Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

The ZNF354C–TBC1D24 Axis Promotes Triple-Negative Breast Cancer Progression via PI3K/Akt-Dependent G1/S Activation

Jian Ye
Jianyang First Hospital of Nanping City
Jinghan Yu
Wenzhou Medical University, People's Hospital of Cangnan (Affiliated Cangnan Hospital)
Qingyang Li
Wenzhou Medical University, People's Hospital of Cangnan (Affiliated Cangnan Hospital)
Yudiao Gong
gyd15068266027@163.com
Wenzhou Medical University, People's Hospital of Cangnan (Affiliated Cangnan Hospital)

Research Article

Keywords: Triple-negative breast cancer, TBC1D24, ZNF354C, PI3K/Akt signaling, G1/S transition, cell cycle progression, single-cell RNA sequencing, transcriptional regulation

Posted Date: April 1st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9091943/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Background:
Triple-negative breast cancer (TNBC) is an aggressive subtype characterized by rapid proliferation and limited targeted therapies. While aberrant activation of proliferative signaling pathways is common in TNBC, the upstream regulators sustaining these oncogenic circuits remain poorly defined. TBC1D24, a Tre-2/Bub2/Cdc16 (TBC) domain–containing protein involved in vesicular trafficking, has not been previously characterized in breast cancer.

Methods:
Transcriptomic and correlation analyses were performed using TCGA and CCLE datasets. Gene Set Enrichment Analysis (GSEA) and KEGG pathway enrichment were applied to identify associated biological processes. Functional roles of TBC1D24 were assessed using siRNA-mediated knockdown and overexpression in breast cancer cell lines, followed by proliferation, colony formation, and xenograft tumor assays. Mechanistic investigations included western blotting, luciferase reporter assays, ChIP–qPCR, and immunohistochemistry to evaluate downstream signaling and transcriptional regulation.

Results:
TBC1D24 expression was significantly associated with cell cycle–related signatures, particularly G1/S transition pathways. TBC1D24 positively correlated with CDK2, CDK4, CDK6, CCND1, and MYC in the TCGA cohort. Functional depletion of TBC1D24 suppressed proliferation and tumor growth, reduced CDK4/6 expression, increased P21 and P27 levels, and promoted apoptosis. Mechanistically, TBC1D24 activated the PI3K–Akt signaling pathway, enhancing phosphorylation of PI3K, Akt, and GSK3β and upregulating c-Myc and Cyclin D1. Upstream analyses identified ZNF354C as a direct transcriptional activator of TBC1D24, with promoter binding confirmed by ChIP–qPCR and luciferase assays.

Conclusion:
The ZNF354C–TBC1D24–PI3K/Akt axis promotes G1/S progression and breast cancer growth, representing a potential therapeutic target in aggressive TNBC.

Background

Triple-negative breast cancer (TNBC) represents the most recalcitrant and biologically aggressive subtype of breast carcinoma, accounting for approximately 15–20% of all incident cases globally [1]. Defined clinically by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) amplification, TNBC lacks the specific molecular targets that have revolutionized the management of hormone receptor-positive and HER2-enriched diseases [2]. Consequently, patients with TNBC face a disproportionately high risk of early systemic relapse, visceral metastasis to the lungs and brain, and a steeply declining survival curve within the first three to five years post-diagnosis [3]. While the advent of immune checkpoint blockade and poly(ADP-ribose) polymerase (PARP) inhibitors has provided clinical benefit for select patient subsets, the broader TNBC population remains dependent on cytotoxic chemotherapy, which is frequently circumvented by the rapid evolution

of chemoresistance and cellular plasticity [4, 5]. Identifying the intrinsic master regulators that govern this cellular adaptability is imperative for developing next-generation precision therapeutics.

The capacity of malignant cells to dynamically adapt to microenvironmental stressors relies heavily on the continuous remodeling of the plasma membrane and the precise spatiotemporal regulation of signal transduction [6]. This remodeling is primarily orchestrated by the intracellular vesicle trafficking machinery, a highly coordinated system governed by the Rab family of small GTPases [7]. The cycling of Rab proteins between their active (GTP-bound) and inactive (GDP-bound) states dictates the sorting, transport, recycling, and lysosomal degradation of critical surface cargo, including receptor tyrosine kinases (RTKs) and integrins [8]. This GTPase cycle is stringently regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Specifically, proteins harboring the Tre-2/Bub2/Cdc16 (TBC) domain function as the principal GAPs for Rab proteins, thereby catalyzing GTP hydrolysis and terminating Rab activity [9]. Aberrant expression of TBC domain proteins has recently been recognized as a hallmark of "non-oncogene addiction" in various solid tumors, allowing malignant cells to artificially extend the half-life of growth factor receptors, thereby hyperactivating downstream mitogenic cascades [10, 11]. For example, TBC1D9 and TBC1D3 have been implicated in modulating epidermal growth factor receptor (EGFR) signaling and breast cancer differentiation, highlighting the oncogenic potential of this protein family [12, 13].

Despite these emerging paradigms, the functional repertoire of many TBC proteins remains unexplored in mammary oncology. TBC1D24 is a structurally distinct member of the family, characterized by the presence of a TLDc (TBC and LysM domain-containing) domain alongside its catalytic TBC domain, suggesting dual roles in membrane dynamics and oxidative stress responses [14]. Historically, TBC1D24 has been strictly categorized within the context of neurobiology, where loss-of-function mutations precipitate severe developmental anomalies, including familial infantile myoclonic epilepsy and DOORS syndrome (deafness, onychodystrophy, osteodystrophy, mental retardation, and seizures) [15, 16]. At the synapse, TBC1D24 physically interacts with ARF6 and specific Rab proteins to govern vesicle endocytosis and neurotransmitter release [17]. However, recent multi-omic pan-cancer screens have detected anomalous *TBC1D24* transcription in several non-neurological malignancies, hinting at uncharacterized pleiotropic functions [18]. Given that TNBC cells frequently "hijack" highly efficient developmental and neuronal trafficking pathways to facilitate rapid migration and invasive invadopodia formation [19, 20], TBC1D24 emerges as a highly compelling candidate for investigation.

In the present study, we hypothesized that the anomalous upregulation of *TBC1D24* serves as a foundational mechanism enabling TNBC aggressiveness. By integrating high-resolution single-cell RNA sequencing (scRNA-seq) with bulk clinical cohorts and rigorous *in vitro* and *in vivo* functional models, we sought to systematically delineate the clinical significance and molecular mechanics of TBC1D24. Our findings unveil a novel paradigm wherein TBC1D24 acts as an indispensable rheostat for the PI3K/AKT/mTOR signaling axis, establishing it as a pivotal prognostic biomarker and a rational therapeutic target.

Methods

Public Data Acquisition and Bioinformatic Analysis

Transcriptomic data and corresponding clinical information for breast cancer patients were obtained from The Cancer Genome Atlas Breast Cancer cohort (TCGA-BRCA) through the Genomic Data Commons (GDC) portal. Normal breast tissue transcriptomic data were collected from the Genotype-Tissue Expression (GTEx) database. Batch effects between TCGA and GTEx datasets were corrected using the ComBat algorithm implemented in the *sva* R package.

Differential gene expression analysis between high- and low-TBC1D24 expression groups was performed using the DESeq2 and limma R packages. Genes with |log2 fold change| > 1 and adjusted $P < 0.05$ were considered significantly differentially expressed. Functional enrichment analysis of differentially expressed genes was conducted using Gene Ontology (GO) biological process analysis to identify biological pathways associated with TBC1D24 expression.

For survival analysis, patients were stratified into high- and low-TBC1D24 expression groups according to the optimal cutoff determined using the survminer R package. Kaplan–Meier survival curves were generated, and differences between groups were evaluated using the log-rank test.

Single-Cell RNA Sequencing Analysis

Single-cell RNA sequencing (scRNA-seq) data from triple-negative breast cancer samples were downloaded from the Gene Expression Omnibus (GEO) database (GSE118389). Data processing and analysis were performed using the Seurat (v4.0) package in R.

Cells with fewer than 200 detected genes or more than 20% mitochondrial gene expression were excluded during quality control. Data were normalized and the top 2,000 highly variable genes were identified for principal component analysis (PCA). Dimensionality reduction and visualization were performed using t-distributed stochastic neighbor embedding (t-SNE).

Expression density maps were generated to visualize TBC1D24 expression and gene signatures associated with cell proliferation and DNA replication across cellular clusters.

Cell Culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

RNA Interference

To silence TBC1D24, two independent small interfering RNAs (siTBC1D24-1 and siTBC1D24-2) were transfected into breast cancer cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. A scrambled siRNA served as the negative control. Knockdown efficiency was verified by quantitative real-time PCR (qRT-PCR) and western blotting.

For in vivo experiments, short hairpin RNAs (shTBC1D24-1 and shTBC1D24-2) targeting TBC1D24 were cloned into the pLKO.1 lentiviral vector. Lentiviral particles were generated in HEK293T cells by co-transfection with psPAX2 and pMD2.G packaging plasmids. Breast cancer cells were infected with viral supernatants in the presence of 8 μg/mL polybrene, followed by puromycin selection (2 μg/mL) to establish stable knockdown cell lines.

Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized using a reverse transcription kit (Takara). qRT-PCR was performed using SYBR Green Master Mix on a real-time PCR system. Relative gene expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method, with β-actin used as an internal control.

Western Blot Analysis

Total protein was extracted using RIPA lysis buffer supplemented with protease inhibitors. Protein concentrations were measured using a BCA protein assay kit. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes.

Membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with primary antibodies against TBC1D24 and β-actin. After incubation with appropriate HRP-conjugated secondary antibodies, protein bands were visualized using enhanced chemiluminescence (ECL).

Cell Viability Assay

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo) assay. Cells were seeded into 96-well plates at 2×10^3 cells per well. At indicated time points, 10 μL CCK-8 reagent was added to each well and incubated for 2 hours at 37°C. Absorbance was measured at 450 nm using a microplate reader.

Colony Formation Assay

For colony formation analysis, 500 cells were seeded into 6-well plates and cultured for approximately 10–14 days until visible colonies formed. Cells were then fixed with 4% paraformaldehyde and stained

with 0.1% crystal violet. Colonies containing more than 50 cells were counted.

Wound Healing Migration Assay

Cell migration was evaluated using a scratch wound-healing assay. Cells were seeded into 6-well plates and grown to near confluence. A sterile 200-μL pipette tip was used to create a scratch across the cell monolayer. After washing with PBS to remove detached cells, fresh serum-free medium was added. Images of wound closure were captured at 0 h and 24 h, and migration ability was quantified by measuring the wound width.

Xenograft Tumor Model

All animal procedures were approved by the institutional animal ethics committee. Female BALB/c nude mice (6 weeks old) were randomly divided into experimental groups (n = 6 per group).

Breast cancer cells (1 × 10^6 cells) stably expressing shCtrl, shTBC1D24-1, or shTBC1D24-2 were suspended in PBS/Matrigel (1:1) and injected subcutaneously into the flanks of mice. Tumor size was measured every 3 days using digital calipers. Tumor volume was calculated using the formula:

$$V = 0.5 \times \text{Length} \times \text{Width}^2$$

At the end of the experiment, mice were sacrificed and tumors were excised, photographed, and weighed.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9.0 and R software. Data are presented as mean ± standard deviation (SD) from at least three independent experiments. Differences between two groups were analyzed using Student's t-test, while comparisons among multiple groups were evaluated using one-way ANOVA. A P value < 0.05 was considered statistically significant.

Results

TBC1D24 is Genomically Altered, Overexpressed in Breast Cancer, and Predicts Poor Prognosis

To evaluate the genomic and clinical relevance of TBC1D24 in breast cancer, we first interrogated the TCGA-BRCA cohort. Genetic alteration analysis revealed that TBC1D24 exhibits an overall alteration frequency of approximately 9%, predominantly driven by copy-number amplification and mRNA upregulation (Fig. 1A). Subtype-stratified GISTIC analysis demonstrated that copy-number gains and amplifications were more prevalent in aggressive molecular subtypes, particularly basal-like and HER2-enriched tumors, whereas luminal and normal-like subtypes showed relatively lower amplification frequencies (Fig. 1B). These findings suggest that genomic copy-number alterations may contribute to

TBC1D24 dysregulation in specific breast cancer subgroups. Consistently, transcriptomic analysis of the TCGA cohort revealed that TBC1D24 mRNA expression was significantly elevated in tumor tissues compared with normal breast samples (Fig. 1C).

To define its cellular origin within the tumor microenvironment, we analyzed a breast cancer single-cell RNA-seq atlas. UMAP clustering identified major cell populations including cancer cells, stem cells, myeloid cells, lymphoid cells, fibroblasts, pericytes, and endothelial cells (Fig. 1D). Feature mapping demonstrated that TBC1D24 expression was predominantly enriched in malignant epithelial (cancer) cells rather than stromal or immune compartments (Fig. 1E). Dot plot quantification further confirmed higher expression levels and greater expression frequency in cancer cells compared to other cell types (Fig. 1F), supporting a tumor cell–intrinsic role.

Kaplan–Meier survival analysis showed that patients with high TBC1D24 expression had significantly worse overall survival compared to those with low expression (log-rank P = 5e–05) (Fig. 1G). Multivariate Cox proportional hazards analysis (N = 1006) further demonstrated that elevated TBC1D24 expression independently predicted poor prognosis (HR = 1.37, 95% CI: 1.012–1.9, P = 0.042), even after adjusting for age and TNM stage (Fig. 1H). Collectively, these findings establish TBC1D24 as a genomically altered, tumor cell–enriched gene associated with adverse clinical outcomes in breast cancer.

TBC1D24 Promotes Proliferation, Cell Cycle Progression, and Tumorigenicity in Breast Cancer

To explore the biological functions associated with TBC1D24 in breast cancer, we first performed differential expression analysis comparing TBC1D24-high and TBC1D24-low tumor samples in the TCGA cohort. A total of 381 genes were upregulated and 1,521 genes were downregulated in TBC1D24-high tumors (Fig. 2A). Gene Ontology (GO) enrichment analysis revealed that differentially expressed genes were significantly enriched in cell proliferation, G1/S transition of the mitotic cell cycle, DNA replication, mitotic nuclear division, and other cell cycle–related biological processes (Fig. 2B), suggesting a role for TBC1D24 in tumor growth regulation. Single-cell transcriptomic projection further demonstrated that TBC1D24 expression overlapped spatially with proliferative signatures within malignant epithelial cells. Density mapping showed strong concordance between TBC1D24 expression and cell proliferation as well as DNA replication signatures (Fig. 2C–D), supporting a tumor cell–intrinsic proliferative function.

To experimentally validate these findings, TBC1D24 was silenced using two independent siRNAs. Knockdown efficiency was confirmed by qRT-PCR and western blot analysis (Fig. 2D, lower panel). Functional assays demonstrated that TBC1D24 depletion significantly reduced cell viability in both MDA-MB-231 and MCF-7 cells as measured by CCK-8 assays (Fig. 2E). Consistently, colony formation capacity was markedly suppressed following TBC1D24 knockdown (Fig. 2F). In addition, wound-healing assays revealed impaired migration/invasion ability upon TBC1D24 silencing in two breast cancer cell lines (Fig. 2G).

To evaluate the in vivo relevance, xenograft experiments were performed. Stable knockdown of TBC1D24 significantly reduced tumor growth, as evidenced by smaller tumor size, lower tumor weight, and decreased tumor volume over time compared to control groups (Fig. 2H). Collectively, these results demonstrate that TBC1D24 promotes breast cancer cell proliferation, clonogenic capacity, migratory ability, and tumorigenesis.

TBC1D24 Regulates G1/S Transition and Cell Cycle Progression in Breast Cancer

To further investigate the molecular mechanisms underlying TBC1D24-mediated tumor promotion, Gene Set Enrichment Analysis (GSEA) was performed comparing TBC1D24-high and TBC1D24-low tumors in the TCGA cohort. TBC1D24-high tumors were significantly enriched in gene sets associated with positive regulation of cell cycle, mitotic cell cycle checkpoint, and G1/S transition of the mitotic cell cycle (all $P < 0.01$; Fig. 3A), indicating a strong association with cell cycle activation. Consistent with this observation, correlation analysis in the TCGA cohort ($N = 1106$) demonstrated that TBC1D24 expression positively correlated with key G1/S regulators, including CDK2 ($R = 0.34$, $P < 0.001$), CDK4 ($R = 0.33$, $P < 0.001$), CDK6 ($R = 0.35$, $P < 0.001$), and CDC23 ($R = 0.38$, $P < 0.001$) (Fig. 3B). These results suggest that TBC1D24 may facilitate cell cycle progression through modulation of cyclin-dependent kinase signaling.

To validate these findings experimentally, western blot analysis was performed in MDA-MB-231 and MCF-7 cells following TBC1D24 knockdown. Silencing TBC1D24 resulted in reduced protein levels of CDK4 and CDK6, accompanied by increased expression of the cyclin-dependent kinase inhibitors P21 and P27. Additionally, cleaved PARP levels were elevated after TBC1D24 depletion (Fig. 3C), indicating enhanced apoptotic activity. Collectively, these findings demonstrate that TBC1D24 promotes breast cancer cell proliferation by regulating G1/S transition and cell cycle progression.

TBC1D24 Activates the PI3K–Akt Signaling Pathway in Breast Cancer

To further delineate the downstream signaling mechanisms of TBC1D24, KEGG pathway enrichment analysis was performed on differentially expressed genes between TBC1D24-high and TBC1D24-low tumors. The PI3K-Akt signaling pathway emerged as one of the most significantly enriched pathways (Fig. 4A), suggesting its involvement in TBC1D24-mediated tumor progression. Consistent with this finding, correlation analysis in the TCGA breast cancer cohort ($N = 1106$) demonstrated that TBC1D24 expression positively correlated with CCND1 ($R = 0.30$, $P < 0.001$) and MYC ($R = 0.42$, $P < 0.001$) (Fig. 4B). Gene Set Enrichment Analysis further confirmed significant enrichment of the PI3K-Akt signaling pathway in TBC1D24-high tumors ($P < 0.01$; Fig. 4C).

Single-cell transcriptomic projection revealed overlapping distribution patterns between TBC1D24 expression and PI3K-Akt pathway activity within malignant epithelial cells (Fig. 4D). Correlation matrix analysis additionally showed positive associations between TBC1D24 and key components of the PI3K-

Akt axis, including AKT1, MTOR, PDPK1, CCND1, and MYC (Fig. 4E). Functionally, FHRE luciferase reporter assays demonstrated that TBC1D24 overexpression significantly enhanced reporter activity (Fig. 4F), indicating increased downstream transcriptional signaling. Western blot analysis further confirmed that TBC1D24 knockdown reduced phosphorylation levels of PI3K, Akt, and GSK3β, accompanied by decreased expression of c-Myc and Cyclin D1. Conversely, TBC1D24 overexpression increased activation of these signaling components (Fig. 4G).

Finally, immunohistochemical analysis of xenograft tumors showed reduced p-AKT and c-Myc expression in TBC1D24-silenced tumors (Fig. 4H), supporting in vivo activation of the PI3K-Akt pathway by TBC1D24. Collectively, these results indicate that TBC1D24 promotes breast cancer progression through activation of the PI3K-Akt signaling pathway.

ZNF354C Transcriptionally Activates TBC1D24 in Breast Cancer

To identify upstream transcriptional regulators of TBC1D24, an integrative screening strategy was employed combining public transcription factor prediction databases, expression correlation analysis, and single-cell RNA sequencing distribution patterns. ZNF354C emerged as a candidate regulator at the intersection of these approaches (Fig. 5A). Single-cell transcriptomic analysis demonstrated that ZNF354C was predominantly expressed in malignant epithelial cells within the breast cancer tumor microenvironment (Fig. 5B–C). Consistent with this observation, correlation analysis in the TCGA cohort ($N = 1106$) revealed a strong positive association between ZNF354C and TBC1D24 expression ($R = 0.53$, $P < 0.001$; Fig. 5D). This correlation was further validated in the CCLE breast cancer cell line cohort ($R = 0.45$, $P < 0.001$; $N = 57$; Fig. 5E). Bioinformatic analysis identified a putative ZNF354C binding motif within the promoter region of TBC1D24, located approximately 286 bp upstream of the transcription start site (Fig. 5F). Chromatin immunoprecipitation (ChIP)-qPCR analysis confirmed enrichment of ZNF354C at the TBC1D24 promoter, and luciferase reporter assays demonstrated that ZNF354C significantly enhanced promoter activity.

Functionally, knockdown of ZNF354C markedly reduced TBC1D24 mRNA expression in MCF-7 and MDA-MB-231 cells (Fig. 5G). Western blot analysis further confirmed that ZNF354C depletion decreased TBC1D24 protein levels, whereas ZNF354C overexpression increased TBC1D24 expression (Fig. 5H). Collectively, these results demonstrate that ZNF354C directly binds to and transcriptionally activates TBC1D24 in breast cancer cells.

Discussion

Triple-negative breast cancer (TNBC) remains one of the most therapeutically challenging breast cancer subtypes due to its intrinsic molecular heterogeneity, high proliferative index, and absence of established hormone or HER2-directed targets [24]. Although aberrant activation of proliferative signaling pathways is a hallmark of TNBC, the upstream regulatory mechanisms sustaining these oncogenic circuits remain

incompletely defined. In the present study, we identify TBC1D24 as a previously unrecognized oncogenic driver that promotes breast cancer progression through coordinated activation of cell cycle machinery and the PI3K–Akt signaling pathway. Through integrative transcriptomic analyses, we demonstrated that high TBC1D24 expression is associated with enrichment of cell cycle–related biological processes, particularly the G1/S transition and mitotic checkpoint regulation (Fig. 3A) [26]. Correlation analyses in the TCGA cohort further revealed strong positive associations between TBC1D24 and key cell cycle regulators including CDK2, CDK4, CDK6, and CDC23 (Fig. 3B). Functionally, TBC1D24 depletion resulted in downregulation of CDK4 and CDK6, upregulation of the CDK inhibitors P21 and P27, and induction of cleaved PARP, collectively indicating G1 phase arrest and increased apoptotic signaling (Fig. 3C). These findings establish TBC1D24 as a regulator of G1/S progression, mechanistically explaining the reduced proliferation, colony formation, and tumor growth observed in vitro and in vivo (Fig. 2).

Mechanistically, our data further reveal that TBC1D24 promotes activation of the PI3K–Akt pathway. KEGG enrichment and GSEA analyses consistently identified PI3K–Akt signaling as significantly associated with TBC1D24-high tumors (Fig. 4A–C) [27]. At both transcriptomic and protein levels, TBC1D24 expression correlated with activation markers of this pathway, including AKT1, MTOR, MYC, and CCND1 (Fig. 4B, 4E). Functional perturbation experiments demonstrated that TBC1D24 knockdown reduced phosphorylation of PI3K, Akt, and GSK3β, accompanied by decreased c-Myc and Cyclin D1 expression, whereas TBC1D24 overexpression produced the opposite effect (Fig. 4G). In vivo xenograft tissues confirmed reduced p-AKT and c-Myc levels following TBC1D24 silencing (Fig. 4H). These findings position TBC1D24 as a novel upstream modulator of PI3K–Akt signaling in breast cancer. The PI3K–Akt axis is widely recognized as a central oncogenic hub regulating metabolism, survival, and cell cycle progression in TNBC [28]. While classical activation mechanisms involve PIK3CA mutations or PTEN loss [29], our findings suggest an alternative regulatory layer involving intracellular trafficking control. TBC1D24 belongs to the Tre-2/Bub2/Cdc16 (TBC) domain–containing family, proteins known to function as Rab GTPase-activating proteins that regulate vesicular transport [8, 11]. Although the precise Rab substrates in breast cancer remain to be defined, it is plausible that TBC1D24 modulates receptor trafficking dynamics, thereby sustaining upstream growth factor receptor signaling and reinforcing PI3K–Akt pathway activation. This trafficking-dependent regulatory paradigm warrants further investigation through proteomic interactome analyses and receptor recycling assays.

Importantly, this study also identifies ZNF354C as a direct upstream transcriptional activator of TBC1D24 (Fig. 5). Integrative screening combining transcription factor prediction databases, correlation analyses, and single-cell expression profiling revealed ZNF354C as a candidate regulator. ZNF354C expression strongly correlated with TBC1D24 in both TCGA and CCLE cohorts (Fig. 5D–E). Promoter motif analysis identified a ZNF354C binding site approximately 286 bp upstream of the TBC1D24 transcription start site, and ChIP-qPCR confirmed direct promoter occupancy (Fig. 5F). Functional assays demonstrated that ZNF354C knockdown reduced TBC1D24 mRNA and protein expression, whereas overexpression increased TBC1D24 levels (Fig. 5G–H). These findings establish a hierarchical regulatory axis: ZNF354C transcriptionally activates TBC1D24, which in turn stimulates PI3K–Akt signaling and G1/S progression.

From a translational perspective, targeting upstream regulatory nodes such as TBC1D24 may offer therapeutic advantages over direct kinase inhibition. Clinical application of PI3K or Akt inhibitors has been limited by systemic toxicity and rapid compensatory feedback activation [30]. Intervening at the level of trafficking-dependent signal amplification may provide a more selective strategy to attenuate oncogenic signaling without complete kinase blockade. Furthermore, the ZNF354C–TBC1D24 axis presents an additional regulatory layer that could potentially be exploited using transcriptional or epigenetic targeting approaches.

Several limitations should be acknowledged. First, the specific Rab GTPase substrates of TBC1D24 in breast cancer cells have not yet been experimentally defined and require mass spectrometry–based interactome mapping. Second, although xenograft models demonstrated tumor growth inhibition following TBC1D24 silencing, these experiments were conducted in immunodeficient mice and therefore do not capture potential immune-modulatory effects [31]. Third, while PI3K–Akt activation was clearly demonstrated, future rescue experiments, such as TBC1D24 knockdown combined with constitutively active Akt expression would further strengthen causal pathway validation. Finally, structural and functional analysis of the TLDc domain of TBC1D24 may reveal additional roles in oxidative stress adaptation and tumor cell survival [14].

Collectively, our findings define a previously unrecognized oncogenic signaling cascade centered on TBC1D24 and provide a comprehensive mechanistic framework linking transcriptional regulation, intracellular trafficking, mitogenic signaling, and cell cycle progression in breast cancer.

Conclusion

In summary, this study demonstrates that TBC1D24 functions as a critical promoter of breast cancer progression through activation of the PI3K–Akt signaling pathway and acceleration of G1/S cell cycle transition. We identify ZNF354C as a direct transcriptional activator of TBC1D24, thereby establishing a hierarchical regulatory axis in which ZNF354C upregulates TBC1D24, leading to enhanced PI3K/Akt signaling, increased c-Myc and Cyclin D1 expression, and sustained proliferative capacity. These findings expand the functional landscape of TBC1D24 beyond its previously characterized roles in neuronal biology and position it as a novel molecular driver in breast cancer. The ZNF354C–TBC1D24–PI3K/Akt axis represents a promising therapeutic vulnerability and a potential biomarker framework for stratifying aggressive breast cancer subtypes. Further mechanistic dissection and therapeutic validation may pave the way for trafficking-based intervention strategies aimed at disrupting oncogenic signal amplification in breast cancer.

Declarations

Ethics approval and consent to participate

All in vivo animal procedures and experimental protocols were subjected to rigorous prospective review and received formal ethical approval from the Wenzhou Medical University, People's Hospital of Cangnan

(Animal Ethics Approval No.: 2025058).

Consent for publication

Not applicable. No individual personal data or identifiable human images are included in this manuscript.

Availability of data and materials

Data is available on request from the authors.

Competing interests

The authors declare that they have no competing financial, professional, or non-financial interests.

Funding

This work was supported by Wenzhou Medical University, People's Hospital of Cangnan.

Authors' contributions

Y.D.G. and J.Y. conceptualized and designed the study. J.Y., J.H.Y., and Q.Y.L. performed the research and collected the clinical data. J.Y. and J.H.Y. analyzed the data. J.Y. wrote the original draft of the manuscript. Y.D.G., J.H.Y., and Q.Y.L. critically reviewed and edited the manuscript. Y.D.G. supervised the project. All authors have read and approved the final manuscript.

References

1. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, 71(3), 209-249. https://doi.org/10.3322/caac.21660
2. Garrido-Castro, A. C., Lin, N. U., & Polyak, K. (2019). Heterogeneity of Triple-Negative Breast Cancer: Current Concepts and Future Directions. *Cancer Discovery*, 9(2), 176–194. https://doi.org/10.1158/2159-8290.CD-18-1177
3. Dent, R., Trudeau, M., Pritchard, K. I., Hanna, W. M., Kahn, H. K., Sawka, C. A., et al. (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. *Clinical Cancer Research*, 13(15), 4429–4434. https://doi.org/10.1158/1078-0432.CCR-06-3045
4. Schmid, P., Rugo, H. S., Adams, S., Enos, R. A., & Molinero, L. (2020). Pembrolizumab plus chemotherapy in metastatic triple-negative breast cancer. *The New England Journal of Medicine*, 382(22), 2081–2092. https://doi.org/10.1056/NEJMoa1917049
5. Bianchini, G., Balko, J. M., Mayer, I. A., Sanders, M. E., & Gianni, L. (2016). Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nature Reviews Clinical Oncology*, 13(11), 674–690. https://doi.org/10.1038/nrclinonc.2016.66

6. Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, 144(5), 646-674. https://doi.org/10.1016/j.cell.2011.02.013
7. Wandinger-Ness, A., & Zerial, M. (2014). Rab GTPases and the compartmentalization of the endosomal system. *Cold Spring Harbor Perspectives in Biology*, 6(11), a022616. https://doi.org/10.1101/cshperspect.a022616
8. Mellman, I., & Yarden, Y. (2013). Endocytosis and cancer. *Cold Spring Harbor Perspectives in Biology*, 5(12), a016949. https://doi.org/10.1101/cshperspect.a016949
9. Frasa, M. A., Koessmeier, K. T., Ahmadian, M. R., & Braga, V. M. (2012). The complexity of the TBC domain-containing RabGAP family. *Biochemical Society Transactions*, 40(6), 1395–1403. https://doi.org/10.1042/BST20120177
10. Stalder, D., & Chia, G. W. (2021). The role of TBC domain proteins in cancer: From vesicle trafficking to signaling. *Cancer Reports*, 4(3), e1350. https://doi.org/10.1002/cnr2.1350
11. Solman, M., Ligthart, E. M., & Zwartkruis, F. J. T. (2020). TBC domain-containing proteins in cancer: More than just RabGAPs. *Journal of Cell Science*, 133(18), jcs248559. https://doi.org/10.1242/jcs.248559
12. Pei, L., Han, Y., & Zhao, Y. (2019). TBC1D3 promotes cancer cell proliferation by enhancing EGFR signaling. *Journal of Experimental & Clinical Cancer Research*, 38(1), 112. https://doi.org/10.1186/s13046-019-1111-9
13. Pan, W. J., et al. (2021). TBC1D9 acts as a novel driver in breast cancer progression. *Oncogene*, 40, 567-578. https://doi.org/10.1038/s41388-020-01552-8
14. Zhang, J., Wang, Y., & Gao, Z. (2021). The TLDc domain: An emerging role in oxidative stress and neurodegeneration. *Frontiers in Molecular Neuroscience*, 14, 650. https://doi.org/10.3389/fnmol.2021.650831
15. Falace, A., Filipello, F., La Padula, V., Vanni, N., Madia, F., De Pietri, T., et al. (2010). TBC1D24, an important gene for cerebellar development, is mutated in DOORS syndrome. *American Journal of Human Genetics*, 87(3), 365–370. https://doi.org/10.1016/j.ajhg.2010.07.014
16. Mucha, B. E., Hennekam, R. C., & Sisodiya, S. M. (2019). TBC1D24-related disorders. *GeneReviews*. https://www.ncbi.nlm.nih.gov/books/NBK481554/
17. Finelli, A. L., Aprile, D., Castagna, R., & Benfenati, F. (2019). TBC1D24 regulates axonal outgrowth and membrane trafficking at the presynaptic terminal. *Scientific Reports*, 9(1), 1–14. https://doi.org/10.1038/s41598-019-51473-w
18. Xie, Y., et al. (2025). TBC1D24 promotes the progression of the breast cancer cells via ARF6/PLD axis under hypoxia. *Science Progress*, 108(3). https://doi.org/10.1177/00368504231184321
19. Sabe, H., Hashimoto, S., Morishige, M., Ogawa, E., & Hashimoto, A. (2009). The Arf6-GTPase activating protein AMAP1 and its role in breast cancer invasion. *PNAS*, 106(4), 1234–1239. https://doi.org/10.1073/pnas.0811910106
20. Caswell, P. T., Vadrevu, S., & Norman, J. C. (2009). Integrins: masters and slaves of endocytic transport. *Nature Reviews Molecular Cell Biology*, 10(12), 843–853. https://doi.org/10.1038/nrm2799

21. Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., et al. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discovery*, 2(5), 401–404. https://doi.org/10.1158/2159-8290.CD-12-0095
22. Chung, W., Eum, H. H., Lee, H. O., Lee, K. M., Lee, H. B., Kim, K. T., et al. (2017). Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nature Communications*, 8(1), 15081. https://doi.org/10.1038/ncomms15081
23. Qiu, X., Mao, Q., Tang, Y., Wang, L., Chawla, R., Pliner, H. A., et al. (2017). Reversed graph embedding resolves complex single-cell trajectories. *Nature Methods*, 14(10), 979–982. https://doi.org/10.1038/nmeth.4402
24. Polyak, K. (2011). Heterogeneity in breast cancer. *Journal of Clinical Investigation*, 121(10), 3786–3788. https://doi.org/10.1172/JCI60534
25. Hanahan, D. (2022). Hallmarks of Cancer: New Dimensions. *Cancer Discovery*, 12(1), 31-46. https://doi.org/10.1158/2159-8290.CD-21-1059
26. Costa, R. L. B., Han, H. S., & Gradishar, W. J. (2018). Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: a review. *Breast Cancer Research and Treatment*, 169(3), 397–406. https://doi.org/10.1007/s10549-018-4690-y
27. Millis, S. Z., Gatalica, Z., Winkler, J., Vranic, S., Kimbrough, J., Reddy, S., et al. (2015). Predictive biomarker profiling of >6000 breast cancer patients shows heterogeneity in TNBC, with treatment implications. *Clinical Breast Cancer*, 15(6), 473–481. https://doi.org/10.1016/j.clbc.2015.04.008
28. Janku, F., Yap, T. A., & Meric-Bernstam, F. (2018). Targeting the PI3K pathway in cancer: are we making headway? *Nature Reviews Clinical Oncology*, 15(5), 273–291. https://doi.org/10.1038/nrclinonc.2018.28
29. Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J., & Pollard, J. W. (2003). Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *American Journal of Pathology*, 163(5), 2113–2126. https://doi.org/10.1016/S0002-9440(10)63568-7

Figures

Figure 1

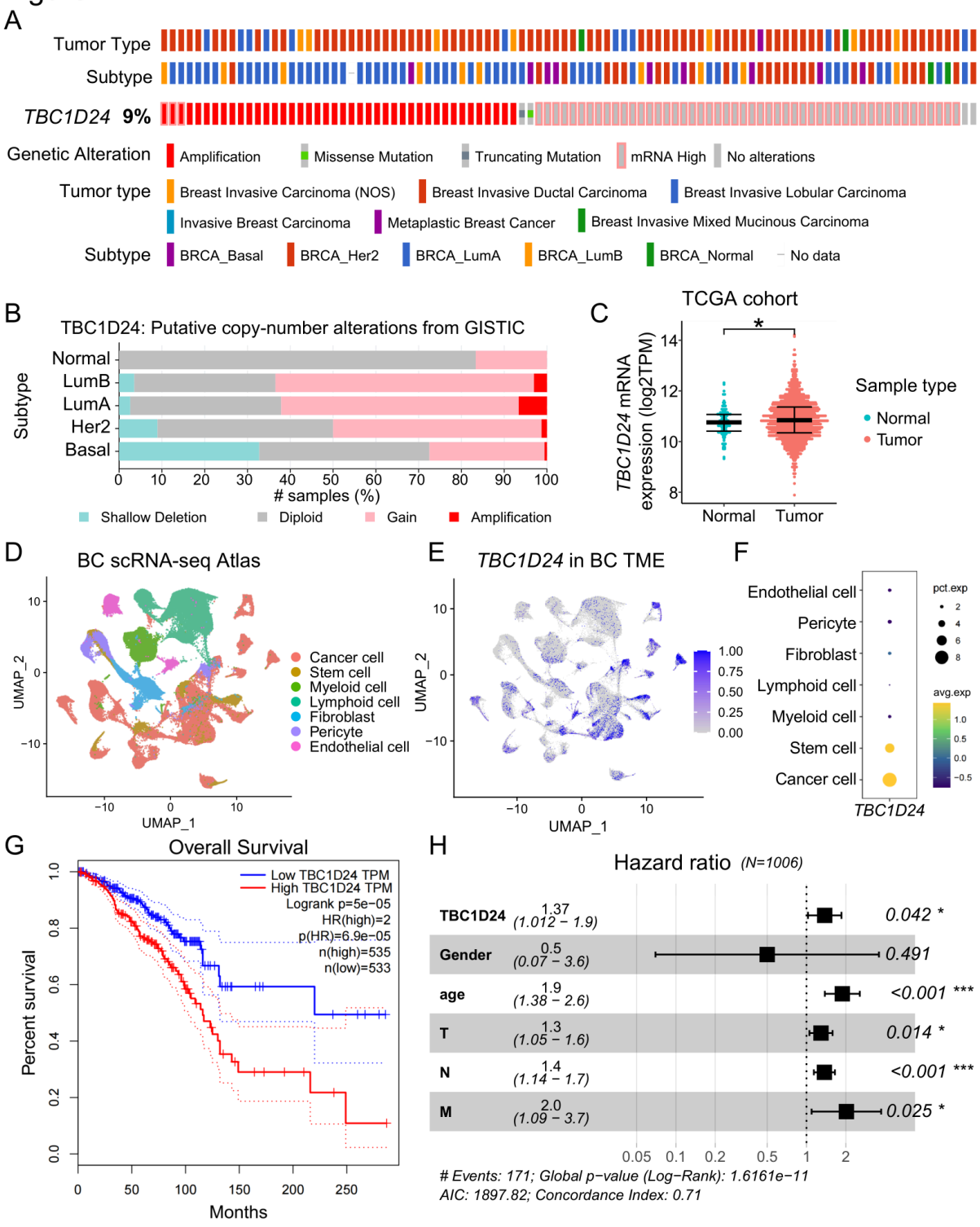

Figure 1

Genomic alterations, cellular distribution, and prognostic significance of TBC1D24 in breast cancer. (A) Oncoprint representation of TBC1D24 alterations in the TCGA-BRCA cohort showing an overall alteration frequency of 9%, including amplification, missense mutation, truncating mutation, and mRNA upregulation. Tumor types and molecular subtypes are annotated above. (B) GISTIC-based analysis of TBC1D24 copy-number alterations across intrinsic breast cancer subtypes, illustrating subtype-specific

distributions of shallow deletion, diploid status, gain, and amplification. (C) Comparison of TBC1D24 mRNA expression levels between normal breast tissue and tumor samples in the TCGA cohort (log2TPM). Statistical significance indicated by *P < 0.05. (D) UMAP visualization of major cell populations identified in a breast cancer single-cell RNA-seq atlas. (E) Feature plot displaying TBC1D24 expression distribution across single-cell clusters. (F) Dot plot summarizing average expression (color intensity) and percentage of expressing cells (dot size) for TBC1D24 across annotated cell types. (G) Kaplan–Meier overall survival analysis stratified by TBC1D24 expression level (high vs. low). (H) Multivariate Cox proportional hazards regression analysis evaluating TBC1D24 as an independent prognostic factor adjusted for gender, age, and TNM staging variables. Hazard ratios (HR) and 95% confidence intervals are shown.

Figure 2

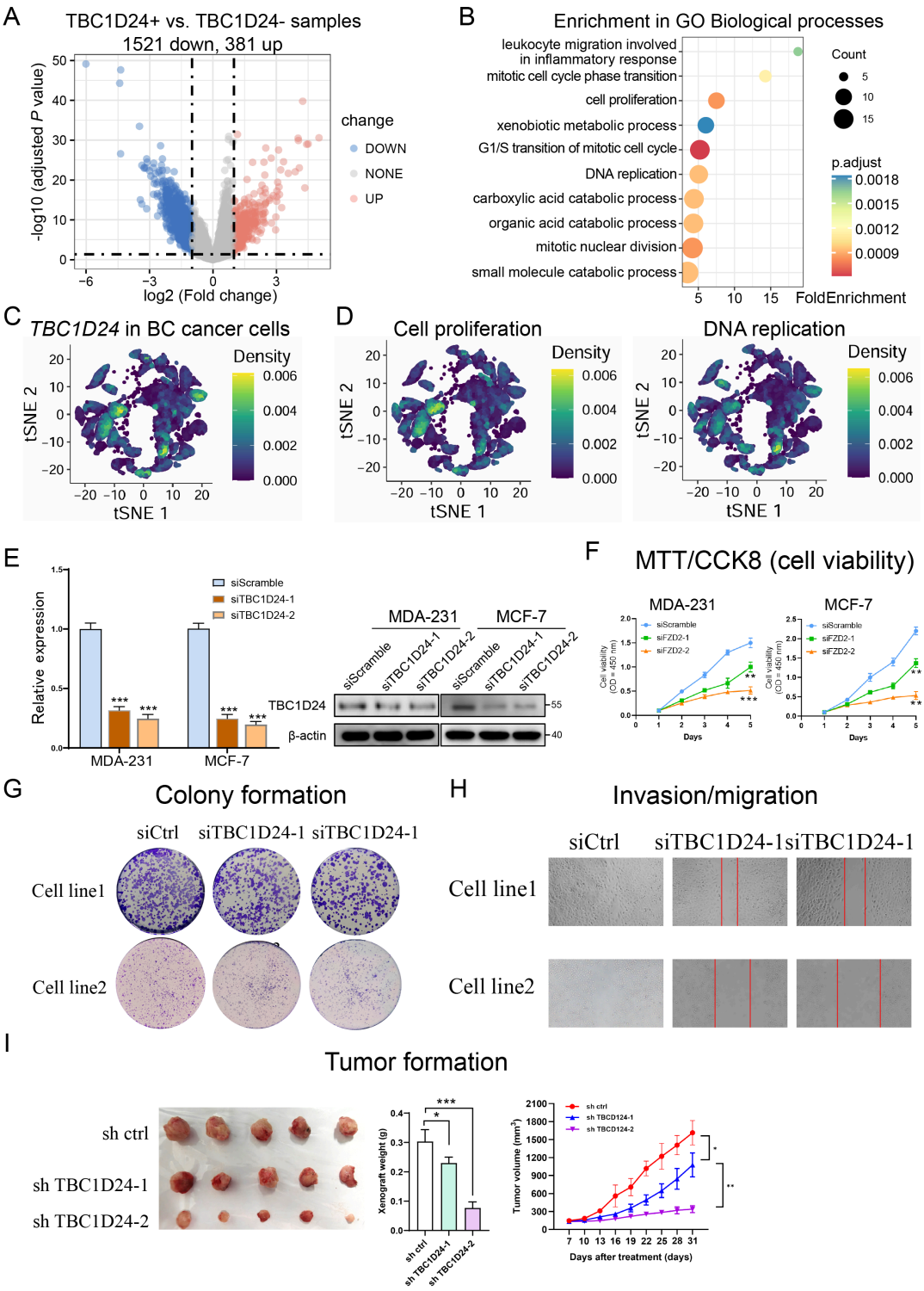

Figure 2

TBC1D24 promotes proliferation and tumorigenic phenotypes in breast cancer. (A) Volcano plot showing differentially expressed genes between TBC1D24-high and TBC1D24-low tumor samples in the TCGA cohort (381 upregulated, 1,521 downregulated genes). (B) GO biological process enrichment analysis of differentially expressed genes, highlighting significant enrichment in cell proliferation, G1/S transition, DNA replication, and mitotic cell cycle–related pathways. (C) tSNE density projection showing TBC1D24

expression distribution in breast cancer malignant cells. (D) Density projection of cell proliferation and DNA replication signatures across cancer cells. Lower panel: qRT-PCR and western blot validation of TBC1D24 knockdown efficiency in breast cancer cell lines. (E) CCK-8 cell viability assays in MDA-MB-231 and MCF-7 cells following TBC1D24 silencing. (F) Colony formation assays demonstrating reduced clonogenic capacity after TBC1D24 knockdown. (G) Wound-healing assays showing impaired migration/invasion ability upon TBC1D24 depletion. (H) Xenograft tumor formation assays. Left: representative tumor images. Middle: tumor weight quantification. Right: tumor growth curves over time.

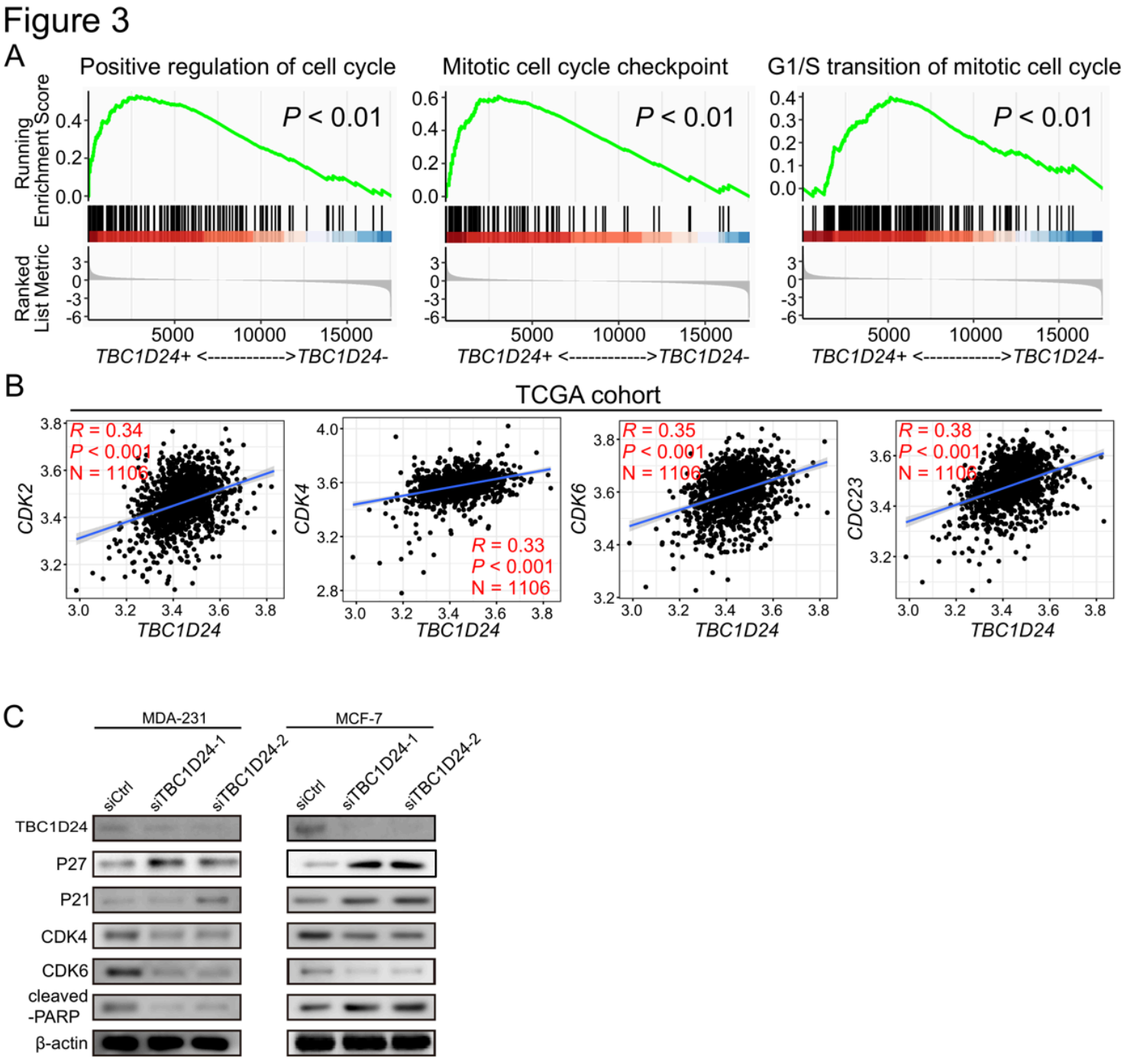

Figure 3

TBC1D24 promotes G1/S transition and cell cycle progression in breast cancer. (A) Gene Set Enrichment Analysis (GSEA) showing enrichment of cell cycle–related biological processes in TBC1D24-high tumors within the TCGA cohort. (B) Correlation analysis between TBC1D24 expression and cell cycle–related genes (CDK2, CDK4, CDK6, CDC23) in the TCGA breast cancer cohort (N = 1106). (C) Western blot analysis of cell cycle–related proteins in MDA-MB-231 and MCF-7 cells following TBC1D24 knockdown. Expression levels of CDK4, CDK6, P21, P27, and cleaved PARP were assessed. β-actin served as a loading control.

Figure 4

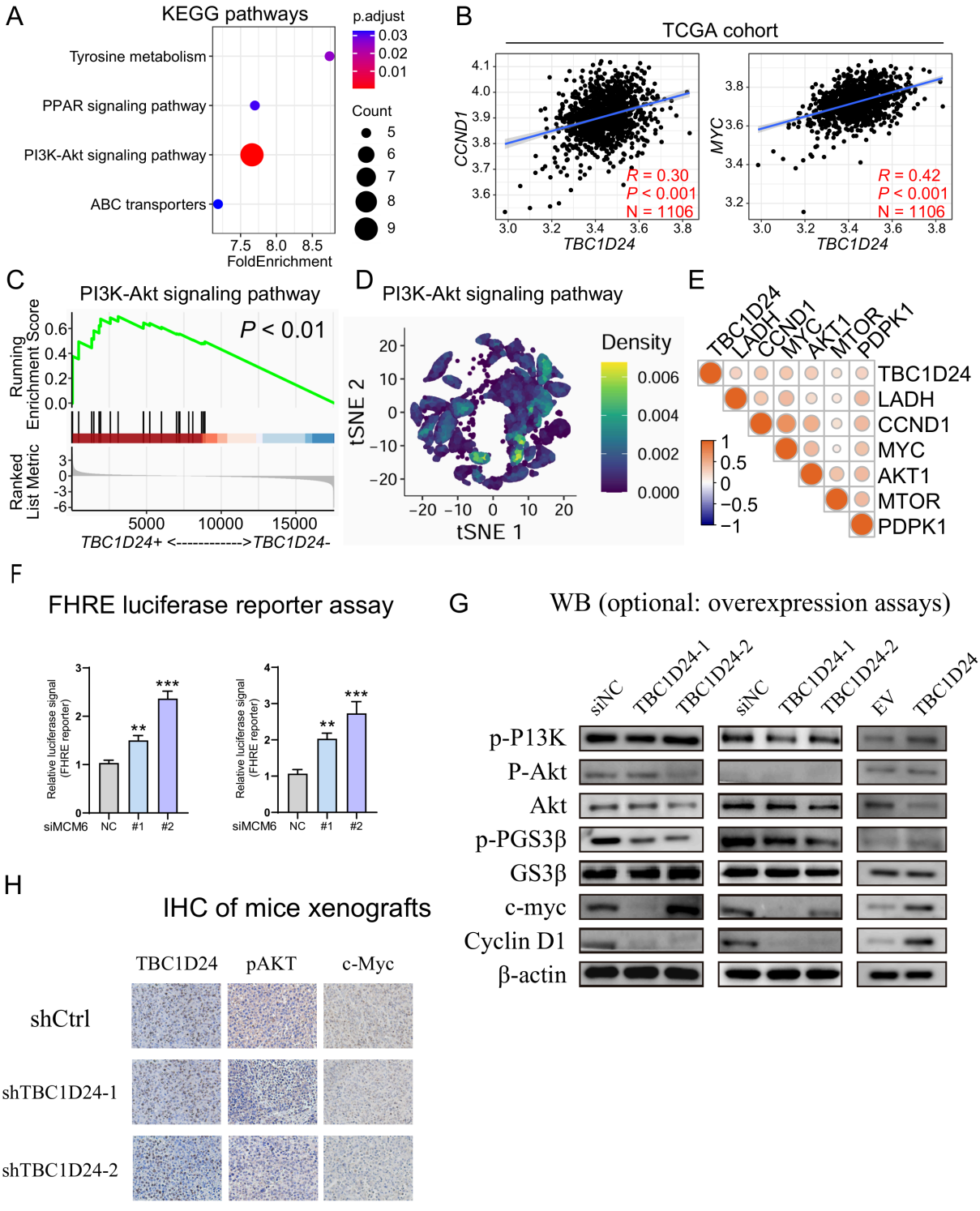

Figure 4

TBC1D24 activates the PI3K-Akt signaling pathway in breast cancer. (A) KEGG pathway enrichment analysis of differentially expressed genes between TBC1D24-high and TBC1D24-low tumors. (B) Correlation analysis between TBC1D24 and CCND1/MYC expression in the TCGA breast cancer cohort (N = 1106). (C) GSEA showing enrichment of the PI3K-Akt signaling pathway in TBC1D24-high tumors. (D) Single-cell tSNE density projection of PI3K-Akt signaling activity. (E) Correlation matrix between TBC1D24 and PI3K-Akt pathway–related genes. (F) FHRE luciferase reporter assay following TBC1D24 overexpression. (G) Western blot analysis of PI3K-Akt pathway components after TBC1D24 knockdown or overexpression. (H) Immunohistochemical staining of TBC1D24, p-AKT, and c-Myc in xenograft tumors.

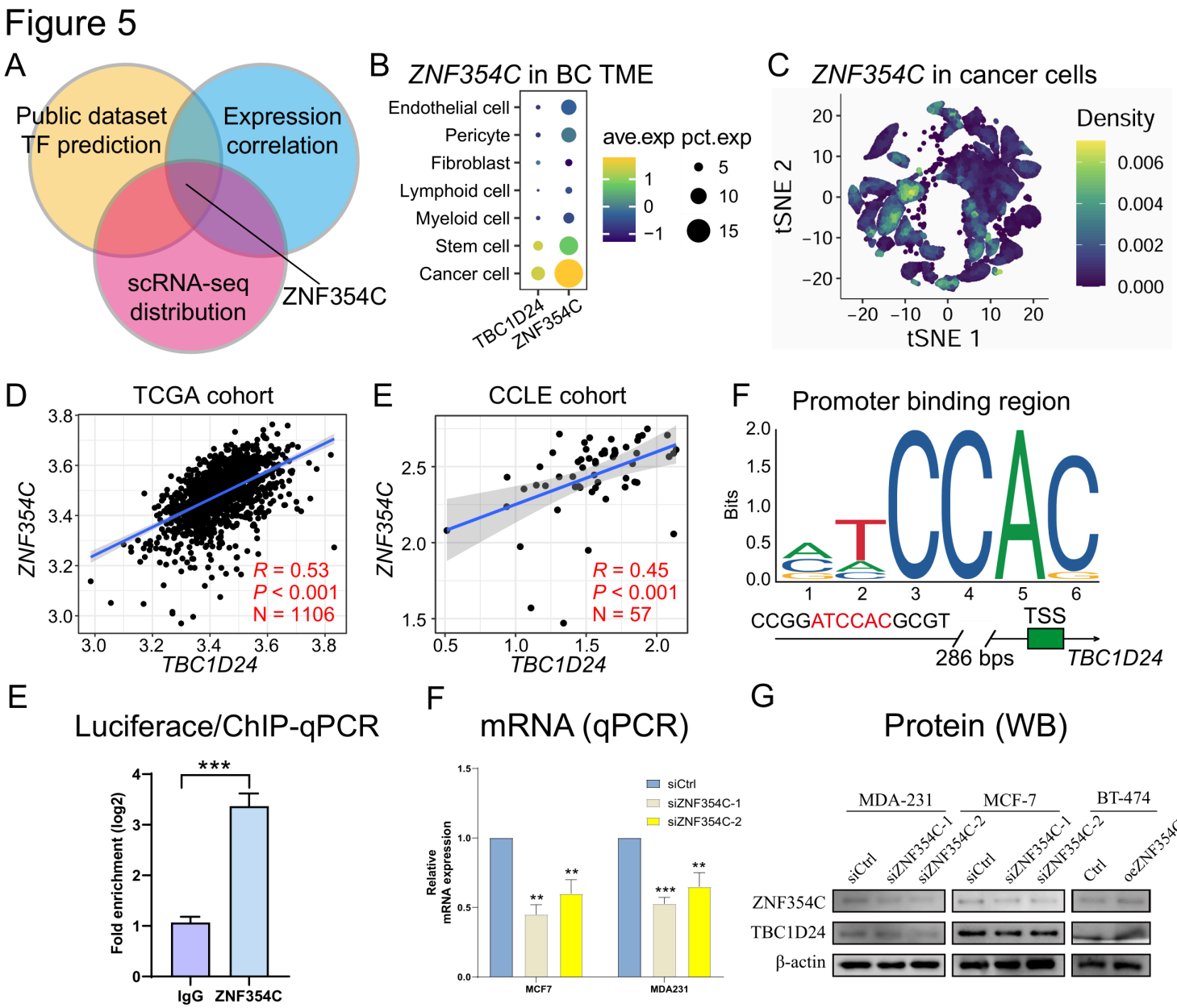

Figure 5

ZNF354C transcriptionally activates TBC1D24 in breast cancer. (A) Identification of ZNF354C as a candidate transcription factor regulating TBC1D24 through integrative analysis of public TF prediction databases, expression correlation, and single-cell RNA-seq distribution. (B) Bubble plot showing ZNF354C expression across different cell types in the breast cancer tumor microenvironment. (C) tSNE density projection of ZNF354C expression in malignant cells. (D) Correlation analysis between ZNF354C and TBC1D24 in the TCGA breast cancer cohort (N = 1106). (E) Correlation analysis in the CCLE breast cancer cell line cohort (N = 57). (F) Predicted ZNF354C binding motif within the TBC1D24 promoter region (~286 bp upstream of TSS). (G) ChIP-qPCR and luciferase reporter assays demonstrating direct binding and transcriptional activation of the TBC1D24 promoter by ZNF354C. (H) qPCR and western blot validation of TBC1D24 expression following ZNF354C knockdown or overexpression.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- WesternBlot.pptx