Long noncoding RNA EIF1AX-AS1 promotes endometrial cancer cell apoptosis by affecting EIF1AX mRNA stabilization

Chengyu Lv1,2 | Jiandong Sun1 | Yuhong Ye3,4 | Zihang Lin1 | Hua Li1,3 | Yue Liu1,3 | Kaien Mo1 | Weiwei Xu1 | Weitao Hu1 | Eman Draz3,5 | Shie Wang1,3

Abstract
Long noncoding RNAs (lncRNAs) have been found to play an important role in the occurrence and development of endometrial carcinoma (EC). Here, using RNA sequencing analysis, we systemically screened and identified the lncRNA eukaryotic translation initiation factor 1A, X-linked (EIF1AX)-AS1, which is aberrantly downregulated in clinical EC tissues and closely correlated with tumor type. EIF1AX-AS1 markedly inhibited EC cell proliferation and promoted apoptosis in vitro and in vivo. Mechanistically, EIF1AX-AS1 interacts with EIF1AX mRNA and poly C binding protein 1 (PCBP1), which promote EIF1AX mRNA degradation. Intriguingly, by interacting with internal ribosome entry site-related protein Y-box binding protein 1 (YBX-1), EIF1AX promotes c-Myc translation through the internal ribosome entry site pathway. c-Myc promotes EIF1AX transcription and thus forms a feed-forward loop to regulate EC cell proliferation. Taken together, these data reveal new insights into the biology driving EC proliferation and highlights the potential of lncRNAs as biomarkers for prognosis and future therapeutic targets for cancer.

KEYWORDS
apoptosis, EIF1AX, endometrial cancer, long noncoding RNA, RNA binding protein

Abbreviations: CHX, cycloheximide; CPAT, coding potential assessment tool; EC, endometrial cancer; EIF1AX, eukaryotic translation initiation factor 1A, X-linked; GO, Gene Ontology; hnRNP, heterogeneous nuclear ribonucleoprotein; IGF2BP1, insulin-like growth factor 2 mRNA binding protein 1; IRES, internal ribosome entry site; KEGG, Kyoto Encyclopedia of Genes and Genomes; KH, K homology; lncRNA, long noncoding RNA; PCBP1, poly C binding protein 1; RACE, rapid amplification of cDNA ends; RBP, RNA-binding protein; RIP, RNA immunoprecipitation; RNA-seq, RNA sequencing; RT-qPCR, quantitative RT-PCR; YBX-1, Y-box binding protein 1.

Chengyu Lv, Jiandong Sun, and Yuhong Ye contributed equally to this work.

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1 | INTRODUCTION

Endometrial cancer is the most common malignanat tumor of the female reproductive system, with new EC cases accounting for 7% of all new malignant tumor cases in 2018. Annual deaths from EC rank sixth among all malignant tumor deaths in female individuals, and deaths from EC are expected to account for 4% of all malignant tumor deaths in 2020. Therefore, clarifying the detailed genetic mechanisms of EC is critical to aid in the development of effective strategies for both diagnosis and treatment.

Long noncoding RNAs are nonprotein-coding RNA molecules with various cellular functions. In the nucleus, IncRNAs can interact with chromatin and participate in transcriptional regulation processes. Long noncoding RNAs in the cytoplasm can regulate mRNA stability, translation, and intracellular signaling. Xu et al found that the IncRNA TLR8-AS1 activates NF-κB signaling and promotes ovarian cancer invasion and metastasis by maintaining TLR8 mRNA stability in the cytoplasm. In addition, IncRNAs often function by recruiting RBPs to aid in the regulation of the expression level of target genes or target proteins. The IncRNA EGFR-AS1 promotes proliferation and metastasis of renal cell carcinoma by recruiting the RBP HuR and maintaining the stability of EGFR mRNA. What interests us is that many studies have confirmed that IncRNAs are closely related to tumor development and progression. Huang et al indicated that silencing of lncRNA firmed that lncRNAs are closely related to tumor development and progression. Huang et al indicated that silencing of IncRNA HOTAIR significantly inhibits proliferation, migration, and invasion of EC cells and arrests the cell cycle in G0/G1 phase. Therefore, the discovery of novel IncRNAs in EC could provide new targets for cancer therapy.

In this study, we undertook RNA-seq and identified the IncRNA EIF1AX-AS1, which is aberrantly downregulated in clinical EC tissues and closely correlated with tumor type of EC patients. Expression of IncRNA EIF1AX-AS1 markedly inhibited cell proliferation and induced apoptosis in vitro and in vivo. Mechanistically, EIF1AX-AS1 interacts with EIF1AX mRNA and PCBP1, which promote EIF1AX mRNA degradation. Intriguingly, through the interaction with the IRES-related protein YBX-1, EIF1AX promotes c-Myc translation through the IRES pathway. In addition, c-Myc promotes EIF1AX transcription and forms a feed-forward loop and EC cell proliferation.

2 | MATERIALS AND METHODS

2.1 | Tissue samples

Endometrial cancer tissues and adjacent noncancerous tissues were retrieved from the Department of Pathology at The First Affiliated Hospital of Fujian Medical University. The protocol for the research project has been approved by a suitably constituted Ethics Committee of the institution within which the work was undertaken and conforms to the provisions of the Declaration of Helsinki. The study approval reference is [2019]-096.

2.2 | RNA sequencing

For RNA-seq, tissues were collected and subjected to RNA extraction (EZNA Total RNA Kit I, Omega). Samples were sent to Allwegene Technology for sequencing.

2.3 | Immunohistochemistry

For immunohistochemical staining, formalin-fixed paraffin-embedded (Sigma, 252549; Sigma, 8002-74-2) EC tissues and adjacent noncancerous tissues were deparaffinized and rehydrated. The specimens were incubated in EDTA buffer (1 mM, pH 8.0) for antigen retrieval using a high-pressure method. Then, tissue sections were incubated overnight at 4°C with EIF1AX antibody (1:500), 3,3′-diaminobenzidine solution (ZSGB-BIO) was used to detect target proteins, which were conjugated with a peroxidase enzyme to form a brown precipitate.

2.4 | RNA FISH

RNA FISH was carried out with an EIF1AX-AS1-specific probe (GenePharma). Cells were fixed with 4% paraformaldehyde for 10 minutes and then incubated with EIF1AX-AS1 probe overnight at 37°C. The cells were then washed and blocked by 3% BSA. The sequences of the EIF1AX-AS1 probe are listed in Table S1.

2.5 | Cell culture, RNA interference, and stable cell lines

Human endometrial cancer EC cell lines HEC-1A and RL95-2 were provided by Cell Bank/Stem Cell Bank, Chinese Academy of Sciences. The cells were cultured in McCoy’s 5A medium or DMEM/F12 medium (Gibco-BRL) containing 10% FBS (Gibco-BRL) at 37°C in humidified atmosphere containing 5% CO2. The siRNAs were transfected in EC cells with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s instructions. To establish stable cell lines, lentivirus-mediated shRNA against EIF1AX (shEIF1AX), EIF1AX-AS1 (shEIF1AX-AS1), and YBX-1 (shYBX-1) and the IncRNA EIF1AX-AS1 overexpression construct designed by Shengzhe Biotechnology were each transfected into EC cells according to the manufacturer’s protocols. Infected cells were selected with puromycin treatment used in experiments.

2.6 | Mitochondrial membrane potential detection by JC-1

HEC-1A and RL95-2 cells were incubated with JC-1 (Beyotime) as previously reported and examined under a Leica TCS SP5 confocal microscope. The relative fluorescence intensity of the red
and green light was calculated as an index reflecting mitochondrial activity.

2.7  |  Cell proliferation assay

The CCK-8 (MedChemExpress) was used to assess cell proliferation ability in accordance with the manufacturer’s instructions. Cells were seeded into 96-well culture plates at a density of 2 × 10^3 cells per well on the day before transfection. After 48 hours, the viability of EC cells was assessed using CCK-8 reagents from five replicates in three independent experiments.

2.8  |  RNA pulldown assay

Biotin-labeled IncRNA-EIF1AX-AS1 and its antisense RNA were in vitro transcribed with the Biotin RNA Labeling Mix (Invitrogen) and SP6/ T7 RNA polymerase (Invitrogen). After treatment with RNase-free DNase I (Roche), biotinylated RNAs were purified with the RNeasy Mini Kit (Qiagen) and incubated with the indicated cell lysates for 1 hour at 4°C. Streptavidin-agarose beads (Invitrogen) were added to each tube and samples were held for 1 hour at room temperature. The retrieved proteins were evaluated by western blot analysis.

2.9  |  RNA immunoprecipitation

We undertook RIP experiments using the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) in accordance with the manufacturer’s instructions. HEC-1A and RL95-2 cells were lysed and cell lysates were incubated with PCBP1 Ab (2 µg Ab/sample). An aliquot of lysate was removed as an input control. RNA enrichment was determined by RT-qPCR and normalized to the input control.

2.10 | 5′ and 3′ RACE

We used 5′ and 3′ RACE to determine the transcriptional initiation and termination sites of IncRNA EIF1AX-AS1 using the SMARTer RACE cDNA Amplification Kit (Clontech) in accordance with the manufacturer’s instructions. The primers used for the PCR of the RACE analysis are listed in Table S2.

2.11 | mRNA decay measurements

The stability of the EIF1AX mRNA was assessed by adding 10 µg/mL actinomycin D into the cell culture medium to inhibit mRNA transcription. At the indicated time points, the relative amount of specific mRNA remaining in each sample could be correlated with mRNA degradation. Total RNA was extracted at indicated hours after actinomycin D (10 µg/mL) treatment.

2.12 | Cycloheximide chase measurements of RNH1 half-life

EIF1AX siRNA was transiently transfected into HEC-1A cells. Cycloheximide (100 ng/mL) was added to the DMEM culture medium, and incubation was continued for the indicated time. The cell lysates were submitted for western blot analysis using mouse anti-His mAb (Invitrogen), and western blot data were quantified using ImageJ software.

2.13 | Flow cytometry

Cells (1 × 10^6) were trypsinized and resuspended to obtain single-cell suspensions. Detached cells were fixed overnight at 4°C in 70% ethanol, then stained with propidium iodide (Cell Cycle Detection kit; KeyGen) and analyzed with a FACScan flow cytometer (BD Biosciences) and ModFit 3.0 software (Verity Software House). To detect apoptosis, cells were stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (Apoptosis Detection kit; KeyGEN) as recommended by the manufacturer and analyzed by flow cytometry. Data were analyzed with FlowJo software (Tree Star).

2.14 | Quantitative RT-PCR analyses

HEC-1A and RL95-2 cells transfected with siRNA were collected and subjected to RNA extraction with an EZNA Total RNA Kit I. Synthesis of cDNA was carried out on a PCR amplifier (AB2720; Applied Biosystems) following the reverse transcription kit manual. Sequences of PCR primers are listed in Table S2.

2.15 | Western blot analysis

Cell lysates were prepared as previously reported. Anti-EIF1AX, anti-YBX-1, and anti-PCBP1 were diluted to 1:1000. Information regarding Abs is listed in Table S1.

2.16 | Animal experiments

Four-week-old athymic nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Experimental protocols concerning mice handling were under the approval of the Institutional Animal Care and Use Committee of Fujian Medical University. Mice were randomly divided into two or three groups, with seven mice in each group. HEC-1A cells (5 × 10^5) with EIF1AX-AS1 stable overexpression, EIF1AX-AS1 and EIF1AX overexpression, or control cells were subcutaneously injected into the flank of mice. Mice were killed 4 weeks after the injection of cancer cells, and tumor weights were analyzed. Expression of EIF1AX in tumors was determined by western blot analysis.
FIGURE 1 Heatmap showing different gene expressions between EC and adjacent normal tissues. A, EIF1AX-AS1 expression between EC samples (EC) and adjacent normal tissues (NT) was compared using PCR analysis (n = 6). B, EIF1AX-AS1 expression between EC and adjacent normal tissues (red) in EC cells compared with adjacent normal tissues (blue; n = 8). C, Schematic illustration showing that EIF1AX-AS1 is derived from the intron region of the EIF1AX gene. D, Full-length sequence of EIF1AX-AS1 was determined using 5′ and 3′ RACE assays. Kaplan-Meier analysis of the overall survival of EC patients with high or low EIF1AX-AS1 expression. E, EIF1AX-AS1 ORF sequences were constructed and transfected into HEC-1A, positive control (pcDNA3.1 + GAPDH ORF), and negative control (pcDNA3.1 + vector). G, H, FISH analysis of EIF1AX-AS1 in HEC-1A and RL95-2 cells using a biotin-labeled RNA probe. Nuclei were stained with DAPI. Scale bar = 100 μm. ImageJ software measured the quantification of RNA in the nucleus and cytoplasm. t test: ns, P > .05; *P < .01. Bars indicate SD.

TABLE 1 Relationship between expression of EIF1AX-AS1 and clinicopathologic characteristics in patients with endometrial cancer

| Characteristic       | IncRNA EIF1A-AS1 | P value |
|----------------------|------------------|---------|
| Specimen type        |                  |         |
| Endometrial carcinoma| 41               | 21      | 20      |
| Normal endometrium   | 30               | 0       | 30      | .000   |
| Tumor type           |                  |         |
| Endometrioid carcinoma| 21              | 7       | 14      |
| Serous carcinoma     | 20               | 14      | 6       | .019   |
| FIGO grade           |                  |         |
| Low                  | 16               | 2       | 14      |
| High                 | 5                | 5       | 0       | .001   |
| FIGO stage           |                  |         |
| I + II               | 29               | 13      | 16      |
| III + IV             | 12               | 8       | 4       | .203   |
| Invasive depth       |                  |         |
| <1/2                 | 21               | 9       | 12      |
| ≥1/2                 | 20               | 12      | 8       | .272   |
| Lymphovascular invasion|              |         |
| Negative             | 24               | 11      | 13      |
| Positive             | 17               | 10      | 7       | .412   |
| Lymph node metastasis|                 |         |
| Negative             | 32               | 16      | 16      |
| Positive             | 9                | 5       | 4       | .768   |
| Ki-67 index          |                  |         |
| Low expression       | 20               | 10      | 10      |
| High expression      | 21               | 11      | 10      | .879   |

Abbreviations: Negative, FISH fluorescence intensity ≤30; Positive, FISH fluorescence intensity >30.

3 | RESULTS

3.1 Long noncoding RNA EIF1AX-AS1 expression is decreased in human EC tissues and associated with tumor type

Long noncoding RNAs play key roles in tumor development. To identify lncRNAs that might play important roles in EC, we undertook RNA-seq analysis using EC and adjacent normal tissues. The results identified 1169 upregulated and 1841 downregulated lncRNAs, and the top 42 differentially expressed IncRNAs were depicted by a heatmap (Figure 1A). To validate the RNA-seq results, we randomly selected four downregulated IncRNAs with greater than 3-fold change (P < .01) and examined their expression in EC by RT-qPCR. Among the four IncRNAs, EIF1AX-AS1 (transcript accession ENST00000424026) was the most decreased lncRNA in EC compared with adjacent normal tissues (Figure S1A). Downregulation of EIF1AX-AS1 was further verified in the others EC and the HEC-1A and RL95-2 cell lines (Figures 1B and S1B). Thus, these two cell lines were chosen for subsequent experiments.

We next evaluated the expression level of lncRNA EIF1AX-AS1 in 40 pairs of EC tissues and adjacent normal tissues. Most tumor tissues (32/40) showed lower levels of IncRNA EIF1AX-AS1 compared with the adjacent normal tissues (Figures 1C and S1C). Moreover, increased IncRNA EIF1AX-AS1 was positively related to tumor type (P = .001), FIGO grade (P = .001), FIGO stage (P = 0.203), and invasive depth (P = 0.272; Table 1).

The RACE assay was used to identify the full sequence of EIF1AX-AS1 in HEC-1A cells according to the primer sequence archived in the database of LNCipedia (https://lncipedia.org/). The results showed that EIF1AX-AS1 is derived from the intron region between exons 1 and 2 of the EIF1AX gene (chrX: 20139547–20141012 reverse strand) (Figure 1D, E). In addition, the potential protein-coding capacity of IncRNA EIF1AX-AS1 was predicted by the online prediction software ORF finder, which searches for ORFs (https://www.ncbi.nlm.nih.gov/orffinder/), and RNA CPAT (http://ilabresearch.bcm.edu/cpat/index.php). The possible coding sequences of EIF1A-AS1, predicted by ORF finder and CPAT, were constructed on the EGFP fluorescent labeled vector, and the protein expression level was detected by immunofluorescence. Compared with the positive control (GAPDH ORF), the fluorescence signal of

2.17 Statistical analysis

Statistical analyses were undertaken using SPSS 20.0 statistical software (IBM) and GraphPad Prism 7.0 (GraphPad Software). Statistical tests used were the t test, one-way ANOVA, and χ² test. P values of less than .05 were considered to be significant.
**Figure 2** EIF1AX-AS1 knockdown suppressed proliferation activities and facilitates apoptosis of endometrial cancer (EC) cells in vitro and in vivo. A, CCK-8 detected the cell proliferation of EC cells at the indicated times. One-way ANOVA: *P < .05; **P < .01. B-D, After EIF1AX-AS1 overexpression or knockdown, apoptosis of HEC-1A and RL95-2 cells was detected by annexin V-FITC and JC-1, respectively. t test: ns, P > .05. **P < .01. E, Nude mice were given xenographs of EIF1AX-AS1 overexpression (LV-EIF1AX-AS1) and control HEC-1A cells (LV-NC) (5 × 10⁶ cells per site). Tumors were dissected and photographed after approximately 4 weeks (n = 5 per group). F, Growth curves of EIF1AX-AS1 overexpression (LV-EIF1AX-AS1) tumors compared to control HEC-1A tumors (LV-NC). One-way ANOVA: **P < .01; ***P < .001. G, Tumor weights were measured after tumor removal. t test: **P < .01. Scale bar = 100 μm. Bars indicate SD.

**Figure 3** EIF1AX-AS1 maintains EIF1AX mRNA instability. A, B, Expression of EIF1AX after EIF1AX-AS1 overexpression vector transfection. Antisense RNA (pcDNA3.1-EIF1AX-AS1 RCS) was used as the negative control. One-way ANOVA: ns, P > .05; *P < .05. C, D, EIF1AX expression after EIF1AX-AS1 knockdown. t test: ***P < .001. E, F, RNA stability assays were undertaken in EC cell lines using actinomycin D (ActD) to disrupt RNA synthesis, and the degradation rate of the EIF1AX mRNA was measured and calculated over 12 h. One-way ANOVA: *P < .05; **P < .01. G, RNA FISH analysis of EIF1AX mRNA (green) and EIF1AX-AS1 (red) in RL95-2 and HEC-1A cells. Far right images show the colocalization of signals between the red signal (EIF1AX-AS1) and the green signal (EIF1AX). Scale bar = 100 μm. Bars indicate SD.
the EIF1A-AS1 ORF group was not detected (Figure 1F), indicating that IncRNA EIF1AX-AS1 was a noncoding RNA. Analysis using RNA FISH revealed that IncRNA EIF1AX-AS1 was mainly located in the cytoplasm of HEC-1A and RL95-2 cells and in cells in clinical EC tissues (Figures 1G,H and S1D).

### 3.2 Long noncoding RNA EIF1AX-AS1 suppresses proliferation and increases apoptosis of EC cells

Based on the association between IncRNA EIF1AX-AS1 and EC prognosis, we next explored the biological role of EIF1AX-AS1 in EC cells in vitro and in vivo. Overexpression of EIF1AX-AS1 significantly impaired the proliferative capacity of HEC-1A and RL95-2 cells (P < .05), as determined by CCK-8 cell proliferation assays (Figures 2A and S2A). However, flow cytometry and EdU assays indicated that the cell cycle and proliferation ability of EC cells was not changed after EIF1AX-AS1 overexpression (Figure S2C,D). Annexin V-FITC and JC-1 staining showed that EIF1AX-AS1 overexpression induced apoptotic cell death compared with the pcDNA3.1 vector group (Figure 2B-D). Collectively, these results indicate that overexpression of EIF1AX-AS1 induced apoptotic cell death and impaired EC cell proliferation but had no influence on cell cycle progression. However, cell cycle progression, apoptosis, and proliferation of EC cells were not changed in cells with EIF1AX-AS1 knockdown compared with the negative siRNA group (Figure S2B,E,F).

To investigate how EIF1AX-AS1 impacts the proliferation of EC cells in vivo, we constructed HEC-1A stable cell lines with EIF1AX-AS1 overexpression mediated by lentivirus (LV-EIF1AX-AS1) (Figure S2G). EIF1AX-AS1 overexpression or control HEC-1A cells were subcutaneously injected into nude mice, and mice were monitored for several weeks. We found that tumor volumes and weights were smaller in the LV-EIF1AX-AS1 group compared with controls (Figures 2E-G and S2H). Together, these findings indicate that IncRNA EIF1AX-AS1 suppresses EC cell proliferation in vitro and in vivo.

### 3.3 EIF1AX-AS1 induces EIF1AX mRNA instability

It was reported that EIF1AX could participate in tumorigenesis. Given the sequence complementarity of EIF1AX with EIF1AX-AS1, we next explored the relationship between their expression levels. Results showed that EIF1AX expression was diminished after EIF1AX-AS1 overexpression in HEC-1A and RL95-2 cells (Figure 3A,B), whereas EIF1AX expression was notably increased when EIF1AX-AS1 was knocked down (Figure 3C,D).

Based on the negative correlation between the expression of IncRNA EIF1AX-AS1 and EIF1AX and previous reports on the relationship between IncRNAs and their complementary mRNAs, we speculated that EIF1AX-AS1 might affect EIF1AX expression by regulating EIF1AX mRNA stability in EC. We observed decreased mRNA levels of EIF1AX after EIF1AX-AS1 overexpression in the presence of actinomycin D, a transcriptional inhibitor, indicating that EIF1AX mRNA stability decreased after EIF1AX-AS1 was overexpressed (Figure 3E). This effect was most significant after 8 hours. Furthermore, loss of EIF1AX-AS1 increased EIF1AX mRNA stability (Figure 3F). Results from RNA FISH analysis indicated that EIF1AX-AS1 colocalized with EIF1AX mRNA in the cytoplasm (Figure 3G). These results indicate that EIF1AX-AS1 regulates EIF1AX mRNA destabilization in EC cells.

### 3.4 EIF1AX-AS1 mediates EIF1AX mRNA instability by binding to PCBP1

We next examined the mechanism by which EIF1AX-AS1 affects EIF1AX mRNA stability. Our data showing that EIF1AX-AS1 was mainly located in the cytoplasm of HEC-1A and RL95-2 cells (Figure 1G) suggested that EIF1AX-AS1 could act as a scaffold to posttranscriptionally regulate EIF1AX mRNA by directly interacting with specific RBPs, as observed with other IncRNAs. Thus, we used catRAPID (http://service.tagtalgialab.com) to estimate potential protein-RNA pairs. We found that HNRNPQ, HNRNPD, and PCBP1 were potential RBPs that might interact with EIF1AX-AS1.
Poly C binding protein 1 belongs to the hnRNP family that contains three hnRNP KH structural domains for binding to specific elements of target mRNAs with AU-rich elements or U-rich elements located in 3'-UTRs and 5'-UTRs and regulates gene expression. To further determine the relationship between EIF1AX, EIF1AX-AS1, and PCBP1, we used the RNA-protein interaction prediction website RPISeq (http://pridb.gdcb.iastate.edu/RPISeq/). The results suggested a potential interaction between EIF1AX, EIF1AX-AS1, and PCBP1 (Figure S3C).

We next undertook RNA pull-down with PCBP1 Abs to evaluate the interaction between EIF1AX-AS1 with PCBP1. As shown in Figure 4A(B), PCBP1 was coprecipitated with synthesized sense EIF1AX-AS1 but not the antisense EIF1AX-AS1 in HEC-1A and RL95-2 cells. RNA immunoprecipitation assay was then carried out using an Ab directly against PCBP1. A significant enrichment of EIF1AX-AS1 or EIF1AX with PCBP1 was identified, and EIF1AX-AS1 knockdown reduced the interaction between PCBP1 and EIF1AX mRNA in HEC-1A and RL95-2 cells (Figure 4C,D).

To determine the regions required for the interactions between EIF1AX, EIF1AX-AS1, and PCBP1, we used RIP assays and found that one of three regions (842-901 nt) of EIF1AX-AS1, predicted by catRAPID, was required for its interaction with PCBP1. We further found that the 805-933 nt region of EIF1AX, but not the region predicted by catRAPID, was required for association with PCBP1 in HEC-1A cells (Figures 4E and S3D,E). These results further illustrate the close regulatory relationship among EIF1AX, EIF1AX-AS1, and PCBP1.

Finally, we examined the effect of PCBP1 on EIF1AX. Downregulation of PCBP1 increased EIF1AX expression in HEC-1A and RL95-2 cells, whereas forced expression PCBP1 decreased EIF1AX expression in HEC-1A and RL95-2 cells (Figures 4F,G and S3F,G). Moreover, results of actinomycin D treatment recommend that forced expression of PCBP1 decreased EIF1AX mRNA levels and facilitated its destabilization (Figure 4H), whereas loss of PCBP1 increased EIF1AX mRNA levels and impeded its destabilization. Additionally, knockdown of EIF1AX-AS1 abrogated the induction of EIF1AX mRNA destabilization caused by PCBP1 overexpression (Figure 4I), and EIF1AX-AS1 overexpression could not eliminate the effect of PCBP1 knockdown on EIF1AX mRNA (Figure 4I), consistent with western blot results (Figure 4K). Based on these results, we conclude that EIF1AX-AS1 causes the destabilization of EIF1AX mRNA by binding to PCBP1.

3.5 EIF1AX-AS1 promotes EC cell apoptosis by downregulating EIF1AX expression

To determine whether EIF1AX-AS1 promotes EC cell apoptosis by regulating EIF1AX expression, we explored the roles of EIF1AX-AS1 and EIF1AX in the proliferation and apoptosis of EC cells. EIF1AX expression abrogated the proliferative capacity of EC cells impaired by EIF1AX-AS1 overexpression, as determined by CCK-8 proliferation assays (Figures S5A and S4A). Annexin V staining assay further showed that EIF1AX overexpression abrogated the apoptotic cell death induced by EIF1AX-AS1 forced expression compared with controls (Figure 5B,C). Both JC-1 and western blot assays further confirmed that EIF1AX overexpression reduced EC cell apoptosis caused by EIF1AX-AS1 overexpression compared with controls (Figures 5D and S4B). These results indicate that EIF1AX-AS1 facilitates EC cell apoptosis by impaired EIF1AX expression, rather than through the cell cycle (Figure S4C,D).

In addition, RT-qPCR and western blot results also showed that overexpression of EIF1AX-AS1 lacking a binding site for PCBP1 (842-901 nt) could not promote EIF1AX mRNA degradation (Figure S5A,B). Compared with the pcDNA3.1-EIF1AX-AS1 group, cell proliferation and cell apoptosis were restored in the pcDNA3.1-EIF1AX-AS1 mut group (Figure S5C-E). RNA pulldown also indicated that EIF1AX mRNA lacking the binding site (805-933 nt) could not combine with PCBP1 (Figure S5F).

We next examined the relation between EIF1AX-AS1 and EIF1AX in EC in vivo. We subcutaneously injected EIF1AX-AS1 overexpression cells or EIF1AX-AS1 and EIF1AX co-overexpression cells into nude mice. After several weeks of observation, we found that tumor volumes and weights were lower in the LV-EIF1AX-AS1 group compared with the other groups (Figure 5E,F). Taken together, these findings indicate that IncRNA EIF1AX-AS1 suppresses the impaired EC cell proliferation caused by EIF1AX in vitro and in vivo.

3.6 EIF1AX increases c-Myc translation by binding YBX-1

To further examine how EIF1AX regulates apoptosis in EC, we undertook coimmunoprecipitation experiments followed by liquid chromatography-tandem mass spectrometry to identify proteins associated with EIF1AX. Significant interactors compared with the IgG control were analyzed by using GO and the KEGG pathway database. Interestingly, except for "protein function about translation initiation", "ribosomal complex", and "regulation of mRNA binding YBX-1".
stability”, GO terms associated with “internal ribosomal entry sites (IRESs)”, caught our attention among these protein biological processes (Figure S6A,B). It has reported that IRES could serve as an alternative mechanism for protein production. Our results identified YBX-1 as a potential binding protein to EIF1AX (50-117 aa) (Figure S6C,D). Previous studies showed that YBX-1 functions as an
IRES trans-acting factor that upregulates c-Myc expression,\textsuperscript{14} and c-Myc is closely associated with apoptosis.\textsuperscript{13} We thus hypothesized that EIF1AX binds to YBX-1 as an IRES trans-acting factor resulting in IRES-mediated translation of c-Myc, which results in apoptosis in EC cells.

We next investigated whether EIF1AX influenced the translation of c-Myc by binding to YBX-1. We confirm that downregulating either EIF1AX or YBX-1 in EC cells resulted in a decrease in c-Myc protein expression levels but not c-Myc mRNA levels (Figure S6E,F). Moreover, the results indicate that knockdown of YBX-1 partially rescued the effect of forced expression of EIF1AX on the expression of c-Myc, BCL-2, and BAX (Figure S6G). Taken together, these results indicate that EIF1AX could regulate c-Myc expression at the translational level rather than the transcriptional level, and EIF1AX increases the translation of c-Myc by binding to YBX-1, affecting apoptosis in EC cells.

To further confirm the hypothesis that EIF1AX regulates c-Myc expression at the translational level, we undertook experiments in EIF1AX forced expression or knockdown cells that were pretreated with the protein synthesis inhibitor CHX. As shown in Figure S6H,I, decreased protein levels of c-Myc were observed in EIF1AX silenced cells that were pretreated with CHX and increased protein levels of c-Myc were observed in cells with EIF1AX forced expression after treatment with CHX. This effect was most significant after 30 minutes, indicating that the translation of c-Myc was likely affected by EIF1AX. To exclude the possibility that EIF1AX affects c-Myc expression through proteasome-dependent degradation, we carried out experiments using the proteasome inhibitor MG132. We confirmed that MG132 treatment resulted in no detectable change in c-Myc protein levels in the EIF1AX forced expression or knockdown cells (Figure S6J,K). Together, these results show that EIF1AX increases c-Myc protein levels independent of degradation or post-translational control.

We next examined whether c-Myc was closely associated with apoptosis in EC cells. Loss of c-Myc increased BAX expression and decreased BCL-2 expression in HEC-1A and RL95-2 cells, whereas forced expression c-Myc decreased BAX expression and increased BCL-2 expression in HEC-1A and RL95-2 cells (Figure S7A-C). Taken together, these results implied that EIF1AX increases the translation of c-Myc by binding to YBX-1, affecting apoptosis in EC cells. Interestingly, the results of ChIP-seq, ChIP-qPCR, and luciferase reporter assay found that the region from -235 to 177 bp contains essential components required for maximal promoter activity, and c-Myc significantly promote EIF1AX promoter activity in this region in EC cells (Figure S7D-G). c-Myc could bind to the EIF1AX promoter and promote the transcription of EIF1AX, establishing a positive feed-forward (feedback) loop.

4 DISCUSSION

Endometrial cancer has continued to show increasing incidence and diagnosis rates.\textsuperscript{16} Recent studies have shown the utility of IncRNAs as potential tumor markers and new targets for therapy of various cancers.\textsuperscript{17} Long noncoding RNAs regulate gene expression and protein function through a variety of molecular mechanisms, with roles in transcriptional interference, RNA splicing, and miRNA deactivation, as well as through direct interactions with transcription factors and RBPs.\textsuperscript{18} Previous studies have revealed key roles for IncRNAs in EC. For example, IncRNA DLEU1 binds to mTOR and increases the expression of the PI3K/AKT/mTOR pathway to promote tumorigenesis and progression of EC.\textsuperscript{19} Therefore, screening differentially expressed IncRNAs in EC and paracancerous tissues by RNA-seq could provide new ideas for the treatment of this tumor. In the current study, we found that the IncRNA EIF1AX-AS1 was expressed at lower levels in EC than in adjacent benign tissue and was correlated with poor survival of EC patients. Overexpression of EIF1AX-AS1 led to a decrease in EIF1AX expression level, accompanied by suppressed proliferation in EC cells. These findings could provide new insights into EC.

EIF1AX promotes the formation of the ternary complex of eIF2/ GTP/Met-tRNA, which binds to the 40S ribosomal subunit to form the 43S preinitiation complex and works with EIF1 to promote ribosome scanning and initiation codon selection to initiate protein translation.\textsuperscript{20,21} Kaplan-Meier survival analysis of 5143 breast cancer cases revealed that elevated EIF1AX expression was positively associated with poor survival in breast cancer patients.\textsuperscript{10} In addition, EIF1AX mutations can lead to the development of uveal melanoma.\textsuperscript{22} These results suggest that EIF1AX is not only involved in protein translation in tumor cells, but might also be closely related to tumor proliferation and migration. Krishnamoorthy et al reported that EIF1AX mutations were accompanied by changes in c-Myc expression in uveal melanoma.\textsuperscript{23} In the present study, decrease of c-Myc protein expression induced EC cell apoptosis after EIF1AX knockdown (Figure S6E,F). Yu et al reported that c-Myc expression was reduced in endometrioid adenocarcinoma and was accompanied by increased expression of caspase-3.\textsuperscript{24} Our data also showed that knockdown of c-Myc expression led to a significant increase in BAX expression, accompanied by a decrease in BCL-2 expression (Figure S7A-C). It suggests that EIF1AX could affect apoptosis through c-Myc in EC cells (Figure 6).

Interestingly, we found that EIF1AX interacts with YBX-1 to promote c-Myc translation through the IRES pathway (Figure S6). Most eukaryotic mRNAs are translated in a cap-dependent fashion. However, under oxygen deprivation and nutrient limitation conditions, the cap-independent translation driven by IRES can serve as an alternative mechanism for protein production.\textsuperscript{25,26} In addition, this mechanism could be exploited by tumor cells to continuously promote their proliferation. EIF1AX also interacts with other IRES-related proteins in addition to YBX-1. We anticipate that future studies will identify other IRES-related proteins that are closely associated with EIF1AX-regulated apoptosis.

Our RIP assay results showed that PCBP1 binds EIF1AX mRNA. Poly C binding protein 1 is an RBP and member of the hnRNP family, which contains three KH structural domains that are essential for binding RNA.\textsuperscript{27,28} Poly C binding protein 1 plays an important role in
mRNA stabilization, translation activation, and translation silencing. Studies have shown that PCBP1 binds AU-rich elements or U-rich elements in the 3′-UTR of target mRNAs to regulate their expression. In addition, PCBP1 deletion can lead to tumorigenesis.

THAP11 regulates CD44 v6 expression through interacting with PCBP1 to inhibit CD44 v6 expression and cell invasion in liver cancer cell lines. Transforming growth factor-β promotes epithelial-to-mesenchymal transition and stemness of prostate cancer cells by inducing PCBP1 degradation.

Our results showed that PCBP1 interacts with both EIF1AX mRNA and EIF1AX-AS1 to promote EC cell apoptosis by promoting EIF1AX mRNA degradation (Figures 4C,D and 6). Notably, Shi et al reported that PCBP1 depletion promotes tumorigenesis through attenuating the length of p27 mRNA stability. Zhang et al reported that PCBP1 regulates p62/SQSTM1 mRNA stability, and we confirm that PCBP1 regulates the stability of EIF1AX mRNA (Figure 6). Insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) acts as an adaptor protein that recruits the CCR4-NOT complex and thereby initiates the degradation of HULC. Zhang et al reported that IGF2BP1 recognizes m6A sites in the 3′-UTR of paternally expressed gene 10 (PEG10) mRNA to enhance its stability, suggesting that RBPs perform different functions in different cell lines. Future investigations are required to elucidate how PCBP1 affects the degradation of EIF1AX mRNA.

Long noncoding RNAs could originate from different regions of the genome, including the sense or antisense strands of protein-coding genes, intron or exon regions of protein-coding genes, or even as independent transcripts within and outside of protein-coding genes. EIF1AX-AS1 is an antisense lncRNA derived from the intron region of EIF1AX. In NCBI and the LNCipedia database, the full length EIF1AX-AS1 is 378 nt and includes two exons (exon 1, 268 nt; exon 2, 110 nt) and one intron (99 nt) between the exons. In this study, the full length of EIF1AX-AS1 was 1466 nt and no intron (Figure 1E). One possible reason for these differences is the use of different cell lines, which might result in different splice variants of lncRNA. For example, Li et al reported that the length of ZEB1-AS1 is 2449 nt and Su et al reported that its length is 2535 nt; both lengths were different from the NCBI database (2568 nt). Although several lncRNAs have been functionally annotated in the LNCipedia database, most lncRNAs remain uncharacterized. This study provides a new perspective about lncRNAs and bioinformatics databases.

ACKNOWLEDGMENTS
The work was supported by the Science and Technology Innovation Foundation of Fujian Province (2017Y9114) and Startup Fund for Scientific Research of Fujian Medical University (2018QH2016). We thank the staff of the public technical service center of Fujian Medical University for technical assistance.

DISCLOSURE
None of the authors have any conflict of interest to declare.
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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Lv C, Sun J, Ye Y, et al. Long noncoding RNA EIF1AX-AS1 promotes endometrial cancer cell apoptosis by affecting EIF1AX mRNA stabilization. Cancer Sci. 2022;113:1277-1291. doi:10.1111/cas.15275