slightly higher than the EIF3H knockdown groups. To further extend the present findings in clinical, we subsequently detected the expression of EIF3H and Snail in the tissue microarray containing 73 paired ESCC tissues to investigate the clinic correlation (Fig. 7b). In concordance with our findings in xenograft model, the expression levels of EIF3H were positively correlated with Snail expression in the ESCC tissue samples (Fig. 7c). Thus, these data in ESCC tissues validated our observations in cell lines and in animal models, lending further support to our hypothesis that the EIF3H is required for the stabilization of Snail and the aggressiveness of ESCC.

**Discussion**

Previous research demonstrated that EIF3H is involved in multiple cancers. Overexpression of EIF3H promotes cancer cell growth [14], results in a malignant phenotype [46], and serves as a prognostic marker of recurrence and metastasis [12, 47]. However, little is known about the role of this molecule in ESCC. Thus, this study aimed to elucidate the function of EIF3H and the molecular mechanism underlying the functional relevance in ESCC.

Validated by the expression difference in human ESCC clinical samples and their adjacent tissues, we observed that EIF3H is highly expressed in ESCC. Additionally, an oncogenic role for EIF3H in tumor progression and metastasis in this research is convincingly demonstrated in vitro and in vivo, which is in alignment with previous studies [15, 48, 49]. EIF3H depletion in ESCC cells significantly decreased the ability of cell proliferation and mobility, examined by CCK8 assay, colony formation assay and transwell assay in vitro and xenograft and tail-vein lung metastatic mouse models in vivo. In this study, we verified that EIF3H is involved in different processes of tumor tumorigenesis and progression. The increase of proliferation rate is significantly higher in EIF3H-overexpression HET1A cells than that of EIF3H-
overexpression KYSE150 and KYSE510 cells but is in nearly the same proportion of EIF3H knockdown KYSE150 and KYSE510 cells. In clinical samples, EIF3H levels in primary tumors are significantly higher than their adjacent normal samples. Taken together, EIF3H might be more essential in the process of tumorigenesis. Besides, it also takes part in EMT process and the knockdown of EIF3H suppressed tumor metastasis in vivo. Whether EIF3H expression levels rise continuously during tumor progression? We still need more clinical samples and in vivo evidence to prove this hypothesis.

Besides, the mechanism of EIF3H mediated cell growth and migration also needs further study. EIF3H is one of the 13 subunits of the elongation initiation factor EIF3 and it connected with EIF3F and other subunits to form the functional octamer core in translational regulation and control [9]. EIF3H also belongs to the JAMM family of deubiquitinating enzymes for its putative non-conserved MPN domain. The uncharacterized deubiquitinating enzyme ability of EIF3H was verified by structural modelling analysis based on its noncanonical metalloprotease motif and in vitro deubiquitination analysis using tetra-ubiquitin cleavage assays biochemically.
The mis-regulation of EIF3H might disrupt this function leading to the cause of human diseases. To address this issue, we focused on the functional partners of EIF3H. We performed a co-IP assay combined with the mass spectrometry analysis and identified that an EMT-associated transcription factor, Snail could interact with EIF3H. In vivo co-IP assays in EIF3H knockdown cells were also carried out to confirm the specificity of the interaction between Snail and EIF3H, but there is no interaction with other EIF3 subunits (EIF3A and EIF3I). Additionally, EIF3H is demonstrated to be responsible for Snail deubiquitination and stability in ESCC cell lines. EIF3H significantly decreased the poly-ubiquitin level of ubiquitinated Snail in an in vitro ubiquitination assay. Therefore, we identified EIF3H as a potential deubiquitinating enzyme of Snail.

It has been reported that Snail plays an eminent role in the tumorigenesis, growth and metastasis of epithelial tumors [21, 51, 52], and one of its main mechanisms involved is the induction of EMT [53]. EMT is a cellular process accompanied by the loss of epithelial phenotypes and the gain of mesenchymal features. EMT is integral in cell development, stemness and contributes pathologically to cancer progression [54]. Mounting evidences also suggest that the acquisition of cancer invasiveness is correlated to EMT [55, 56] and tumor metastasis is the major cause of mortality in cancer patients [57]. EMT is featured by loss of E-cadherin expression and gain of N-cadherin and Vimentin [58]. Snail is a well-known transcriptional repressor of E-cadherin during EMT, and it is also shown to activate the expression of invasion-associated genes and the migratory phenotype [59]. A recent publication in lung adenocarcinoma demonstrated that EIF3H overexpression could also induce EMT signaling pathway [16]. Our results exhibited that ectopic expression of EIF3H promoted ESCC metastasis with the increase of Snail, N-Cadherin and Vimentin, and decrease of E-cadherin, while knockdown of EIF3H exerted an opposite effect. Knockdown of Snail strikingly reversed the promoting effects of ectopic expression of EIF3H. The IHC staining of the lung metastatic nodules in tail-vein injection mice model indicated that knockdown of EIF3H correlated with Snail downregulation and it mediated EMT switch in vivo. Clinically, the levels of EIF3H were positively correlated with Snail expression. Therefore, our study demonstrates a critical EIF3H-Snail signaling axis in EMT process and tumor metastasis in ESCC. Snail expression is regulated tightly during development, and posttranslational regulation of Snail has been a frontier issue in recent years. Protein ubiquitination is a reversible posttranslational modification that regulates a broad range of biological processes [60]. The balance between ubiquitination and deubiquitination contributes to ubiquitin homeostasis, which disruption leading to tumor initiation and progression [61]. It is quite common that several deubiquitinating

Fig. 7 EIF3H and Snail levels positively correlate in ESCC. a Representative HE staining, EIF3H, Snail, E-cadherin, N-cadherin and Ki67 immunohistochemistry staining in lung tissues of shControl, shEIF3H#1 and shEIF3H#2 groups described in Fig. 3 (e). Scale bars, 100 μm. b Representative staining of EIF3H and Snail in ESCC samples. Scale bars, 100 μm. c The positive correlation was obtained in ESCC samples between EIF3H and Snail protein expression.
enzymes, working like isozymes, regulate the same sub-
strate under different circumstances, especially in the
regulation of vital transcription factors. The stability and
ubiquitination of p53 are modulated by diverse deubi-
quitinating enzymes under different cancer types and
stimuli, such as USP42, USP49, PHD3, OTUD1, OTUD5, ATXN3 [62–67]. The protein level of Snail is also
reported to be precisely regulated by ubiquitination
and deubiquitination [32]. Some deubiquitinating en-
zeymes were reported to regulate its stability, such as
DUB3, PSMD14, OTUB1 and USP26 [27, 36, 38, 40].
Here, we also identified Snail as a novel substrate of
EIF3H. EIF3H interacts with and deubiquitinates Snail
to prolong its half-life. How these enzymes collaborate
with each other to control Snail expression precisely re-
 mains still unknown and needs further investigation.
Meanwhile, as a potential deubiquitinating enzyme of
Snail, EIF3H might be an efficient target for ESCC thera-
pic treatment. Specific small molecular inhibitors tar-
geting EIF3H would be our next research direction.

In summary, we demonstrated that EIF3H is respon-
sible for tumorigenesis, tumor growth and metastasis of
ESCC through stabilizing Snail, thus promoting the
EMT phenotype in ESCC cells. We revealed the novel
modulating mechanism of the Snail stability by EIF3H in
ESCC. Our study also has further implications in the
development of potential new therapeutic strategy for
ESCC.

Conclusions
In this study, we investigated the mechanism underlying
EIF3H as an oncogene in ESCC. We found overexpres-
sion of EIF3H promotes tumorigenesis, tumor growth
and metastasis in ESCC. Moreover, we found that EIF3H
might be a bona fide deubiquitinating enzyme to
stabilize Snail protein level and Snail is the novel identi-
fied substrate of EIF3H. Knockdown of EIF3H inhibits
the EMT process and cell mobility induced by Snail. In
ESCC clinical samples, EIF3H protein levels correlates
positively with Snail levels. Our study reveals an essential
EIF3H-Snail signaling axis in tumor aggressiveness in
ESCC and provides EIF3H as a promising biomarker for
ESCC treatment.

Additional Information

In this study, we investigated the mechanism underlying
EIF3H as an oncogene in ESCC. We found overexpres-
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and metastasis in ESCC. Moreover, we found that EIF3H
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ESCC and provides EIF3H as a promising biomarker for
ESCC treatment.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13046-020-01678-9.

Additional file 1 Table S1. LC-MS/MS results of co-
immunoprecipitation assay.

Additional file 2 Figure S1. EIF3H increases the proliferation and
transforming potential of HET1A cell line. (A-B) EIF3H was stably
overexpressed in HET1A cells and the efficacy was detected by
immunoblotting and RT-qPCR. (C) Co-IP proliferation assay was per-
formed in these established cells. (D-E) Overexpression of EIF3H promotes
colonies formation ability in plate and soft agar colony formation assay. (F) Representative views of soft agar colony formation assay. Scale bar,
500 μm.

Additional file 3 Figure S2. EIF3H specifically interacts and
deuubiquitinates with Snail. (A-B) In vivo co-IP experiments of EIF3H-
knockdown HEK293T (A) and KYSE510 (B) were performed using anti-
Snail antibody. EIF3H were immunoprecipitated by Snail, but EIF3A and
EIF3I were not detected. (C) An in vitro ubiquitination assay of Snail-FLAG
and EIF3H-MYC purified from HEK293T cells.

Abbreviations

CHX: Cycloheximide; co-IP: Co-immunoprecipitation; EIF3H: Eukaryotic
translation initiation factor 3H; EMT: Epithelial-mesenchymal transition;
ESCC: Esophageal squamous cell carcinoma; IHC: Immunohistochemistry; LC-
MS/MS: Liquid chromatography tandem mass spectrometry; Snail: SNAIL
family transcriptional repressor 1

Acknowledgements

We thank Professor Shimada Yutaka, from Kyoto University for kindly
providing us the ESCC cell lines and Professor Cao Xiufeng from Nanjing
First Hospital for providing us the human ESCC tissues.

Authors’ contributions

Conceptualization: XBG and ZHL; Methodology: XBG, RZ, APL, HHZ and FD;
Validation: XBG, RZ and ZHL; Formal analysis: XBG, RZ and HXY; Writing the
manuscript: XBG and RZ; Supervision: ZHL; Funding acquisition: XBG and
ZHL; Approval of the final version of the manuscript: all authors.

Funding

This research was funded by National Key R&D Program of China, grant
number 2016YFC1302100; the CAMS Innovation Fund for Medical Sciences,
grant number 2016-I2M-1-001 and 2019-I2M-1-003; and Natural Science
Foundation of Inner Mongolia Autonomous Region, grant number 2019MS08078.

Availability of data and materials

All data generated or analyzed during this study are included in this
published article and its supplementary information files.

Ethics approval and consent to participate

The ethical committee of Nanjing First Hospital and Chinese Academy of
Medical Sciences Cancer Hospital approved this experiment process. De-
identified ESCC tissue samples were obtained from patients after signed in-
fomed consent in Nanjing First Hospital. The animal experiments conducted
strictly in line with the Animal Study Guidelines of Chinese Academy of Med-
ical Sciences.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Received: 27 February 2020 Accepted: 17 August 2020
Published online: 31 August 2020

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer
statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide
for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.
2. Rustgi AK, El-Serag HB. Esophageal carcinoma. N Engl J Med. 2014;371:
2499–509.
3. Pennathur A, Gibson MK, Jobe BA, Luketic JD. Oesophageal carcinoma. Lancet. 2013;381:1400–12.
4. Zeng H, Zheng R, Guo Y, Zhang S, Zou X, Wang N, et al. Cancer survival in China, 2003-2005: a population-based study. Int J Cancer. 2015;136:9120–31.
5. Enzinger PC, Mayer RJ. Esophageal cancer. N Engl J Med. 2003;349:2241–52.
6. Okumura H, Uchikado Y, Matsumoto M, Owaki T, Kita Y, Omoto I, et al. Prognostic factors in esophageal squamous cell carcinoma patients treated with neoadjuvant chemoradiation therapy. Int J Clin Oncol. 2013;18:329–34.
7. Liu J, Xie X, Zhou C, Peng S, Rao D, Fu J. Which factors are associated with actual 5-year survival of esophageal squamous cell cancer? Eur J Cardiothorac Surg. 2012;41:e7–11.
8. Pick E, Hofmann K, Glickman MH. PC complexes: beyond the proteasome, CSN, and eIF3 troika. Mol Cell. 2009;35:260–4.
9. Masutani M, Sonenberg N, Yokoi S, Imataka H. Reconstitution reveals the functional core of mammalian eIF3. EMBO J. 2007;26:3373–83.
10. Cappuzzo F, Varella-Garcia M, Rossi E, Gajapathy S, Valente M, Drabkin H, et al. MYC and EIF3H Coamplification significantly improve response and survival of non small-cell lung cancer patients (NSCLC) treated with gefitinib. J Thorac Oncol. 2009;4:472–8.
11. Mahmoud SF, Gruel N, Chapeaublanc E, Lesoude A, Jones T, Peyal F, et al. A siRNA screen identifies RAD21, EIF3H, CHC1AC and TANK as driver genes within the 8q23, 8q24.3 and 17q23 amplicons in breast cancer with effects on cell growth, survival and transformation. Carcinogenesis. 2014;35:670–82.
12. Okamoto H, Yasui K, Zhao C, Arii S, Inazawa J. PTK2 and EIF3S3 genes may be amplification targets at 8q23-q24 and are associated with large hepatocellular carcinomas. Hepatology. 2003;38:1242–9.
13. Hutter CM, Chang-Claude J, Slattery ML, Pflugeisen BM, Lin Y, Duggan D, et al. Characterization of gene-environment interactions for colorectal cancer susceptibility loci. Cancer Res. 2012;72:2036–44.
14. Savinainen KJ, Helenius MA, Lehtonen HJ, Visakorpi T. Overexpression of EIF3S3 promotes cancer cell growth. Prostate. 2006;66:1144–50.
15. Hong S, Liu Y, Xiong H, Cai D, Liu Q, Wang L, Ramirez-Moya J, et al. EIF3S3 promotes cancer cell growth. Prostate. 2018;78:839–49.
16. Hu Y, Wei X, Lv Y, Xie X, Yang L, He J, et al. EIF3S3 interacts with PDCD4 enhancing eIF3H suppression inhibits osteocarcinoma cell growth and tumorigenesis. Exp Ther Med. 2016;14:4095–2011.
17. Hu Y, Wei Y, Xie X, Yang L, He J, et al. EIF3S3 interacts with PDCD4 enhancing eIF3H suppression inhibits osteocarcinoma cell growth and tumorigenesis. Exp Ther Med. 2016;14:4095–2011.
18. Choe J, Lin S, Zhang W, Liu Q, Wang L, Ramírez-Moya J, et al. mRNA circulation by METTL3-eIF3H enhances translation and promotes oncogenesis. Nature. 2018;561:556–61.
19. Thiery JP, Acquone H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139:871–90.
20. Mani SA, Guo W, Liao M, Eaton EN, Ayyanan A, Zhou YF, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;135:187–200.
21. Moon H, Ju HL, Chung SJ, Cho KJ, Eun JW, Nam SW, et al. Transforming growth factor-beta promotes liver tumorigenesis in vivo via up-regulation of snail. Gastroenterology. 2017;153:1378–91 e6.
22. Wang J, Ye Q, Cao Y, Guo Y, Huang X, Mi W, et al. Snail determines the therapeutic response to mTOR kinase inhibitors by transcriptional repression of 4E-BP1. Nat Commun. 2017;8:2007.
23. Massoumi R, Kuphal S, Hellerbrand C, Haas B, Wild P, Spruss T, et al. Down-regulation of CYLD expression by snail promotes tumor progression in malignant melanoma. J Exp Med. 2009;206:2211–32.
24. Dong C, Yuan T, Wu Y, Wang Y, Fan TW, Miyala S, et al. Loss of FBX1 by snail-mediated repression provides metabolic advantages in basal-like breast cancer. Cancer Cell. 2013;23:3316–31.
25. Kudo-Saito C, Shirako H, Takeuchi T, Kawamura Y. Cancer metastasis is accelerated through immunosuppression during snail-induced EMT of cancer cells. Cancer Cell. 2009;15:195–206.
26. Zheng X, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, et al. Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature. 2015;527:525–30.
27. Liu Y, Ju D, Ding M, Yin Y, Zhang H, Luo K, et al. CDK4/6-dependent activation of DUB3 regulates cancer metastasis through SNAIL1. Nat Commun. 2017;8:13923.
28. Zhao BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, et al. Dual regulation of snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol. 2004;6:931–40.
52. Lee JH, Jung SM, Yang KM, Bae E, Ahn SG, Park JS, et al. A20 promotes metastasis of aggressive basal-like breast cancers through multimonoubiquitylation of Snail1. Nat Cell Biol. 2017;19:1260–73.

53. Wang Y, Shi J, Chai K, Ying X, Zhou BF. The role of snail in BMT and tumorigenesis. Curr Cancer Drug Targets. 2013;13:963–72.

54. Lamouille S, Xu J, Deynck K. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15:178–96.

55. Ye X, Weinberg RA. Epithelial-Mesenchymal plasticity: a central regulator of Cancer progression. Trends Cell Biol. 2015;25:675–86.

56. Davidson B, Trope CG, Reich R. Epithelial-mesenchymal transition in ovarian carcinoma. Front Oncol. 2012;233.

57. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147:275–92.

58. Eastham AM, Spencer H, Soncin F, Ritson S, Merry CL, Stern PL, et al. Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. Cancer Res. 2007;67:11254–62.

59. Nieto MA, Huang RY, Jackson RA, Thiery JP. EMT: 2016. Cell. 2016;166:21–186.

60. Komander D, Clague MJ, Urbe S. Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol. 2009;10:550–63.

61. Luo J, Lu Z, Lu X, Chen L, Cao J, Zhang S, et al. OTUD5 regulates p53 stability by deubiquitinating p53. PLoS One. 2013;8:e77682.

62. Piao S, Pei HZ, Huang B, Baek SH. Ovarian tumor domain-containing protein 1 deubiquitinates and stabilizes p53. Cell Signal. 2017;33:22–9.

63. Liu H, Li X, Ning G, Zhu S, Ma X, Liu X, et al. The Machado-Joseph disease Deubiquitinase Ataxin-3 regulates the stability and apoptotic function of p53. PLoS Biol. 2016;14:e2000733.

64. Hock AK, Vigneron AM, Carter S, Ludwig RL, Vousden KH. Regulation of p53 stability and function by the deubiquitinating enzyme USP42. EMBO J. 2011;30:4921–30.

65. Rodriguez J, Herrero A, Li S, Rauch N, Quintanilla A, Wynne K, et al. PHD3 regulates p53 protein stability by Hydroxylating Proline 359. Cell Rep. 2018;24:1316–29.

66. Tu R, Kang W, Yang X, Zhang Q, Xie X, Liu W, et al. USP49 participates in the DNA damage response by forming a positive feedback loop with p53. Cell Death Dis. 2018;9:553.

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