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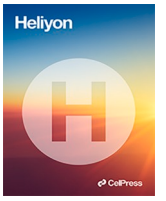

Research article

The circZEB1/miR-337–3p/*OGT* axis mediates angiogenesis and metastasis via O-GlcNAcylation and up-regulating *YBX1* in breast cancer

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ABSTRACT

Background: A growing corpus of research has revealed that circular RNAs (circRNAs) have become increasingly important for the growth of malignancies in recent years. CircRNAs as ideal candidates for breast cancer (BC) therapeutic targets is still absent.

Methods: In our study, the dysregulated circRNAs in BC progression were explored, we analysed the BC's circRNA expression profiles using publicly available datasets (GSE101124 and GSE101122). The expression of circZEB1 in BC and cell lines was investigated by qPCR. RNase and actinomycin D were used to examine the features of circZEB1. The function of circZEB1 was subsequently investigated through the utilisation of colony formation, tube formation, transwell assays, and xenograft animal models.

RNA immunoprecipitation (RIP), luciferase reporter assays, immunoprecipitation (co-IP) test in conjunction with LC-MS, and ChIP-seq assay to investigate the molecular mechanism underlying the biological activity of circZEB1 in BC.

Results: Among the circRNAs, we were particularly interested in hsa_circ_0000228, which is spliced from the oncogene *ZEB1*. In BC cell lines, CircZEB1 expression was upregulated. CircZEB1 knockdown prevented BC cells from migrating and invading, as well as HUVECs from forming tubes and developing. By sponging miR-337–3p, functional testing revealed that circZEB1 promoted O-GlcNAcylation, increased *YBX1*, and *OGT* expression. Moreover, circZEB1 overexpression is reversible, in contrast to *YBX1* knockdown, which mostly results in the downregulation of multiple oncogenes.

Conclusion: Our study indicate that circZEB1 had oncogenic function in BC by focusing on circZEB1/miR-337–3p/*OGT* and *YBX1*. It might be inferred that circZEB1 could be a promising new target for BC treatment.

Abbreviations: SLE, systemic lupus erythematosus; FBS, foetal bovine serum; PBS, phosphate buffered saline; ChIP, chromatin immunoprecipitation; PVDF, polyvinylidene difluoride.

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1. Introduction

Breast cancer (BC) has exceeded all other malignancies in terms of prevalence worldwide, according to the most recent estimates on cancer burden for 2020 [1]. BC typically affects women between the ages of 40 and 60 and typically occurs around menopause [2]. In recent years, the illness has tended to present at younger ages, and women over 25 are seeing an increase in occurrence [3,4]. In the last 20 years, research on BC has helped us better understand the illness and developed a number of treatment choices, such as targeted therapy, surgery, chemotherapy, and so forth. Notwithstanding the remarkable advances made possible by these BC trials, early detection, diagnosis and treatment remain critical therapeutic tenets. Therefore, further understanding of the genetic characteristics is necessary to identify potential therapeutic targets for BC.

A class of noncoding RNA molecules known as circular RNAs (circRNAs) are results from exon skipping or back-splicing of precursor mRNA, unlike the more common linear RNA molecules [5]. CircRNAs have attracted significant interest in molecular biology and genomics for their exceptional stability and evolutionary conservation. Their remarkable properties make them promising biomarkers for various biological processes [6]. CircRNAs are becoming more well acknowledged as essential controllers of gene expression and cellular functions [7]. CircRNAs have been linked to numerous cancers, including colorectal, lung, breast, and cervical cancers, as well as other tumors, according to earlier research [8–11]. Li et al. demonstrated that circNDUFB2 controlled the ubiquitination and degradation of proteins and triggering antitumor immune responses in non-small cell lung cancer [12]. Another study found that the *E2F1*- and *EIF4A3*-mediated circSEPT9/miR-637/*LIF* axis enhances the establishment of triple-negative breast cancer [13]. However, with BC, the circRNAs underlying chemical process is still unknown. Therefore, more research needs to be done to provide more accurate and complete information about practical molecular targets for BC.

O- glycosylation modification is among the posttranslational changes that have been investigated the most of proteins and is important for numerous biological activities [14]. Studies have indicated that O-glycosylation is a flexible and reversible process [15]. Unlike other posttranslational protein modifications, only O-glycosylation is regulated by O-glycosyltransferase (*OGT*) and O-glycosylase (*OGA*) [16]. *OGT*, an essential enzyme for the O-GlcNAcylation of proteins, can attach the O-GlcNAc group to the protein's serine or threonine hydroxyl region. It is the only enzyme capable of facilitating the glycosylation-mediated modification of proteins in mammals. To complete the modification of O-GlcNAc [17], the UDP-G1cNAc was then linked to those exposed hydroxyl groups. Recent research has demonstrated that *OGT* is important for controlling O-GlcNAc glycosylation and contributes to the emergence of cancers. For instance, past studies have shown that thyroid papillary carcinoma malignant growth is enhanced by *YAP* activation caused by *OGT*-regulated O-GlcNAcylation [18]. Li et al. claim that *OGT* activates the *JNK/c-jun/AP-1* pathway, which expedites the onset of liver cancer associated with fatty liver [19]. Additionally, research by Harri M. Itkonen et al. has demonstrated that prostate cancer cell proliferation requires strong OGT activity [20]. Several *OGT* inhibitors have entered preclinical and clinical trials. By reducing *OGT* activity, these inhibitors reduce O-GlcNAc modification of tumor cells and inhibit their proliferation and metastasis [21]. Little is known about the specific chemical process by which *OGT* controls O-GlcNAcylation in BC. In particular, it is currently unclear what role circRNA and *OGT* play in the emergence of BC at the molecular level. To determine whether circRNA and *OGT* interactions could affect tumor invasion and proliferation, we concentrated on the biological functions of OGT and circRNA in BC.

In this investigation, we demonstrated that circZEB1 was expressed at higher quantities in BC. CircZEB1 knockdown prevented BC cells from migrating and invading, as well as HUVECs from forming tubes and developing. By sponging miR-337–3p, functional testing revealed that circZEB1 promoted O-GlcNAcylation, increased *YBX1*, and boosted *OGT* expression. The significance of the circZEB1/miR-337–3p/OGT axis for BC has been established, and it presents a distinct therapeutic target for BC management.

2. Materials and methods

2.1. Bioinformatics data extraction and analysis

Annotations and sequences of circRNAs were culled from publicly available databases (GEO database and circBase). GSE101124 and GSE101122 are the circRNA expression profile data. The starBase 3.0 database indicated that circZEB1 and miR-337–3p would interact. The O-GlcNAcylation site of *YBX1* was predicted by YinOYang version 1.2.

2.2. qRT−PCR

The total RNA of the cells was extracted using the TRIzol reagent (Invitrogen, California USA). The A260/A280 ratio was then used to determine the RNA concentration and purity. The subsequent procedure, which consisted of performing reverse transcription with a Promega reverse transcription kit to generate cDNA, followed. Afterwards, we used the SYBR Green PCR Kit (Takara, Tokyo, Japan) to conduct qPCR. The endogenous control was GAPDH. A list of primers is provided in Table S1.

2.3. Cell culture and transfection

The American Type Culture Collection's MCF-10 A, MCF-7, ZR-75-1, MDA-MB-361, UACC812, MDA-MB-468, HCC-1937, MDA-MB-231, and MDA-MB-436 BC cell lines were utilized in this investigation (ATCC, Virginia, USA). Cell lines for breast cancer, ZR-75-1, MDA-MB-361, MDA-MB-231, and MDA-MB-436, were grown in DMEM-F12 media (Thermo, California, USA), with 10 % FBS (Clark Bioscience, Virginia, USA) added as a supplement.

MCF-7and MCF-10 A cells were cultivated in MEM (Thermo, California, USA). MDA-MB-468 and UACC812 cells were grown in L-

15 media (Thermo Fisher Scientific, California, USA). HUVECs were maintained in basal mammary epithelial cell media (Cambrex, USA). Every one of these cells was kept at 37 °C with 5 % CO_2 at all times. Cycloheximide (CHX) (MCE, China), a protein synthesis inhibitor, was incubated with BC cells for the indicated times to determine protein stability.

Using LipofectamineTM 2000 Transfection Reagent (Invitrogen, California,USA), short interfering RNA (RiboBio, Guangzhou, China) was transfected into BC cells. To induce circZEB1 overexpression, we followed the procedures outlined by the manufacturer of the pLCDH-ciR (Geenseed Biotech Co., China).

2.4. Colony formation assay

A 6-well plate was used, and 1000 cells were planted into each well to perform the colony formation test. These plates were incubated for 12 days, washed with PBS, fixed for 15 min in 75 % ethanol, and then stained.

2.5. Migration and invasion assay

In summary, Transwell chambers were utilized for experiments involving cell invasion or migration. A transwell (Corning, NY, USA) was used, and 1.5×10^5 BC cells were introduced to the upper chamber 24 h after transfection. Media containing 10 % FBS was then in the lower well, and then incubated for 24 h. The infiltrating cells were stained for 10 min. Finally, the cells were counted five times with a microscope.

2.6. Tube formation assay

In a nutshell, 50 μL of Matrigel (Corning, NY, USA) was put into each well of a 96-well plate. Then, 4×10^4 HUVECs were resuspended in supernatant that was taken from the circZEB1 knockdown or control group, and 37 °C was used for incubation. Each well received 300 μL of supernatant after that. Tube development was seen under a microscope 8 h later.

2.7. Xenograft animal studies

In July 2021, the Yiwu Maternity and Children Hospital's Ethical Committee evaluated and authorized the animal-related studies (grant ID: A00076).

To create our xenograft tumor model, we randomly split 10 mice into 2 groups. Then, we implanted 2×10^6 different types of MDA-MB-231 cells subcutaneously into female BALB/c mice (HFK Bioscience, Beijing, China). The mice were put to sleep after five weeks of food, and the tumors were taken out so they could be studied further. Six mice in total were randomly assigned to two groups for the lung metastasis model, and 1×10^6 cells were injected into the tail vein.

2.8. RNA immunoprecipitation (RIP) assay

The RIP assays were conducted using the Magna RIPTM RNA Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA), adhering to the manufacturer's instructions. To sum up, cells were lysed and then treated for an overnight period at 4 °C with either rabbit lgG or Ago2 antibodies (Abcam, UK). RT-qPCR was used the next day to determine the isolated RNAs. Using rabbit lgG as a negative control was employed.

2.9. Co-immunoprecipitation (Co-IP) assay

As directed by the manufacturer, co-immunoprecipitation (co-IP) tests were performed using a DynabeadsTM Co-Immunoprecipitation Kit (14321D, ThermoFisher Scientific, California, USA).

2.10. Chromatin immunoprecipitation (ChIP)-seq assay

As per the guidelines provided by the manufacturer, cells were subjected to ChIP-seq assay, as previously described [22]. Put briefly, 500 μl of lysis buffer was added to a collection of formaldehyde-immobilized cells. After that, the lysate was treated with ultrasonic technology. The supernatant was carefully combined with protein A/G magnetic beads after being diluted. Next, 5 μg of YBX1 antibody was added. Following washing, elution buffer was used to incubate the beads for 2 h the following day, and then for 10 min at 95 °C. Next, the DNA was isolated from the eluent in preparation for RT-qPCR or sequencing.

2.11. Western blotting analysis

Using RIPA buffer (Keygen Biotech, Nanjing, China), the total protein of cells was extracted. Following this, 30 μg of protein were separated by 12 % SDS-PAGE and placed onto a PVDF membrane (Millipore, USA), and than blocked for 2 h at room temperature using 5 % skim milk powder (Licor, Milan, Italy). Following blocking, membranes were incubated at 4 °C for a whole night with primary antibodies, and then with a secondary antibody that was labeled with HRP. Ultimately, chemiluminescence was used to study the bands.

2.12. Statistical analysis

GraphPad Prism 9 and SPSS 22.0 for analysis. Student's *t*-test to determine statistical significance and differences between two data groups. The analysis findings were expressed as a P value in each case (*P < 0.05; **P < 0.01; ***P < 0.001; n. s. P > 0.05).

3. Results

3.1. Identification of upregulated circRNAs in BC

To explore the dysregulated circRNAs of BC, we analysed the circRNAs profiles by publicly available datasets (GSE101124 and GSE101122). Fig. 1A displays the top 10 circRNAs that are upregulated in TNBC tissue, and Fig. 1B displays the top 10 circRNAs that are upregulated in TNBC cells (MDA-MB-231) (log2fold change>1, P value < 0.05). Among them, hsa_circ_0000228 aroused our great interest because it is upregulated in both TNBC tissues and cells (Fig. 1C). By using CircBase database annotation software, we found that hsa_circ_0000228 is spliced from the oncogene *ZEB1,* and the spliced length is 461 bp (Fig. 1D). Moreover, the correlation of *ZEB1* expression with the prognosis of BC patients was investigated by the UALCAN online dataset (based on TCGA) (Fig. S1). Next, circZEB1 and ZEB1 expression in various BC cell lines was determined. ZEB1 and circZEB1 were expressed more in TNBC cells than in luminal A and luminal B cells, as Fig. 1E illustrates. Next, the characteristics of circZEB1 in BC were analysed. For localization of circRNA, the results of the RNA fractionation assay demonstrated a predominantly cytoplasmic localization of circZEB1 (Fig. 1F). The transcription inhibitor actinomycin D and RNase R were applied to both circZEB1 and the linear mRNA ZEB1 in order to further identify the characteristics of circZEB1. CircRNA is not impacted by ribonuclease R; it can only break down linear RNA. The findings indicated that circZEB1 was more stable than linear ZEB1 since it was more resistant to actinomycin D (Fig. 1H) and RNase R (Fig. 1G). To verify that circZEB1 is circular, oligo (dT) 18 primers and random hexamer were used, as indicated in Fig. 1I. The findings demonstrated that the absence of a polyA tail in circZEB1 considerably decreased its reverse-transcription efficacy when using oligo dT primers (Fig. 1I). When combined, our findings showed that circZEB1 may be a carcinogenic molecule and a stable cytoplasmic circRNA derived from the oncogene *ZEB1*.

3.2. CircZEB1 promotes BC progression

To knockdown the expression level of circZEB1, siRNA targeting circZEB1 was constructed and further verified by qRT−PCR (Fig. S2). To further explore the molecular function of circZEB1 during BC progression, we performed multiple cell function assays to

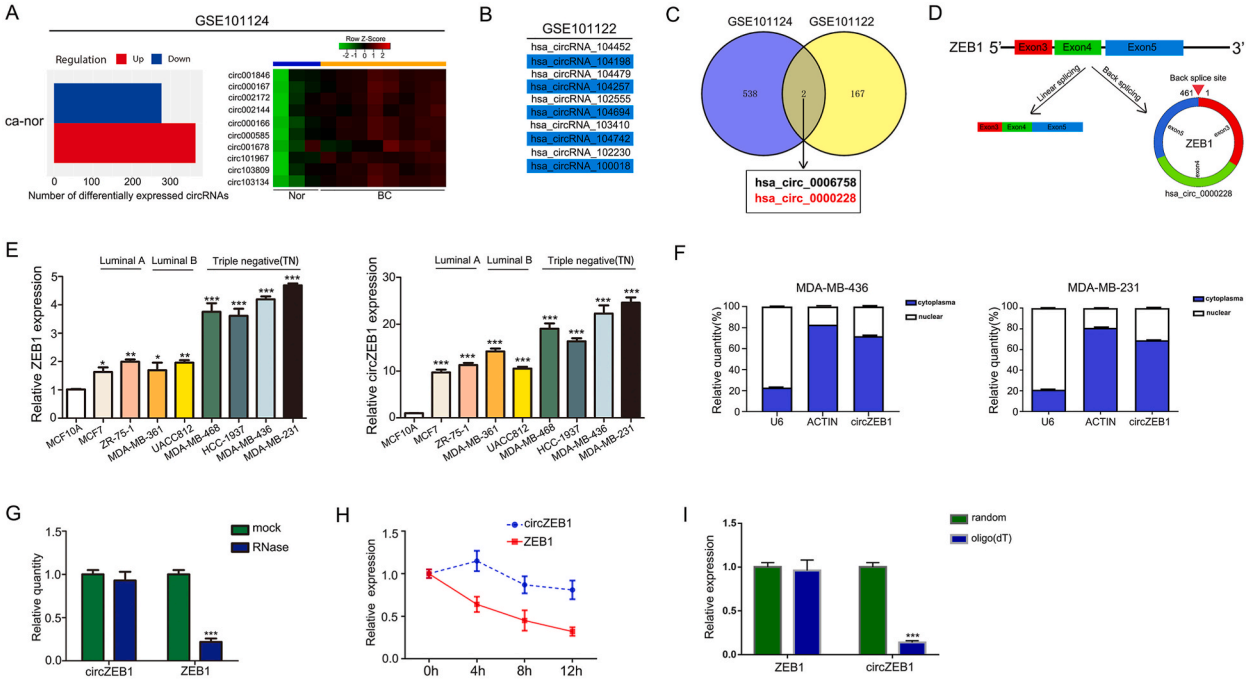

Fig. 1. The circZEB1 expression was up in BC. (A–C) Analysis of circRNAs differentially expressed in BC tissues (GSE101124) and cells (GSE101122) using the GEO database. (D) The diagram illustrates circZEB1 was generated from 3 to 5 exons of the *ZEB1* gene. (E) The ZEB1 and circZEB1 expression in BC cells was evaluated. (F) Subcellular fractionation was utilized to pinpoint the location of circZEB1 in BC cells. (G–H) The expression of circZEB1 and linear *ZEB1* mRNA were tested after treated with RNase R (G) and actinomycin D. (H). (I) The circZEB1 and linear ZEB1 mRNA expression was checked after reverse transcription with oligo (dT)18 primers.

test circZEB1 carcinogenic potency. As shown in Fig. 2A, following circZEB1 knockdown, MDA-MB-231 cells' capacity to proliferate was reduced. Assays for cell invasion and migration were used to determine how circZEB1 affected BC cell metastasis. As shown in Fig. 2B and C, circZEB1 knockdown largely decreased BC cell invasion and migration. HUVECs were used in tube formation assays to determine whether circZEB1 is involved in cell angiogenesis. The results demonstrated that when circZEB1-knockdown cells were used to condition the media for HUVEC cultivation, tube formation and growth were severely reduced (Fig. 2D). The expression of markers of EMT transition and angiogenesis in cell are shown in Fig. 2E. Next, using xenograft tests and a lung metastasis model, the impact of circZEB1 on BC cell metastasis and proliferation in vivo was assessed. The formation of tumorspheres was dramatically decreased by the deletion of circZEB1, as demonstrated by the xenograft assay (Fig. 2F). Furthermore, most mice in the circZEB1 knockdown group formed less lung metastasis than those in the NC group (Fig. 2G). In summary, our results demonstrated that circZEB1 participated in BC progression by regulating cell proliferation, metastasis and angiogenesis.

3.3. *OGT is regulated by circZEB1 as a mir-337–3p sponge*

MDA-MB-231 cells were transfected with si circZEB1 and negative control, we used next-generation sequencing (NGS) to elucidate the aberrant expression of target genes associated with TNBC metastasis and circZEB1 dysfunction, finding 1889 upregulated and 319 downregulated genes (Fig. 3A and B). Among them, as a crucial regulator in O-glycosylation modification process, *OGT* arouse our great interest. In Fig. 3C, the O-glycosylation level was decreased after circZEB1 knockdown in BC cells. Moreover, the expression of *OGT* was test after circZEB1 knockdown (Fig. 3D). Since circZEB1 is primarily found in the cytoplasm, we hypothesized that circZEB1 would function as a miRNA sponge to tangentially up the OGT mRNA level. RIP assays were run to verify the combination of circZEB1 and Ago2 protein in BC cells in order to test this theory (Fig. 3E). Four candidate miRNAs were identified as potential targets of both circZEB1 and OGT by circBase and starBase miRNA target prediction results that overlapped (Fig. 3F). By using starBase database pan-cancer analysis (based on TCGA), the miR-337–3p expression was down in BC tissues (Fig. S3). Consequently, following circZEB1 overexpression, miR-337–3p, the most downregulated miRNA, was chosen for additional examination (Fig. 3G). Furthermore, we discovered that miR-337–3p reduced cell proliferation and invasion (Fig. 3H-I). The next step involved using luciferase reporter assays to find out if circZEB1 and OGT specifically target miR-337–3p. Following their generation in dual-luciferase reporter pmiRGLO vectors, the full-length wild-type circZEB1/OGT or mutant circZEB1/OGT (MUT) were co-transfected into BC cells alongside miR-

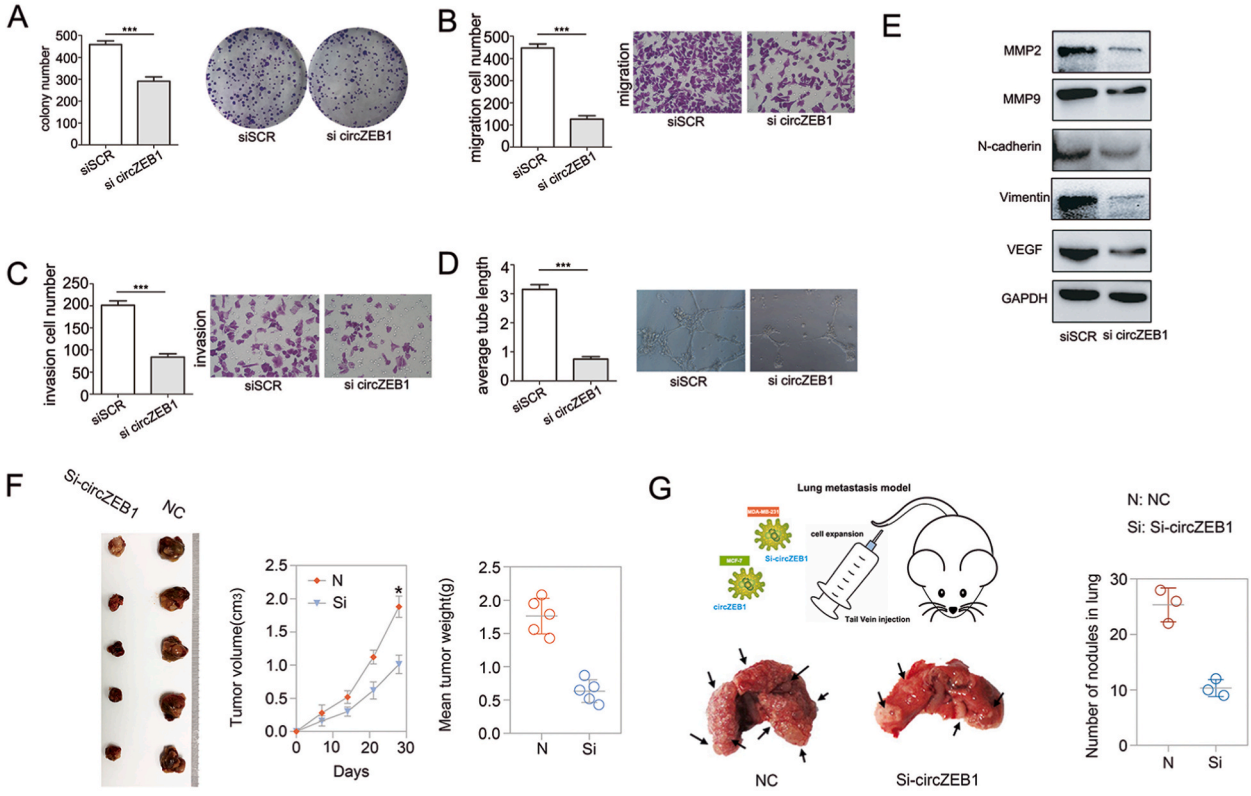

Fig. 2. circZEB1 knockdown reduced cell proliferation and invasion. (A) The effect of circZEB1 on cell proliferation was detected by colony formation assay. (B–C) The effect of circZEB1 on cell migration and invasion was measured by transwell assays. (D) The effect of circZEB1 was detected by cell angiogenesis. (E) The EMT markers protein levels were detected. (F) Subcutaneous tumor growth was used as a marker for the effect of circZEB1 on cell proliferation. (G) Using a lung metastasis model, the effect of circZEB1 on cell invasion was assessed.

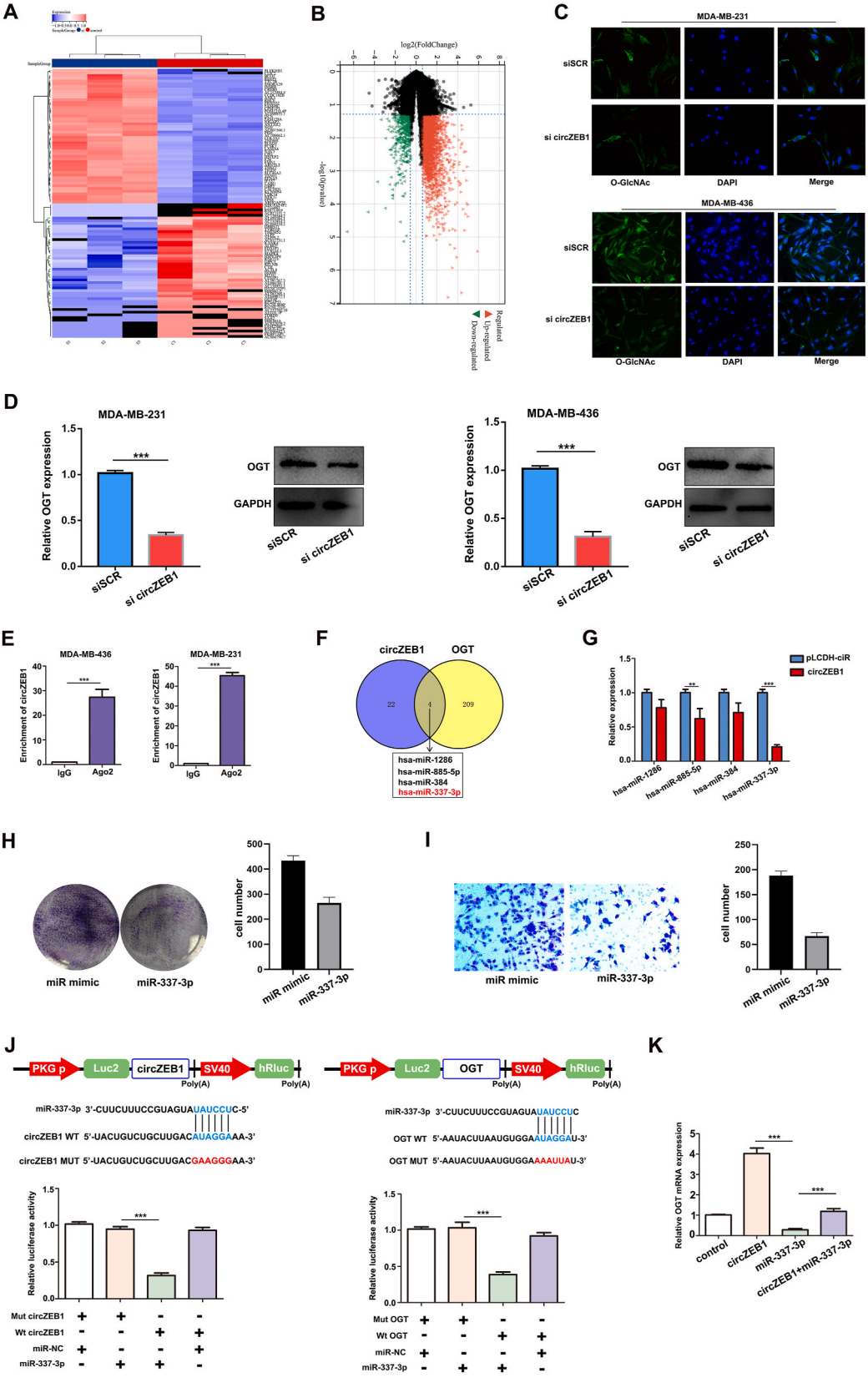

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Fig. 3. *OGT* is regulated by CircZEB1 via miR-337-3p. (A–B) The heatmap and volcano map display different genes in TNBC cells following circZEB1 knockdown. (C) The O-GlcNAcylation level was detected by Lectin staining. (D) *OGT* expression was evaluated in cells using qRT−PCR after circZEB1 was knocked down. (E) CircZEB1 and Ago2 interacted, as demonstrated by the RIP experiment. (F) The circZEB1 and *OGT*-possible-binding miRNAs were identified by the starBase and circRNA interactome databases. (G) The expression of putative miRNAs was evaluated after circZEB1 was overexpressed. (H) The effect of miR-337–3p on cell proliferation was detected. (I) The effect of miR-337–3p on cell invasion was measured by transwell assays. (J) Luciferase reporter activity was measured in TNBC cells transfected with either wild-type (WT) or mutant (Mut) circZEB1 and a miR-337–3p mimic. (K) Once circZEB1 and miR-337–3p were co-transfected, the *OGT* expression was evaluated.

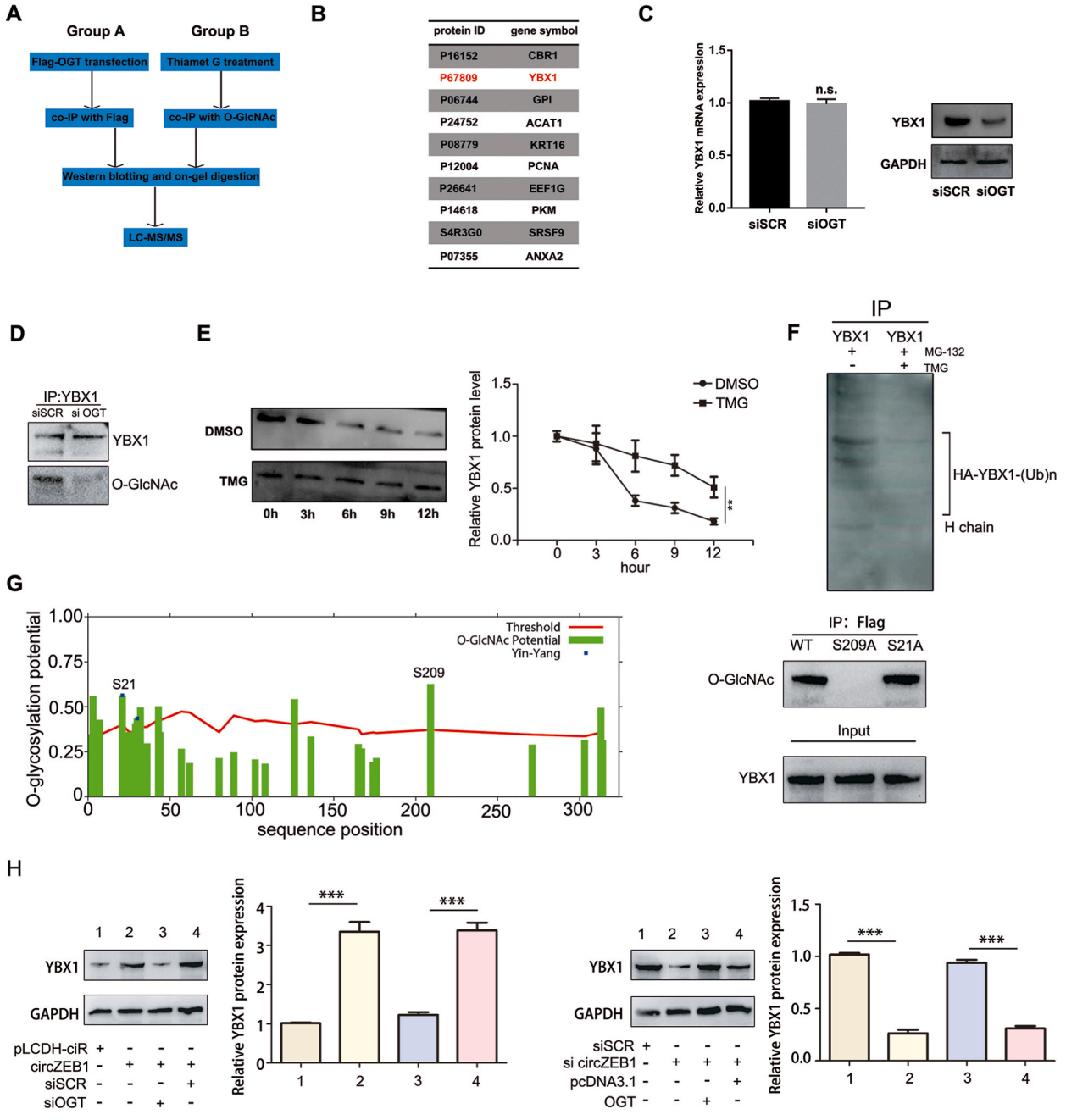

Fig. 4. *YBX1* was a molecular target of *OGT* and O-GlcNAcylation. (A–B) To determine how *OGT* interacted with the likely targets in the sample, mass spectrometry and co-IP were used. (C) The mRNA and protein level of *YBX1* after *OGT* knockdown were determined. (D) IP was performed to validate the O-GlcNAcylation of *YBX1*. (E) The level of *YBX1* protein to ascertain after TMG therapy. (F) The ubiquitylation of *YBX1* was measured by co-IP assay after TMG treatment in MDA-MB-231 cells. (G) YinOYang software predicted that *YBX1* would be O-GlcNAcylated and further validated by co-IP assay. (H) The *YBX1* expression was measured after circZEB1 and siOGT co-transfection.

337–3p mimics. Reductive luciferase reporter activity was very high in cells co-transfected with WT and miR-337–3p mimics, but not with the mutant vector (Fig. 3J). Moreover, overexpression of circZEB1 significantly increased *OGT* expression at the mRNA level, and these effects were weakened by miR-337–3p mimic transfection (Fig. 3K). Finally, our results validated that circZEB1 regulated the intracellular O-GlcNAcylation process by functioning as a miR-337–3p sponge to indirectly increase *OGT*.

3.4. *OGT regulates the O-GlcNAcylation of YBX1 in TNBC*

To further look for O-GlcNAcylated proteins in TNBC cells, we next applied a co-IP assay combined with LC−MS/MS to examine the protein that could interact with *OGT* (Fig. 4A). As shown in Fig. 4B–a novel oncogene, *YBX1,* was detected by LC−MS/MS. Additionally, we discovered that OGT affects YBX1 at the mRNA and protein levels. Results from Western blot and qRT-PCR showed that suppressing OGT significantly decreased OGT protein expression, while the mRNA level of *OGT* did not change after *OGT* knockdown (Fig. 4C). Additionally, OGT overexpression raised YBX1's protein level (Figs. S4A–4B). Moreover, to verify whether *YBX1* was modified by O-GlcNAcylation, the co-IP assay results shown that knockdown of *OGT* reduced the O-GlcNAcylation level of *YBX1* (Fig. 4D). Additionally, CHX analysis showed that blocking OGT might make YBX1 less stable and encourage its degradation (Fig. 4E), indicating that OGT maintains YBX1 stability in TNBC cells by means of O-GlcNAc. Meanwhile, the ubiquitylation of *YBX1* was decreased after TMG treatment (Fig. 4F). Next, the O-GlcNAcylation site of *YBX1* was further predicted by bioinformatics analysis and validated by co-IP assays (Fig. 4G). The result indicated that the O-GlcNAcylation site of *YBX1* was S209. We also detected the effect of circZEB1 and *OGT* on *YBX1* expression. Knockdown of *OGT* reduced the effect of circZEB1 overexpression on *YBX1* in TNBC cells (Fig. 4H). Therefore, *OGT* regulates the O-GlcNAcylation and stability of *YBX1* in TNBC.

3.5. *The circZEB1/miR-337–3p/OGT axis regulates multiple oncogene transcription processes via YBX1*

To explore the target of the circZEB1/miR-337–3p/*OGT* axis and *YBX1*, a ChIP-seq assay was performed with *YBX1*, which is a very important transcription factor (Figs. S5A–S5D). The YBX1-bound genes in MDA-MB-231 cells were linked to biological process items of tube formation, negative control of cell death, positive regulation of epithelial cell proliferation, and many other things, according to a GO analysis from the ChIP-seq data (Fig. 5A). Then, we confirmed the ChIP-seq results by ChIP−qPCR. As shown in Fig. 5B, *YBX1* was enriched in the promoters of several oncogenes, including *FZD1*, *FGF10*, *NRP1* and *HES5*. Additionally, we found that circZEB1 had an impact on the expression of FZD1, FGF10, NRP1, and HES5. As shown in Fig. 5C, the rescue experiment confirmed that circZEB1 overexpression reversed the impact of YBX1 knockdown on the expression of the aforementioned genes. In addition, as shown in

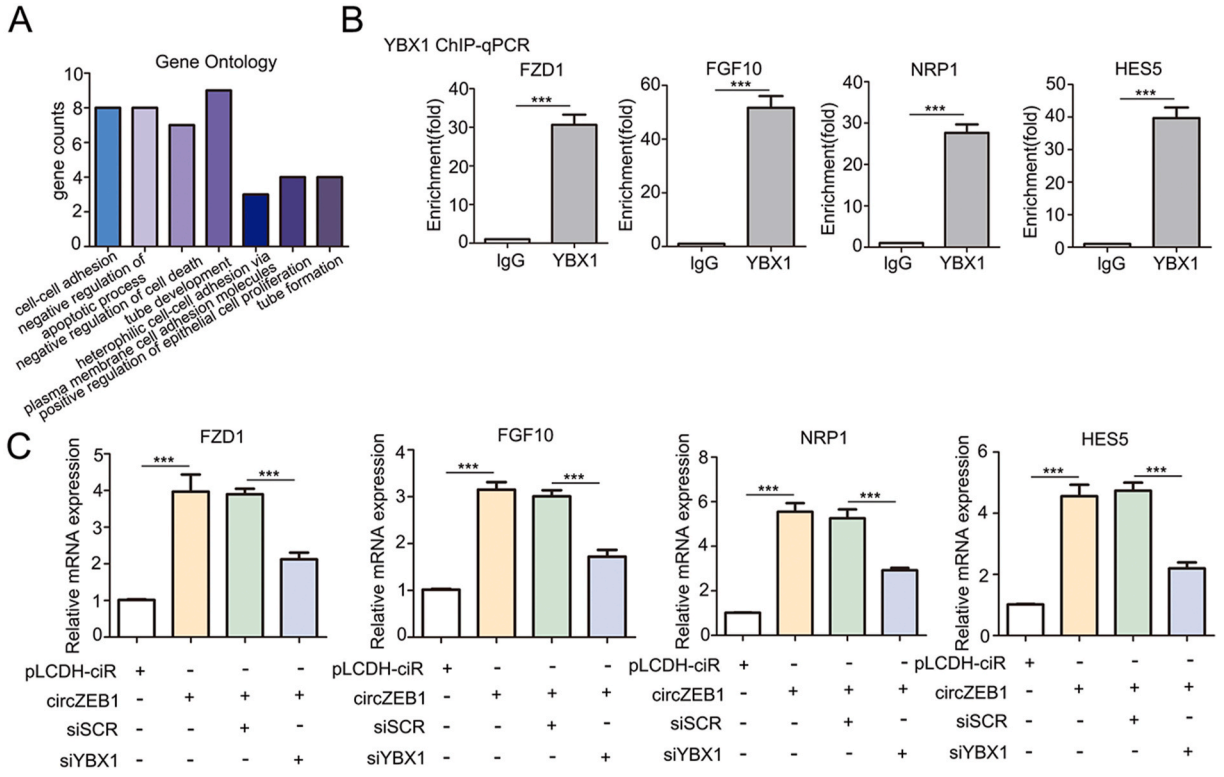

Fig. 5. The downstream target of *YBX1*. (A) The GO enrichment of ChIP-seq data was shown. (B) ChIP−qPCR was employed to validate the results of the ChIP-seq data. (C) The expression of NRP1, FGF10, HES5, and FZD1 was determined by qRT−PCR.

Fig. 6A-C, the decrease of TNBC cells proliferation and migration induced by circZEB1 knockdown was rescued by *OGT* overexpression. Meanwhile, circZEB1 overexpression also prevented si OGT's inhibitory effect on cell migration and proliferation (Figs. S6A–6B). As expected, the expression of *MMP2*, *MMP9* and *Vimentin* was also downregulated in circZEB1 knockdown cells and could be reversed by *OGT* overexpression (Fig. 6D). Moreover, these EMT markers were also up-regulated in circZEB1 overexpression cells and reversed by *OGT* knockdown (Fig. S6C). Taken together, our study demonstrated that the circZEB1/miR-337–3p/*OGT* axis regulated the stability of *YBX1* via O-GlcNAcylation, resulting in the activation of downstream targets such as *FZD1*, *FGF10*, *NRP1* and *HES5* (Fig. 7).

4. Discussion

BC is the leading cause of cancer-related death among women worldwide [23]. BC pathogenesis is a complex biological process that is triggered by the activation of multiple genes or tumor-related pathways [24]. Finding new targets for BC therapy and detection is therefore essential. CircRNAs can be used as therapeutic or diagnostic targets because they have been linked to the onset and spread of

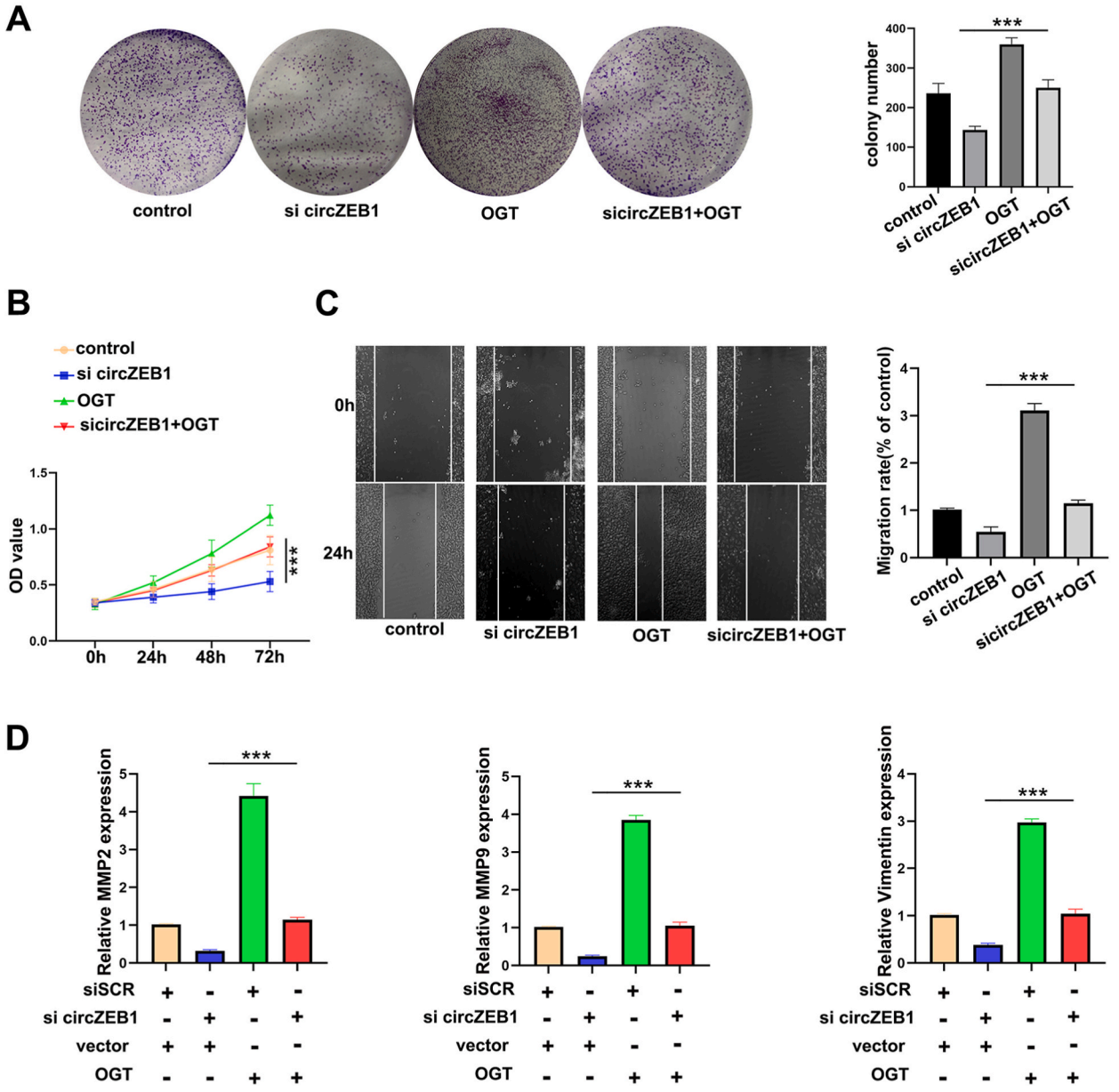

Fig. 6. CircZEB1 promoted TNBC progression via *OGT*. (A–B) The colony formation and CCK-8 assays to measure the effect of circZEB1/*OGT* on cell proliferation. (C) The migration of TNBC cells was detected after si circZEB1 and *OGT* co-transfection. (D) The MMP2, MMP9 and Vimentin expression were detected.

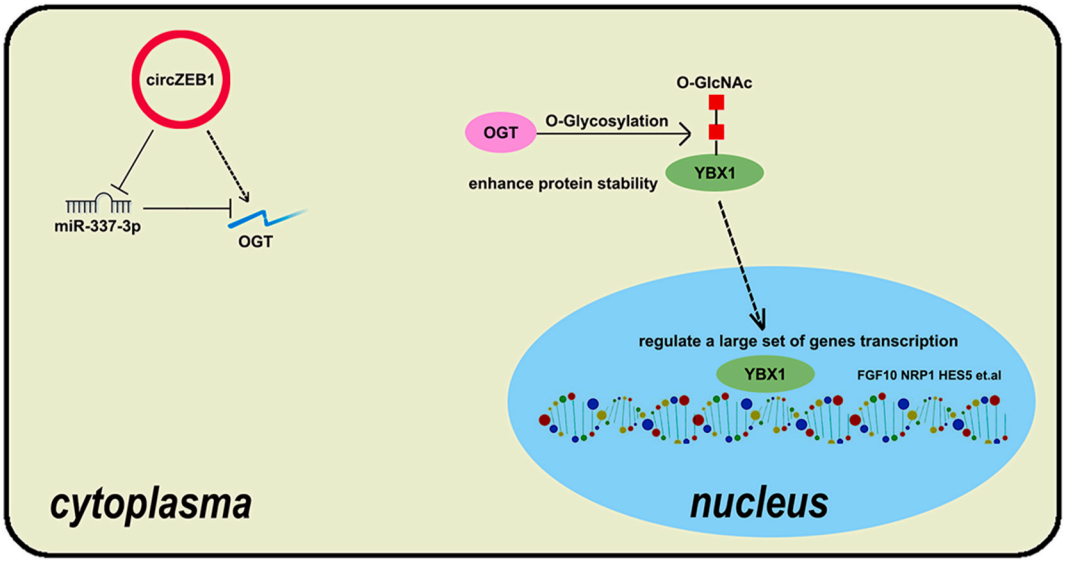

Fig. 7. The controlling function of circZEB1 is depicted in the emergence of TNBC.

a number of malignant tumors, according to earlier studies [25]. CircIKBKB has been demonstrated by Xu et al. to support the NF-κB/bone remodelling factor signaling pathway, which promotes BC metastasis [26]. According to a different study, circACTN4 activates the transcription of the proto-oncogene *MYC*, which both contribute to the spread and growth of BC [27]. CircZEB1 was one of the novel molecules that caught our attention in this study, which examined dysregulated circRNA expression in BC using the GSE101124 and GSE101122 datasets. The expression of circZEB1 was higher in TNBC cells than in luminal A and luminal B cells. To explore the function of circZEB1 during BC progression, we performed multiple cell function assays to test circZEB1 carcinogenic potency. The function of circZEB1 in stimulating the growth and metastasis of BC cells.

Several studies have demonstrated that circRNAs carry out regulatory tasks by sponging miRNAs [28]. The overregulation of circRNA-101237, which speeds up the progression of non-small cell lung cancer, targets miRNA-490–3p, claim Zhang et al. [29]. Furthermore, Yan et al. discovered that circ-104566 might function as miR-338–3p sponge to prevent hepatocellular carcinoma from progressing [30]. Here, we utilized the starBase database for miRNA target prediction. We discovered that the sole miR-337–3p that was thought to be a potential target of both circZEB1 and OGT was downregulated in BC tissues. The next step involved using luciferase reporter assays to find out if circZEB1 and OGT specifically target miR-337–3p. After a number of functional studies, miR-337–3p was also confirmed as a sponge miRNA for circZEB1. Therefore, we successfully found a circRNA-miRNA−mRNA axis, including circZEB1, miR-337–3p, and *OGT*, which might help to elucidate the relevant regulatory mechanisms of circZEB1 in BC. Here, we applied a co-IP assay combined with LC−MS/MS to examine the protein that could interact with *OGT* in TNBC cells. Our first findings suggested that OGT might have an interaction with YBX1, and bioinformatics study further predicted YBX1's O-GlcNAcylation location. The CHX test was then used to determine how O-GlcNAc affects the half-life of the *YBX1* protein. Our results provide novel evidence that O-GlcNAc increased the synthesis of the protein by reducing the rate of YBX1 breakdown.

A protein called YBX1, or YB-1, may attach itself to both DNA and RNA, and is essential for controlling the transcriptional and posttranscriptional phases of gene expression [31]. Research has demonstrated a connection between YBX1 expression and a high recurrence rate and worse prognosis in several prevalent malignancies. For instance, it has been demonstrated that YBX1 accelerates the growth of gastric cancer via taking part in the HoxC-AS3-mediated regulation of gene transcription [32]. Another investigation has shown that *YBX1* promotes renal cell carcinoma cell growth and invasion while inhibiting renal cell carcinoma cell death [33]. However, the exact mechanism by which *YBX1* contributes to BC is currently unknown. It has previously been demonstrated that kinases such p90 ribosomal S6 subunit kinase (p90RSK) can phosphorylate YBX1, which can subsequently translocate and boost transcription of genes linked to malignant tumors [34]. *AKT*, *Raf1*, *ERK*, *c-Myc*, *TGF-1*, *HER2/ErbB2*, *EGFR*, and other genes have all been identified as *YBX1* targets. It was discovered that numerous target genes, including FZD1, FGF10, NRP1, and HES5, are transcriptionally regulated by YBX1. We performed rescue experiments to learn more about the molecular targets of circZEB1/-miR-337–3p/*OGT* and *YBX1*. When *YBX1* was suppressed, the expression of *FZD1*, *FGF10*, *NRP1*, and *HES5* was downregulated. Nevertheless, overexpression of circZEB1 can make this tendency disappear. These results suggest that YBX1 is key for upregulating the transcription of FZD1, FGF10, NRP1, and HES5.

According to our research, circZEB1 could be a useful new treatment target for BC. Based on the biological characteristics of circRNAs, they are considered highly promising therapeutic targets in cancer treatment. For example: 1. CircRNAs have a closed circular shape in vivo and less prone to exonuclease destruction. Their specific expression patterns make them ideal therapeutic targets, allowing for efficient and precise cancer treatment by targeting specific circRNAs [35]. 2. CircRNAs have multiple functions and can regulate gene expression, protein functions, and cellular signaling pathways through various mechanisms. Therefore,

therapeutic strategies targeting circRNAs have broad applicability and can be used in the treatment of cancers [36]. 3. The specific expression patterns and mechanisms of circRNAs make them low-toxicity therapeutic targets. However, despite the significant potential of circRNAs in cancer treatment, their clinical application still faces several challenges: 1. The effective delivery of circRNA inhibitors or gene editing tools to tumor sites remains a technical challenge. There is a need to develop efficient and safe delivery systems. 2. The production and optimization of circRNA delivery systems need to be continuously improved in future research to enhance the feasibility of using circRNAs for cancer treatment. In future studies, continuous optimization of the delivery and production of circular RNAs will enhance their feasibility for use in cancer therapy.

To sum up, our research demonstrates the very critical role of circZEB1, which enhances O-GlcNAcylation, stabilizes YBX1 expression, and induces miR-337–3p to support BC formation. Our study indicate that circZEB1 had oncogenic function in BC by focusing on circZEB1/miR-337–3p/*OGT* or *YBX1*. It might be inferred that circZEB1 could be a promising new target for BC treatment.

Data availability

The corresponding author can provide the datasets used and/or analysed in the current work upon reasonable request.

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Ethics approval

The Yiwu Maternity and Children Hospital's Ethical Committee examined and approved the animal-related studies (grant ID: A00076), ensuring that they adhered to the institution's norms for the care and use of animals. The maximum tumor volume is less than 2000 mm3, and no tumor size was surpassed in this experiment—the entire tumor size was approved by the ethics committee.

CRediT authorship contribution statement

Dongying Wang: Methodology. **Dengyi Chen:** Formal analysis, Data curation. **Leilei Liang:** Project administration, Methodology. **Jialei Hu:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Jialei Hu reports financial support was provided by Zhejiang Provincial Natural Science Foundation of China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34079.

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