

# WNT11 is a direct target of early growth response protein 1

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**WNT11 is a member of the non-canonical *Wnt* family and plays a crucial role in tumor progression. However, the regulatory mechanisms underlying *WNT11* expression are unclear. Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) is a major inflammatory cytokine produced in the tumor microenvironment and contributes to processes associated with tumor progression, such as tumor invasion and metastasis. By using site-directed mutagenesis and introducing a serial deletion in the 5'-regulatory region of *WNT11*, we observed that TNF $\alpha$  activates the early growth response 1 (EGR1)-binding sequence (EBS) in the proximal region of *WNT11* and that the transcription factor EGR1 is necessary for the TNF $\alpha$ -induced transcription of *WNT11*. EGR1 bound directly to the EBSs within the proximal 5'-regulatory region of *WNT11* and ectopic expression of EGR1 stimulated *WNT11* promoter activity, whereas the knockdown of *EGR1* expression by RNA interference reduced TNF $\alpha$ -induced *WNT11* expression in T47D breast cancer cells. We also observed that mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase mediated TNF $\alpha$ -induced transcription of *WNT11* via EGR1. Our results suggest that EGR1 directly targets *WNT11* in response to TNF $\alpha$  stimulation in breast cancer cells. [BMB Reports 2020; 53(12): 628-633]**

## INTRODUCTION

Wnt signaling pathways represent a group of highly evolutionarily conserved signal transduction pathways that control various physiological responses, including embryonic body axis formation, cell fate specification, cell proliferation, and cell migration (1). To date, at least 19 Wnt proteins have been

identified in humans. All Wnt proteins bind and activate the Frizzled receptors, which transduce biological signals to the appropriate downstream targets (2). Wnt signaling is characterized by the  $\beta$ -catenin-dependent canonical pathway and the  $\beta$ -catenin-independent non-canonical pathway (3). Emerging evidence has implicated canonical and non-canonical Wnt signaling pathways in the development of human cancers, including gastrointestinal cancers, hepatocellular carcinoma, leukemia, melanoma, and breast cancers (4, 5).

WNT11 plays a crucial role in several morphological processes associated with embryogenesis, including anterior-posterior axis elongation through a  $\beta$ -catenin-dependent canonical or -independent non-canonical pathway (6). WNT11 promotes the proliferation and transformation of intestinal epithelial cells (7) and the downregulation of its receptor, frizzled-7, apart from reducing the survival, invasion, and metastasis of colon cancer cells (8). Consistent with the above, *WNT11* overexpression promotes the proliferation and migration of several tumor cells, including cervical, breast, prostate, and colon cancer cells (9, 10). These results suggest that WNT11 signaling plays a crucial role in cell proliferation, invasion, and metastasis during carcinogenesis (11).

The transcription of *WNT11* is controlled by multiple factors, including lymphoid enhancer-binding factor 1 (LEF1), GATA transcription factor (12), and E26 transforming sequence (ETS)-related gene (ERG), an ETS family transcription factor involved in hematopoiesis (13). In breast cancer cells, Chromatin immunoprecipitation-linked target site cloning was used to characterize *WNT11* as an estrogen receptor target gene (14).

Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) is a major inflammatory cytokine produced by tumor cells, tumor-associated fibroblasts, and infiltrated inflammatory cells in the tumor microenvironment (15). TNF $\alpha$  stimulates various inflammatory cytokines and chemokines and plays a crucial role in processes associated with tumor progression, including tumor invasion and metastasis (15-18). However, the role of TNF $\alpha$  in the regulation of *WNT11* expression remains largely elusive. Owing to the role of TNF $\alpha$  in tumor progression, we hypothesized that TNF $\alpha$  may control *WNT11* transcription. To evaluate this possibility, we isolated the 5'-regulatory region of the *WNT11* gene and evaluated its expression levels in response to TNF $\alpha$  stimulation in the T47D breast cancer cell line. We observed that TNF $\alpha$  stimulates *WNT11* transcription by activating the

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EGR1-binding cis-acting element within the 5'-regulatory region of *WNT11*. We also observed that the three major mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase, mediated TNF $\alpha$ -induced *WNT11* expression via EGR1 upregulation.

## RESULTS AND DISCUSSION

### TNF $\alpha$ upregulates *WNT11* expression in T47D breast cancer cells

We first examined the basal mRNA expression levels of *WNT11* in various breast and colon cancer cells. MCF-7 and T47D breast cancer cells expressed relatively high levels of *WNT11* mRNA, whereas no expression or low levels of expression were observed in non-transformed MCF10A breast epithelial cells and MDA-MB-231 breast cancer cells (Fig. 1A, left panels). Among the different colon cancer cells, the basal levels of *WNT11* mRNA were relatively high in SW620 and HT29 cells, while the same was not observed in SW480 cells (Fig. 1A, right panels). We selected and characterized T47D

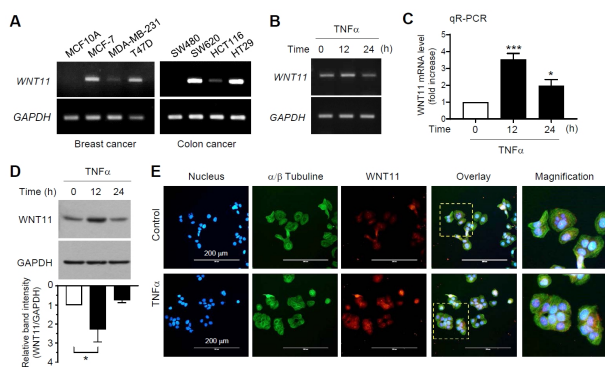
cells exhibiting moderate *WNT11* expression levels to investigate *WNT11* mRNA expression following TNF $\alpha$  stimulation. An increase in the mRNA levels of *WNT11* was detected within 12 h, followed by a reduction at 24 h after TNF $\alpha$  stimulation, whereas the mRNA levels of the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) remained unaltered, as revealed by reverse transcription (RT)-PCR (Fig. 1B) and quantitative real-time PCR (qR-PCR) (Fig. 1C). Similar results were obtained in MDA-MB-231 breast cancer cells (Supplementary Fig. S1A) and HCT116 colon cancer cells (Supplementary Fig. S2B). TNF $\alpha$ -induced elevation of the *WNT11* protein levels was confirmed by immunoblotting in T47D cells (Fig. 1D) and MDA-MB-231 cells (Supplementary Fig. S1B). Immunofluorescence studies also showed *WNT11* antibody-staining after TNF $\alpha$  stimulation; however, no staining was observed in the PBS-treated control (Fig. 1E). These data suggest that *WNT11* expression can be regulated by TNF $\alpha$  in T47D breast cancer cells.

### TNF $\alpha$ stimulates *WNT11* promoter activity by activating the EGR1-binding cis-acting element in the proximal promoter region of the 5'-regulatory region

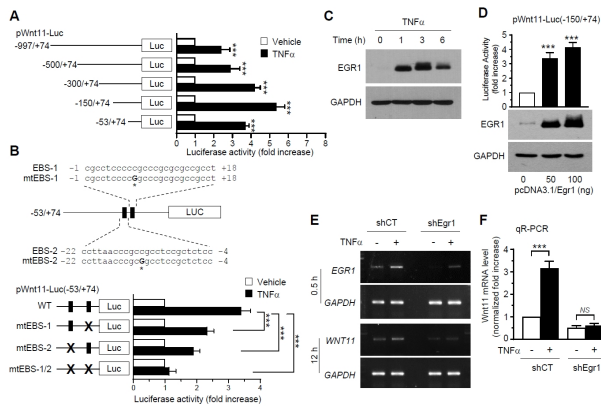
To investigate whether TNF $\alpha$  stimulates *WNT11* transcription, a fragment of the 5'-regulatory region spanning nucleotides -997 to +74 was isolated, and the effect exerted by TNF $\alpha$  on the activation of the *WNT11* promoter was assessed. We observed that TNF $\alpha$  significantly enhanced the promoter-reporter activity (Fig. 2A), suggesting that TNF $\alpha$  enhances *WNT11* expression at the transcriptional level. To delineate the promoter region that is responsible for TNF $\alpha$ -induced *WNT11* promoter activation, we designed a series of deletion constructs and mapped the TNF $\alpha$  response region. Upon TNF $\alpha$  stimulation, the shortest reporter construct (-53/+74) continued to exhibit induction (Fig. 2A), suggesting that the TNF $\alpha$ -inducible region of the *WNT11* promoter is located between nucleotides -53 and +74.

To identify the functional cis-acting element responsible for TNF $\alpha$ -induced *WNT11* gene transcription, we analyzed the transcription factor regulatory sequences between nucleotides -53 and +74 using a web-based MatInspector transcription factor search tool (<http://www.genomatix.de>). We found two putative early growth response protein 1 (EGR1)-binding sequences (EBSs) spanning nucleotides -22 to +18 (named EBS-1, -22 to -4, and EBS-2, -1 to +18) (Fig. 2B). EGR1, also known as zinc finger protein 225 (Zif268) or nerve growth factor-induced clone A (NGFI-A), is a Cys<sub>2</sub>His<sub>2</sub>-type zinc finger protein that exhibits Fos-like induction kinetics in response to various mitogenic stimuli and DNA damage signals (19). The target genes of EGR1 are associated with multiple physiological responses, including cell proliferation, differentiation, apoptosis, and inflammation, in a variety of cell types (19, 20). However, the role of EGR1 in *WNT11* transcription remains elusive.

To assess the functional role of these putative EBSs in



**Fig. 1.** Effect of TNF $\alpha$  on *WNT11* expression. (A) Basal expression of *WNT11* mRNA in various cancer cells. (B, C) T47D cells were treated with 10 ng/ml TNF $\alpha$  for 12 and 24 h. Total RNA was isolated, and the levels of *WNT11* mRNA were determined using RT-PCR (B) and quantitative real-time PCR (qR-PCR) (C). The levels of *GAPDH* mRNA were determined as an internal control. In (C), the 0-time mRNA level was set to 1 after adjustment to the *GAPDH* levels. Data are presented as mean + S.D. ( $n = 3$ ). \* $P = 0.0131$ ; \*\*\* $P < 0.001$  by Dunnett's multiple comparisons test. (D) *WNT11* levels were measured by immunoblotting. *GAPDH* levels were determined as an internal control. Band intensities were measured using the ImageJ software. After adjustment to the *GAPDH* levels, the 0-time band intensity was set to 1. Data are presented as mean + S.D. ( $n = 3$ ). \* $P = 0.0127$  ( $n = 3$ ) by Dunnett's multiple comparisons test. (E) T47D cells were treated with PBS (control) or 10 ng/ml TNF $\alpha$ , and then incubated with antibodies against  $\alpha/\beta$ -tubulin or *WNT11* for 2 h, followed by incubation with Alexa Fluor 488-conjugated (for  $\alpha/\beta$ -tubulin; green signal) and Alexa Fluor 555-conjugated (for *WNT11*; red signal) secondary antibodies for 30 min. Nuclear DNA was stained with 1  $\mu$ g/ml Hoechst 33258 for 10 min (blue staining). White bar size, 50  $\mu$ m. Arrows, *WNT11*-stained spots.

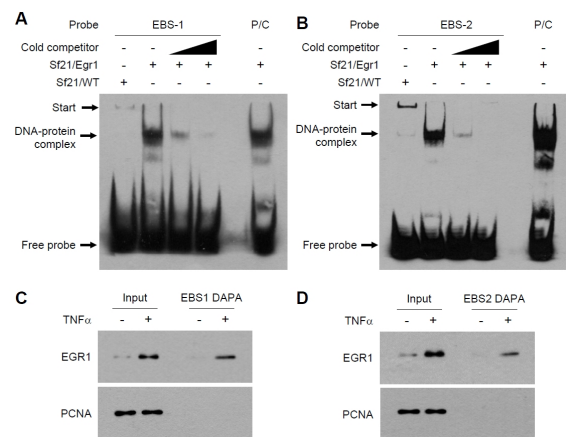


**Fig. 2.** Role of EGR1 in TNF $\alpha$ -induced WNT11 expression. (A) HEK293 cells were transiently transfected with 0.2  $\mu$ g of a series of 5'-deletion constructs. After treating with vehicle (PBS) or 10 ng/ml TNF $\alpha$ , luciferase activities were measured. (B) Sequence elements of EBS between -22 to +18 in the -53/+73 construct (top sub-figure). HEK293 cells were transiently transfected with site-specific mutants derived from the -53/+74 construct. After treatment with the vehicle (PBS) or with 10 ng/ml TNF $\alpha$ , luciferase activities were measured. EBS, EGR1-binding sequence; mtEBS, mutation of EBS. Asterisk (\*) indicates a mutation site. Luciferase activity data are presented as mean  $\pm$  S.D. ( $n = 3$ ). \*\*\* $P < 0.001$  by Sidak's multiple comparisons test. (C) T47D cells were treated with 10 ng/ml TNF $\alpha$  for 0, 3, and 6 h. After preparing the nucleus-enriched fractions, EGR1 levels were measured by immunoblotting. (D) HEK293 cells were transiently cotransfected with 0.2  $\mu$ g of the -150/+74 construct and EGR1 expression plasmid (pcDNA3.1/Egr1). Forty-eight hours later, the cells were harvested, and luciferase activities were measured. Data are presented as mean  $\pm$  S.D. ( $n = 3$ ). The transfected EGR1 levels were confirmed by immunoblotting. (E and F) T47D transfectants stably expressing control scrambled shRNA (shCT) or EGR1 shRNA (shEgr1) were treated with 10 ng/ml TNF $\alpha$  for 0.5 or 12 h. Expression levels of the EGR1 and WNT11 mRNAs were measured by RT-PCR (E), and WNT11 mRNA levels were quantitated by quantitative real-time PCR (qR-PCR) (F). qR-PCR data are presented as mean  $\pm$  S.D. ( $n = 3$ ). NS, not significant; \*\*\* $P < 0.001$  by Sidak's multiple comparisons test.

TNF $\alpha$ -induced WNT11 transcription, we introduced site-directed mutations into the EBSs. Damage to the EBS-1 (mtEBS-1) or EBS-2 (mtEBS-2) motifs in the pWnt11-Luc(-53/+74) significantly reduced TNF $\alpha$ -induced reporter activity compared to that in the wild-type (WT) construct (Fig. 2B, bottom graph). Damage to both EBS-1 and EBS-2 sites resulted in an almost complete loss of TNF $\alpha$  inducibility.

TNF $\alpha$  induces EGR1 expression in various cell types (21-24). Consistently, we observed that EGR1 expression increased in a time-dependent manner upon TNF $\alpha$  stimulation (Fig. 2C). To determine whether EGR1 transactivates WNT11, we transfected the -150/+74 construct into T47D cells, along with an expression plasmid for EGR1. Exogenous overexpression of EGR1 enhanced the WNT11 promoter-reporter activity in a plasmid concentration-dependent manner (Fig. 2D). These results suggest that EGR1 can transactivate WNT11 in T47D cells.

To further investigate the role of EGR1 in TNF $\alpha$ -induced



**Fig. 3.** Binding of EGR1 to putative EBS motifs of WNT11. (A, B) EBS-1 (A) and EBS-2 (B) probes were incubated with lysates of Sf21 cells expressing EGR1 (Sf21/Egr1) or with the wild-type (Sf21/WT) in the presence or absence of an unlabeled probe (cold competitor). Samples were separated by non-denaturing 6% acrylamide gel electrophoresis and visualized using streptavidin-conjugated horseradish peroxidase. (C and D) T47D cells were treated with 10 ng/ml TNF $\alpha$  for 1 h or were left untreated. Nuclear extracts were prepared and incubated with biotinylated EBS-1 (C) or EBS-2 (D), followed by the addition of streptavidin-conjugated agarose beads. After 1 h, the agarose beads were washed and boiled for 5 min with 2  $\times$  Laemmli sample buffer. Immunoblotting was performed using anti-EGR1 or anti-PCNA antibodies. Input, an aliquot of the nuclear extract; DAPA, DNA-affinity precipitation assay.

WNT11 transcription, we established T47D transfectants expressing lentiviral shRNA against EGR1 (T47D/shEgr1) and a scrambled control (T47D/shCT). The stable knockdown of basal and TNF $\alpha$ -induced expression of EGR1 mRNA was confirmed by RT-PCR after TNF $\alpha$  treatment (Fig. 2E, top panels). The ability of TNF $\alpha$  to induce the mRNA expression of WNT11 was substantially attenuated when EGR1 expression in T47D/shEgr1 was reduced compared to that in T47D/shCT cells (Fig. 2E, bottom panels). qR-PCR analysis revealed that TNF $\alpha$  elevated WNT11 mRNA expression by  $3.17 \pm 0.306$ -fold in T47D/shCT cells, while it reduced WNT11 mRNA expression by  $0.600 \pm 0.100$ -fold in T47D/shEgr1 cells (Fig. 2F). These results suggest that the EBS motifs in the promoter region are necessary for the TNF $\alpha$ -induced transcription of WNT11.

### EGR1 binds to the EBS motif and transactivates WNT11 transcription

To determine whether the EBS motif can serve as a binding site for EGR1, we performed the non-radioactive electrophoretic mobility shift assay (EMSA) using Sf21 insect cell lysates overexpressing EGR1 (Sf21/Egr1) and biotinylated EBS oligonucleotide probes. Biotinylated consensus EBS oligonucleotides were used as a positive control. We observed that the biotinylated EBS-1 (Fig. 3A) and EBS-2 (Fig. 3B) probes formed

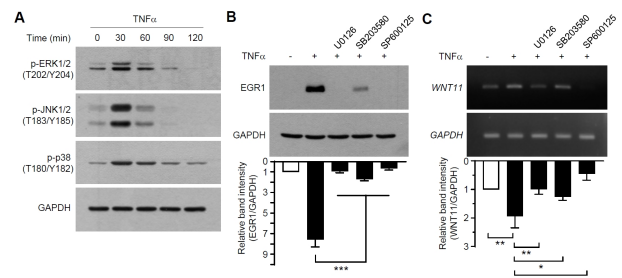
DNA-protein complexes. These complexes competed with a molar excess of an unlabeled probe. These data suggest that EGR1 binds specifically to the EBS motifs within the proximal *WNT11* promoter region.

To further corroborate the binding of EGR1 to the EBS motifs in *WNT11*, we adopted an alternative approach using the DNA-affinity precipitation assay (DAPA). Biotinylated EBS-1 and EBS-2 oligonucleotides were incubated with nuclear extracts from T47D cells. Upon TNF $\alpha$  stimulation, the levels of EGR1 and nuclear protein PCNA increased in the whole nuclear extract (input) (Fig. 3C and D; bottom panels). After pull-down with streptavidin-agarose beads, oligonucleotide-binding proteins were eluted and immunoblotted with an anti-EGR1 or anti-PCNA antibody. We observed that while EBS-1 and EBS-2 bound EGR1 in response to TNF $\alpha$  stimulation, they did not bind PCNA (Fig. 3C and D; top panels). These data suggest that the proximal EBS sites located between nucleotides -22 and +18 in the *WNT11* promoter region bind EGR1; however, they do not exhibit any off-target effect.

#### MAPKs mediate TNF $\alpha$ -induced *WNT11* expression via EGR1 upregulation

Transcription factor ETS like-1 protein (ELK1) and serum response factor (SRF) complex transactivate the serum response element (SRE) in the 5'-regulatory region of the *EGR1* gene. MAPK pathways control *EGR1* expression via phosphorylation of ELK-1 in response to TNF $\alpha$  stimulation in several cell types (21, 25, 26). To determine which MAPK pathways are responsible for EGR1-mediated *WNT11* expression, we validated the active status of TNF $\alpha$ -induced MAPKs in serum-starved T47D cells. The phosphorylation levels of ERK1/2 at Thr-202/Tyr-204, JNK1/2 at Thr-183/Tyr-185, and p38 kinase at Thr-180/Tyr-182 increased within 30 min in response to TNF $\alpha$  stimulation (Fig. 4A). Pretreatment with MAPK inhibitors, including the MAPK kinase (MEK) inhibitor U0126, JNK inhibitor SP600125, and p38 kinase inhibitor SB203580, substantially abrogated the TNF $\alpha$ -induced accumulation of EGR1 proteins (Fig. 4B). Consistent with EGR1 expression results, all MAPK inhibitors significantly reduced TNF $\alpha$ -induced *WNT11* expression; in particular, U0126 and SP600125 showed a strong inhibitory effect compared to SB203580 (Fig. 4C). These data suggest that all three major MAPK pathways contribute to EGR1-mediated *WNT11* expression in response to TNF $\alpha$  exposure in T47D breast cancer cells.

The current study identified the role of EGR1 in TNF $\alpha$ -induced *WNT11* expression in T47D human breast cancer cells. In response to TNF $\alpha$  stimulation, EGR1 directly bound to the EBS in the *WNT11* promoter region and stimulated *WNT11* transcription. All three major MAPKs, ERK, JNK, and p38 kinases, were involved in TNF $\alpha$ -induced EGR1 upregulation. We conclude that *WNT11* is a direct target of the EGR1 in response to TNF $\alpha$  stimulation, further expanding our understanding of the regulatory mechanism of *WNT11* expression in the tumor microenvironment. As *WNT11* activates



**Fig. 4.** Role of MAPKs in TNF $\alpha$ -induced *WNT11* expression. (A) T47D cells were treated with 10 ng/ml TNF $\alpha$  for various durations (0-1200 min). Immunoblotting was performed using a phospho-specific antibody against ERK1/2 (Thr202/Tyr204), JNK1/2 (Thr183/Tyr185), or p38 kinase (Thr180/Tyr182). GAPDH levels were determined as an internal control. (B, C) T47D cells were pretreated with U0126 (10  $\mu$ M), SB203580 (20  $\mu$ M), or SP600125 (20  $\mu$ M) for 30 min and then stimulated with 10 ng/ml TNF $\alpha$ . After 1 and 12 h, the levels of EGR1 (B) and *WNT11* mRNAs (C) were measured by immunoblotting and RT-PCR, respectively. The band intensities were measured relative to the GAPDH levels using the ImageJ software. Bars represent the mean  $\pm$  S.D. ( $n = 3$ ). \* $P = 0.0149$ ; \*\* $P = 0.0017$ ; \*\*\* $P < 0.001$  by Dunnett's multiple comparison test.

cancer cell motility and metastasis (27) and reduction of *WNT11* expression decreases the ability of breast cancer cells to migrate (9), EGR1-regulated *WNT11* expression can be a promising therapeutic target for suppressing metastasis of breast cancer. Further *in vivo* studies are warranted to verify the clinical relevance of targeting the TNF $\alpha$ -EGR1-*WNT11* axis to prevent tumor metastasis in breast cancer.

## MATERIALS AND METHODS

### Cell and reagents

Detailed cells and reagents used in this study were described in Supplementary materials.

### Reverse transcription-PCR (RT-PCR)

Total RNA was isolated using a TRIzol RNA extraction kit (Invitrogen). First-strand cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: hold for 5 min at 94 $^{\circ}$ C, followed by 30 cycles of denaturation at 94 $^{\circ}$ C (30 s), annealing at 55 $^{\circ}$ C (30 s), and elongation at 72 $^{\circ}$ C (1 min). The gene-specific PCR primers were as follows: *WNT11* forward, 5' GAT CCC AAG CCA ATA AAC TGA TGC GTC T-3'; *WNT11* reverse, 5'-GTC TTG TTG CAC TGC CTG TCT TGT GTC C-3'; *GAPDH* forward, 5' CCAAGGAGTAAGAAACCCTGGAC-3'; *GAPDH* reverse, 5'-GGGCCGAGT TGGGATAGG G-3'. The amplified products were electrophoresed in 1% agarose gel.

### Immunoblotting

Immunoblot analysis was performed as described previously

(21). Detailed experimental methods were described in Supplementary materials.

### Immunofluorescence

T47D cells cultured on coverslips were either treated with the vehicle (PBS) or 10 ng/ml TNF $\alpha$  for 18 h, followed by fixation, permeabilization, and incubation with primary antibodies against WNT11 and  $\alpha/\beta$ -tubulin (for counterstaining). After 2 h, the cells were incubated for 30 min with secondary antibodies conjugated with Alexa Fluor 555 (for  $\alpha/\beta$ -tubulin; red signal) and Alexa Fluor 488 (for WNT11, green signal). Nuclear DNA was stained using 1  $\mu$ g/ml Hoechst 33258 for an additional 10 min (blue signal). Fluorescently-stained cells were examined under an EVOS FL fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA).

### Construction and mutagenesis of human WNT11 promoter-reporter constructs

Detailed experimental methods for the generation of the human WNT11 promoter-reporter constructs and primer sequences were described in Supplementary materials.

### WNT11 promoter-reporter assay

HEK293T cells cultured in 12-well plates were transfected with 0.2  $\mu$ g WNT11 promoter constructs using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Detailed experimental methods for promoter-reporter assay were described in Supplementary materials.

### Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a LightShift Chemiluminescence EMSA kit, according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA). Biotin-labeled deoxyoligonucleotide probes specific for EBS-1 (5'-CCT TAA CCC GCC GCC TCC GCT CTC C-biotin-3'), EBS-2 (5'-AGG CGG CGC GCG GGC GGG GGA GGC G-biotin-3'), and the consensus EBS sequence (5'-GGA TCC AGC GGG GGC GAG CGG GCG CGA-biotin-3') were obtained from Macrogen. For the competition assay, 1 and 2.5 pmole of the unlabeled EBS-1 and EBS-2 probes (competitor) were added, respectively. DNA-protein complexes were electrophoresed in non-denaturing 6% polyacrylamide gels and visualized using an ECL chemiluminescence system (GE Healthcare Life Science).

### DNA-affinity precipitation assay (DAPA)

DAPA was performed as reported previously (28). Briefly, nuclear extracts (35  $\mu$ g) were incubated with 500 pmol of biotinylated EBS-1 (5'-biotin-CCT TAA CCC GCC GCC TCC GCT CTC C-3') or EBS-2 (5'-biotin-AGG CGG CGC GCG GGC GGG GGA GGC G-3') oligonucleotide and streptavidin-conjugated agarose beads (Invitrogen) for 1 h, as described previously (29). After washing thrice with PBS, the pellets were boiled with 2  $\times$  Laemmli sample buffer and immunoblot analysis was performed using antibodies against EGR1 or PCNA

(off-target control).

### EGR1 silencing using RNA interference

T47D cells were incubated with lentiviral shRNA (TRCN\_0000273850; MISSION<sup>®</sup> shRNA; Sigma-Aldrich) targeting EGR1, according to the manufacturer's instructions. After 2 wk, the silencing of EGR1 expression was determined by immunoblotting.

### Statistical analysis

Statistical significance of results was determined using one-way analysis of variance (ANOVA), followed by Sidak's multiple comparisons test or Dunnett's multiple comparisons test using the GraphPad Prism version 8.3.1 software (GraphPad Software Inc., La Jolla, CA, USA). A P-value < 0.05 was considered statistically significant.

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### CONFLICTS OF INTEREST

The authors have no conflicting interests.

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