

## **ZNF526 Promotes Triple-Negative Breast Cancer Progression through SHMT1-Mediated Serine Metabolism and Enhanced Antioxidant Defense**

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### **Abstract**

Triple-negative breast cancer (TNBC) is a particularly aggressive cancer type marked by few effective therapies and generally unfavorable survival rates. Although rs3810151 (p.Val94Ala) in the ZNF526 gene has been linked to breast cancer risk as a susceptibility variant, its precise biological contributions and mechanisms of action in TNBC remain unclear. Here, we report that ZNF526 is strongly upregulated in TNBC specimens, and higher levels of this protein are associated with significantly worse patient survival. Through a series of functional tests, we determined that increasing ZNF526 levels drives enhanced growth of TNBC cells, while reducing its expression curbs tumor development. At the molecular level, ZNF526 promotes SHMT1 expression, thereby increasing activity in the serine-glycine-one-carbon (SGOC) metabolic pathway. This change elevates glutathione (GSH) synthesis, lowers reactive oxygen species (ROS) buildup, and improves the cell's ability to handle oxidative stress, all of which help advance TNBC progression. Overall, our data position ZNF526 as an important new factor controlling redox status in TNBC by influencing SHMT1 and associated metabolism, making it a candidate for future targeted treatments.

**Keywords:** ZNF526, TNBC, SHMT1, GSH, Serine metabolism

### **Introduction**

Triple-negative breast cancer (TNBC) stands out due to its rapid progression, limited long-term survival prospects, and frequent spread to distant sites including the liver, brain, and lungs, posing a serious risk to women's well-being [1]. Currently, chemotherapy is the main treatment approach, helping to improve surgical outcomes and achieve complete pathological responses in some cases [2]. Even so, many patients experience

persistent microscopic disease and later relapse, restricting the overall effectiveness of existing strategies [3]. Discovering new molecular insights into TNBC growth is therefore critical for broadening the available drug targets and treatment options.

Proteins belonging to the zinc finger (ZNF) family are key regulators of gene transcription and play a role in controlling cell movement, programmed cell death, cell division, and metastatic behavior in tumors [4]. The ZNF526 gene encodes a Krüppel-type zinc finger protein with 13 zinc finger motifs and 679 amino acids. This protein is expressed broadly in both mature and developing tissues, reaching particularly high amounts in the ovary and showing substantial presence in the lung, pancreas, spleen, testis, spinal cord, and fetal liver [5]. In addition, alterations in the coding regions of ZNF526 have been shown to have clear associations with the development of Hodgkin lymphoma [6]. Earlier research

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by Zhang *et al.* [7] identified the single-nucleotide polymorphism rs3810151 (Val94Ala) in ZNF526 as a notable genetic factor increasing breast cancer susceptibility, calling for deeper examination of its functional effects.

To support continuous proliferation, cancer cells extensively alter their metabolism. This reprogramming enables them to meet demands for energy, building materials, signaling, and protection against oxidative damage in changing tumor microenvironments [8, 9]. The serine-glycine-one-carbon (SGOC) network forms a central component of these adaptations. It links serine and glycine processing with one-carbon transfers from folate and methionine cycles, supplying precursors for nucleotide production, epigenetic regulation, and glutathione generation. In doing so, it bridges biomass needs with redox maintenance and tumor advancement [10]. Because many aggressive cancers overexpress enzymes in this pathway, the SGOC network represents an attractive point for therapeutic attack [11-13].

Serine hydroxymethyltransferases (SHMT) are essential enzymes in the SGOC system, reversibly converting serine and tetrahydrofolate into glycine and 5,10-methylene tetrahydrofolate. The family includes the cytosolic SHMT1 and mitochondrial SHMT2 isoforms [10, 14]. These enzymes support cancer cell survival by enabling de novo nucleotide synthesis for growth and by sustaining antioxidant systems that counteract oxidative stress, as observed in several cancer types [15, 16]. One illustration comes from non-small cell lung cancer (NSCLC), where simultaneous loss of LKB1 and KEAP1 tumor suppressors ramps up SHMT activity to meet heightened requirements for one-carbon units needed for antioxidant defense [17]. In TNBC specifically, the epigenetic modulator KDM2B has been found to strengthen protection against oxidative stress by boosting SGOC pathway flux [18].

Our work shows that ZNF526 is elevated in TNBC and is closely associated with poor clinical outcomes. At the mechanistic core, ZNF526 reshapes SGOC metabolism by raising SHMT1 transcription. The resulting metabolic boost increases intracellular glutathione (GSH) production, efficiently neutralizes reactive oxygen species (ROS), and fortifies antioxidant capabilities, collectively fueling tumor expansion. These discoveries establish the ZNF526/SHMT1/GSH cascade as a major pathway governing redox balance and aggressive behavior in TNBC, suggesting strong therapeutic targeting potential.

## Materials and Methods

### *Data collection and analysis*

Information on ZNF526 expression together with matching clinical details was downloaded from the TCGA pan-cancer collection using the UCSC Xena browser (<https://xenabrowser.net/datapages/>) and the UALCAN resource (<https://ualcan.path.uab.edu/index.html>). We compared ZNF526 levels between TCGA tumor samples and normal control tissues. Analyses were performed in R (version 4.0.3) while adhering to strict bioinformatics practices and quality controls common in cancer genomics studies.

### *Patients*

Tumor tissues from breast cancer cases were gathered during surgical procedures at the Department of Breast Surgery, East Hospital, Tongji University. The Clinical Research Ethics Committee at East Hospital, Tongji University approved the study. All individuals or their legal representatives provided written informed consent before participation. The entire project followed the ethical principles outlined in the Declaration of Helsinki and relevant institutional rules.

### *Cell culture*

TNBC lines MDA-MB-231, MDA-MB-468, BT20, and HCC1806 were acquired from the Cell Resource Center of the Shanghai Academy of Biological Sciences, Chinese Academy of Sciences. MDA-MB-231 and MDA-MB-468 cells were grown in L15 medium with 10% fetal bovine serum (FBS) plus penicillin (50 U/ml) and streptomycin (50 µg/ml) at 37 °C in a humidified atmosphere without additional CO<sub>2</sub>. BT20 cells were cultured in MEM, and HCC1806 cells in RPMI, both supplemented with 10% FBS and the same antibiotics, at 37 °C under 5% CO<sub>2</sub>.

### *Plasmids, siRNA, and lentivirus preparation*

A codon-optimized version of the full wild-type human ZNF526 coding sequence was synthesized and cloned into the PCDH-Puro lentiviral vector, yielding PCDH-Puro-ZNF526. The V94A point mutant construct (PCDH-ZNF526-V94A) was derived from the wild-type plasmid with the Q5® Site-Directed Mutagenesis Kit (E0554S, New England Biolabs) following the supplier's guidelines. SHMT1 promoter segments were synthesized and inserted upstream of the luciferase gene in pGL3.0-

Basic vectors for reporter studies. Lentiviral shRNA plasmids against ZNF526 were built in the PLKO.1-puro system. Knockout plasmids LentiCRISPR-V2-sgZNF526 and LentiCRISPR-V2-sgSHMT1 were prepared using the LentiCRISPR-V2 backbone, with sgRNA.

Lentiviruses were packaged in HEK293T cells (ATCC) by co-transfecting the transfer plasmid with psPAX2 and pMD2.G at a 1:1:1 ratio via Lipofectamine 3000 (Invitrogen). Culture supernatants containing virus were collected 48 and 72 hours later, pooled, and filtered (0.45 µm). Target cells seeded in 6-well plates were infected with lentivirus in the presence of 8 µg/mL polybrene (Sigma-Aldrich) for 24 hours. Medium was then changed to standard growth conditions for another 24 hours, followed by selection with 2.0 µg/mL puromycin (InvivoGen) for 48 hours. Stable lines were maintained at 1 µg/mL puromycin.

Transient gene silencing was achieved by transfecting cells with SHMT1-targeted siRNAs or control siRNA (NC-siRNA) using Lipofectamine RNAiMAX (13778150, Invitrogen). Functional experiments were conducted 48 to 72 hours post-transfection.

#### *Animal studies*

Six-week-old female C57BL/6 nude mice were purchased from Shanghai Model Organisms Center in Shanghai, China. Tumor cells suspended in 100 µL volume were inoculated directly into the mammary fat pad of each animal. Visible and stable tumors appeared approximately one week after inoculation. Mice were humanely euthanized 2 to 4 weeks after tumor establishment. Serum and tumor samples were harvested under sterile conditions for further processing and analysis. In the SHIN1 treatment experiments, once subcutaneous tumors reached appropriate size, animals were randomly allocated into four experimental groups. They received intraperitoneal injections of either vehicle or SHIN1 (100 mg/kg body weight) three times per week [19]. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji University and were conducted in accordance with AAALAC International guidelines.

#### *Cell proliferation assay*

Between 1,000 and 2,000 cells were plated per well into 96-well plates containing roughly 100 µL of medium. Each treatment condition was tested in 4-6 replicate

wells, along with parallel control wells. Cultures were maintained at 37 °C in a humidified incubator for the appropriate time period. Medium was then removed, and fresh medium supplemented with 10% CCK-8 solution (A311-01/02, Vazyme) was added. Plates were incubated for an additional 1–2 hours at 37 °C. Absorbance readings at 450 nm were obtained using a microplate reader (model 1410101, Thermo). Proliferation curves were generated from the resulting data using GraphPad software.

Before the CCK-8 assay, cells were transfected using 20 nM SHMT1-specific siRNA (si-SHMT1) or treated with 10 nM SHIN1 (HY-112066, MCE) for 72 hours. Rescue or supplementation experiments employed the following concentrations of relevant metabolites: N-acetylcysteine (NAC, 5 mM), purine nucleosides (5 µM adenosine + 5 µM guanosine), pyrimidine nucleosides (5 µM each of cytidine, thymidine, and uridine), reduced L-glutathione (5 mM), H<sub>2</sub>O<sub>2</sub> (100 µM), or S-adenosylmethionine (SAM, 100 µM). Nucleosides and H<sub>2</sub>O<sub>2</sub> were obtained from Sigma-Aldrich; SAM was sourced from MedChemExpress (MCE).

#### *Colony formation assay*

Cells were seeded at optimized densities in 6-well plates (800 cells per well for MDA-MB-231; 2,000 cells per well for MDA-MB-468) and allowed to grow for 12 days, with complete medium replacement every 3–4 days. After the growth period, medium was discarded, and cells were gently washed twice with PBS. Colonies were fixed with 4% paraformaldehyde (BL539A, Biosharp) for 10–15 minutes, washed again, and stained with crystal violet solution (548629, Macklin) for 10–30 minutes. Repeated PBS washes removed excess stain. Colonies were photographed under a microscope and analyzed quantitatively with ImageJ software (National Institutes of Health). Images were converted to 8-bit grayscale, then adjusted for brightness/contrast and globally thresholded to produce binary images. The Analyze Particles function was used with calibrated size cutoffs based on colony dimensions and a circularity range of 0.00–1.00. Colony numbers were counted, and formation efficiency was expressed as (colonies formed / cells seeded) × 100%.

#### *Cell cycle analysis via flow cytometry*

For cell cycle profiling, roughly  $1 \times 10^6$  cells were collected, rinsed twice in ice-cold PBS, and fixed overnight in 70% ethanol at 4 °C. Fixed cells were

pelleted by centrifugation at 300 g for 5 minutes, then resuspended in 500  $\mu$ L of PBS containing 50  $\mu$ g/mL propidium iodide (Sigma-Aldrich), 100  $\mu$ g/mL RNase A (Thermo Fisher Scientific), and 0.1% Triton X-100. Samples were incubated in the dark at 37 °C for 30 minutes before immediate analysis.

Data acquisition was performed on a BD FACS Canto II instrument using a 488-nm laser line. PI emission was collected through a 585/42 nm filter, recording a minimum of 10,000 events per sample. Cell cycle distribution was calculated using FlowJo version 10.9. Experiments were repeated three times independently, each in triplicate.

#### *Annexin V/PI staining for apoptosis detection*

Apoptosis rates were determined with the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, Cat. #556547) following the supplier's instructions. In short,  $1 \times 10^6$  cells were harvested per sample, washed twice with ice-cold PBS, and resuspended in 100  $\mu$ L of  $1 \times$  binding buffer. Cells were labeled by adding 5  $\mu$ L Annexin V-FITC and 5  $\mu$ L PI (50  $\mu$ g/mL), gently mixing, and incubating for 15 minutes at room temperature in the dark. Before measurement, 400  $\mu$ L of  $1 \times$  binding buffer was added.

Flow cytometry analysis was performed on a Beckman Coulter CytoFLEX within 1 hour. FITC signal was detected in the FL1 channel (525/540 nm filter), and PI in the FL3 channel (610/620 nm filter). Quadrant gating distinguished live, early apoptotic, late apoptotic, and necrotic populations using FlowJo v10.9 software. Appropriate controls included unstained cells, single-color stained samples, and positive apoptosis controls treated with 10  $\mu$ M camptothecin for 6 hours.

#### *Measurement of GSH and GSSG levels*

Intracellular and serum concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using commercial ELISA kits (KTB1600 for GSH and KBT1610 for GSSG, Abbkine). All samples were assayed with 3–6 technical replicates.

GSH protocol: Absorbance was read at 412 nm. Blank absorbance was subtracted from all wells to calculate  $\Delta A$ . A standard curve was plotted with standard concentration on the y-axis versus  $\Delta A$  on the x-axis. Sample GSH concentration ( $\mu$ g/mL) was determined by interpolating the test  $\Delta A$  value into the linear equation.

GSSG protocol: Absorbance at 412 nm was recorded and blank-subtracted to obtain  $\Delta A$  values. The standard curve

was constructed with concentration (y-axis) against  $\Delta A$  (x-axis). Sample GSSG concentration ( $\mu$ M) was derived by substituting the corresponding  $\Delta A$  into the regression equation.

#### *Untargeted metabolomics profiling*

Cells were washed with PBS, and intracellular metabolites were extracted with 800  $\mu$ L ice-cold acetonitrile: methanol (1:1 v/v). Extracts were centrifuged at  $14,000 \times g$  for 20 minutes, and the supernatants were dried and redissolved in 100  $\mu$ L of acetonitrile: water (1:1) for LC-MS analysis. Quality control injections were inserted every fifth sample. Analysis was conducted on an Agilent 1290 UHPLC coupled to a Sciex TripleTOF 6600 Q-TOF mass spectrometer using a BEH Amide column ( $2.1 \times 100$  mm). Mobile phase A was 25 mM ammonium acetate/ammonium hydroxide, and mobile phase B was acetonitrile. The elution gradient ran as follows: 95% B for 0.5 min, decreased to 65% B at 6.5 min, to 40% B at 7.5 min, and re-equilibrated to 95% B for 3 min. ESI source parameters were  $\pm 5500$  V spray voltage, 600 °C source temperature, Gas1/Gas2 at 60, and curtain gas at 30. Full-scan spectra covered 60–1000 Da (0.20 s), with auto-MS/MS acquisition from 25–1000 Da at collision energy  $35 \pm 15$  eV.

Raw spectral data were converted to mzXML format using ProteoWizard and processed with XCMS (centWave algorithm; 10 ppm tolerance, 10–60 s peak width). Peaks were grouped with 5 s bandwidth and 0.025 m/z tolerance. CAMERA-annotated features present in > 50% of samples within a group were sum-normalized. Metabolites were identified when mass accuracy was < 10 ppm and MS/MS spectra matched in-house library standards. Multivariate modeling (PCA and OPLS-DA) was performed with the ropls package in R, with model validation via 7-fold cross-validation and permutation tests. Significantly altered metabolites were identified by VIP score > 1 and Student's t-test p-value < 0.05, with additional Pearson correlation analysis.

#### *Targeted analysis of one-carbon unit metabolites*

All reagents were HPLC-grade (MeCN and MeOH from Merck, water from Millipore,  $\text{NH}_4\text{Ac}$  from Sigma). Stock solutions of metabolite standards (1 mg/mL in methanol) from Sigma or IsoReag were stored at  $-20$  °C. Cell pellets were resuspended in 100  $\mu$ L water. A 50  $\mu$ L aliquot was mixed with 250  $\mu$ L precooled methanol ( $-20$  °C), vortexed vigorously for 2 minutes at 2500 rpm, and

subjected to three freeze-thaw cycles (5 min in liquid nitrogen followed by 5 min on ice). After centrifugation (12,000 rpm, 10 min, 4 °C), 200 µL of the supernatant was incubated at -20 °C for 30 min and then centrifuged again. The resulting 180 µL clarified supernatant was filtered and analyzed by LC-MS.

Chromatographic separation was performed using an ExionLC AD UPLC system (SCIEX) connected to a QTRAP 6500+ triple quadrupole mass spectrometer in positive electrospray ionization mode. An Accucore 150 Amide HILIC column (100 × 2.1 mm, 2.6 µm) was maintained at 40 °C. Mobile phases were 10 mM NH<sub>4</sub>Ac in water (A) and acetonitrile (B), with a flow rate of 0.66 mL/min. Gradient conditions: 95% B held for 0–1 min, linear decrease to 50% B by 6 min, maintained at 50% B until 7.5 min, then returned to 95% B by 10 min. Injection volume was 2 µL. ESI settings included 5500 V, a source temperature of 550 °C, and 35 psi of curtain gas. Quantification relied on optimized multiple reaction monitoring (MRM) transitions with specific declustering potentials and collision energies. Data processing included principal component analysis (prcomp in R with unit variance scaling), hierarchical clustering with heatmaps (pheatmap package using Pearson correlation), and OPLS-DA modeling (MetaboAnalystR, mean-centered, validated with 200 permutations). Metabolites were considered significant if they met  $VIP > 1$  and  $|\text{Log}_2 \text{ fold change}| > 1$  criteria. Pathway mapping was performed against the KEGG database, and metabolite set enrichment analysis (MSEA) employed the hypergeometric test.

#### *Immunohistochemistry (IHC)*

Tumor specimens were fixed in paraffin, embedded, and cut into thin sections. These sections were processed through standard histological steps: deparaffinization, rehydration, heat-induced antigen retrieval, and serum blocking, following conventional procedures. Primary antibodies targeting SHMT1 (1:200) and ZNF526 (1:100) were applied and incubated overnight. After development with the DAB chromogen, protein expression was visualized under a light microscope, with stronger brown staining indicating higher abundance. The percentage of positive staining was quantified using image analysis software. For tissue microarray slides, the Pattern Quant module in Quant Center 2.3 software was employed to separate brown-positive staining from blue counterstain. The Mask Area tool was used to precisely outline tissue boundaries. Histo Quant analysis then

calculated the area and grayscale intensity of positively stained regions at the appropriate brown threshold, from which optical density values were derived.

#### *Western blotting and antibodies*

Cells were harvested and lysed in RIPA buffer (P0013B, Beyotime Biotechnology), after which standard Western blot (WB) analysis was conducted. The complete WB protocol has been reported in detail elsewhere [20]. The following primary antibodies were utilized: anti-ZNF526 (54686, ThermoFisher), anti-Cyclin D1 (60186-1-Ig, Proteintech), anti-P21 (10355-1-AP, Proteintech), anti-RB1 (10048-2-Ig, Proteintech), anti-Phospho-RB1 (84692-1-RR, Proteintech), anti-SHMT1 (A12489, Abclonal), and anti-GAPDH (2118, Cell Signaling Technology). Secondary detection used HRP-conjugated Goat Anti-Rabbit IgG (A21020, Abbkine). Chemiluminescent signals were captured with a C-Digit scanner (9120, LI-COR).

#### *Quantitative real-time RT-PCR*

Total cellular RNA was isolated using TRIZOL reagent (15596026CN, Invitrogen). One microgram of RNA was converted to cDNA with a Reverse Transcription kit (15662ES, Yeasen). Real-time quantitative PCR was performed on an ABI 7500 system (Applied Biosystems) using SYBR Green I Master Mix (11200ES, Yeasen). Target gene mRNA levels were normalized to the housekeeping gene GAPDH and quantified using the  $2^{-\Delta\Delta Ct}$  method.

#### *RNA sequencing*

Total RNA was extracted from three biological replicates each of ZNF526-overexpressing (ZNF526-OE) cells and vector-control cells using TRIzol. RNA quality was verified on an Agilent Bioanalyzer with the RNA Nano chip, requiring an RNA integrity number (RIN) greater than 8.0. Strand-specific mRNA libraries were prepared with the Illumina TruSeq Stranded mRNA Kit and sequenced on a NovaSeq 6000 platform (150 bp paired-end reads, approximately 40 million reads per sample). Raw sequencing reads were quality-checked with FastQC, trimmed and filtered using Trimmomatic, and aligned to the GRCh38/hg38 human genome with HISAT2. Gene-level counts were obtained via featureCounts using GENCODE annotation. Differential expression analysis was performed with DESeq2 applying median-of-ratios normalization. Genes were classified as significantly differentially expressed if they

showed  $|\log_2 \text{fold change}| > 1$  and an FDR-adjusted P-value  $< 0.05$ . Visualization included volcano plots generated in ggplot2, plotting  $-\log_{10}(\text{FDR})$  against  $\log_2\text{FC}$ , with upregulated genes highlighted in red, downregulated in blue, and non-significant genes in grey.

#### *Intracellular ROS measurement with DCFH-DA*

Reactive oxygen species (ROS) levels in cells were evaluated using a 10  $\mu\text{M}$  DCFH-DA probe (KTB1910, Abbkine). Cells were maintained at a density below  $5 \times 10^5$  cells/mL and pre-incubated with test compounds in serum-free medium at 37 °C, protected from light (duration varied by compound). Positive control wells received 50–250  $\mu\text{M}$  of the appropriate agent. After the treatment and washing steps, cells were loaded with DCFH-DA for 30 minutes at 37 °C in the dark (minimum volume: 1 mL per well in 6-well plates or 100  $\mu\text{L}$  per well in 96-well plates), then washed. For cells in suspension, probing was performed after washing at cell densities of  $1.0 \times 10^6$  to  $2.0 \times 10^7$  cells/mL.

Fluorescence was quantified by two approaches: (1) Fluorescence microscopy: Adherent cells were imaged in serum-free medium or PBS, while suspension cells (25–50  $\mu\text{L}$ ) were placed on slides. Images were acquired using FITC filter sets (excitation 488 nm/emission 525 nm), the background was subtracted, and mean fluorescence intensity (IntDen/Area) was calculated in ImageJ. (2) Flow cytometry: Cells were detached by trypsinization ( $0.5\text{--}1.5 \times 10^5$  cells/mL) and analyzed at 488/525 nm either in real time or at fixed intervals using FlowJo v10.9 software.

#### *Luciferase reporter assay*

HEK293T cells were plated at  $5 \times 10^4$  cells per well in 24-well plates and grown for 24 hours to reach suitable density. Transient co-transfection was performed using Lipofectamine 3000 reagent according to the manufacturer's guidelines. Each well received 500 ng of firefly luciferase reporter plasmid harboring the target promoter sequence, along with 50 ng of Renilla luciferase plasmid for normalization. After 24 hours of incubation at 37 °C with 5%  $\text{CO}_2$ , cells underwent the specified treatments. Luciferase activity was measured using the Promega Dual-Luciferase Reporter Assay System according to protocol instructions. Firefly and Renilla signals were sequentially read on a GloMax Navigator luminometer. Relative promoter activity was expressed as the firefly/Renilla ratio. All experiments included at least three biological replicates; data were

normalized to control conditions and reported as mean  $\pm$  SEM.

#### *Chromatin immunoprecipitation (ChIP) assay*

Cells were crosslinked with formaldehyde, and chromatin was isolated and sonicated under optimized conditions to generate DNA fragments of 200–500 bp. Immunoprecipitation was conducted overnight at 4 °C using anti-ZNF526 antibody (54686, ThermoFisher) or isotype-matched IgG control (2729, Cell Signaling Technology), followed by capture with Protein A/G Magnetic Beads (Thermo Fisher). Crosslinks were reversed, and enriched DNA was purified. Quantitative PCR analysis of ChIP DNA was performed with SYBR Green detection. All reactions were run in technical triplicate and normalized to input DNA. Full methodological details were performed as previously established [20].

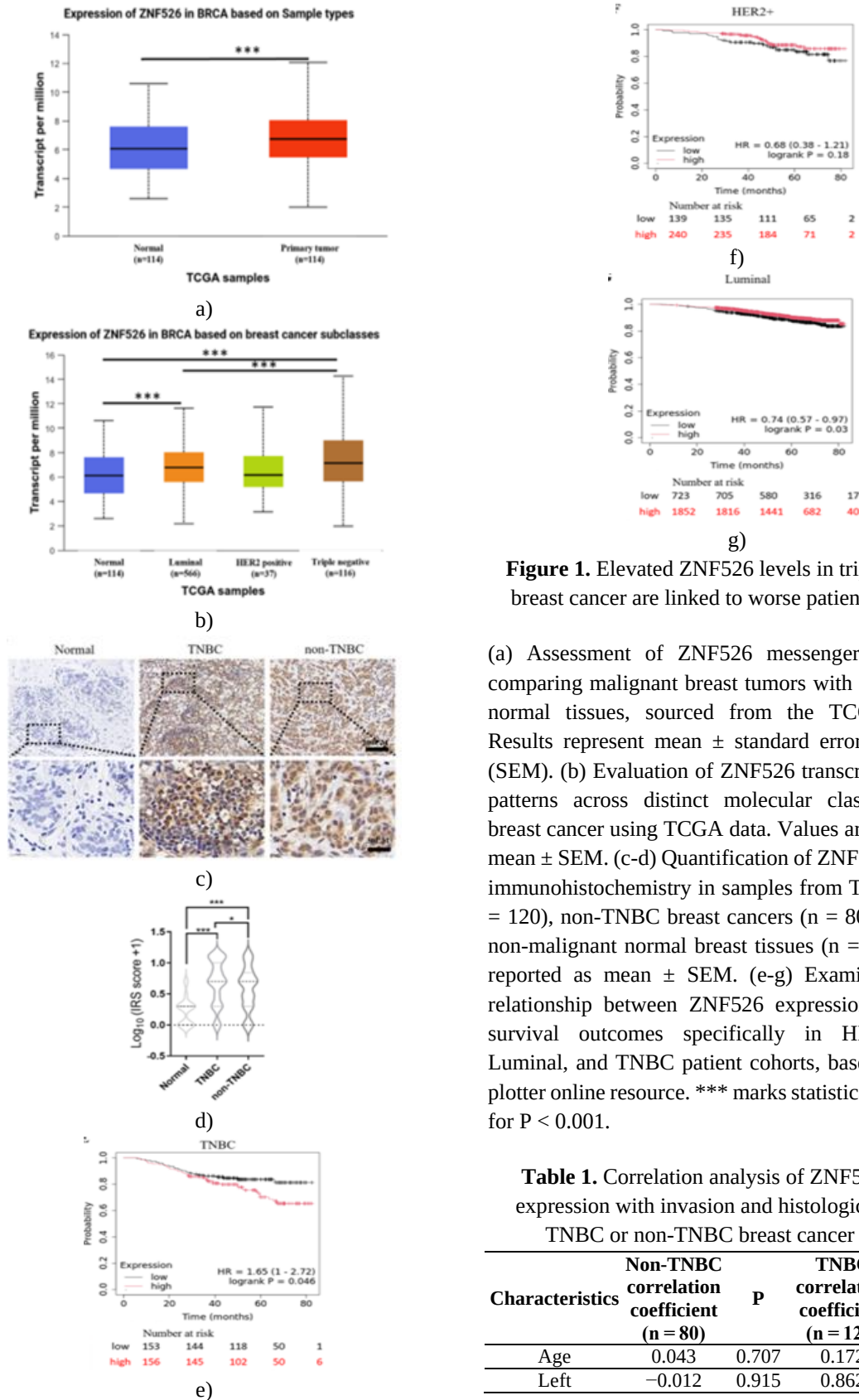
#### *Statistical analysis*

Differences between groups were evaluated using Student's t-test or one-way ANOVA, with post-hoc Mann-Whitney U test where appropriate. All statistical computations were carried out in SPSS version 23.0.

## **Results and Discussion**

### *ZNF526 shows elevated expression in triple-negative breast cancer tissues and is linked to poor clinical outcomes*

We first examined ZNF526 mRNA levels across different breast cancer subtypes. Expression was markedly higher in cancerous tissues compared with normal breast tissue (**Figure 1a**). Among subtypes, triple-negative breast cancer (TNBC) exhibited the strongest ZNF526 upregulation (**Figure 1b**). Protein-level confirmation by immunohistochemistry on clinical samples demonstrated significantly increased ZNF526 staining in both TNBC ( $n = 120$ ) and non-TNBC ( $n = 80$ ) tumors relative to normal controls ( $n = 35$ ), with the highest levels observed in TNBC (**Figures 1c and 1d**). Clinicopathological correlation analysis further indicated that high ZNF526 protein expression was specifically associated with higher histological grade and increased invasiveness in TNBC patients. Still, it showed no such relationship in non-TNBC cases (**Table 1**).



**Figure 1.** Elevated ZNF526 levels in triple-negative breast cancer are linked to worse patient prognosis.

(a) Assessment of ZNF526 messenger RNA levels comparing malignant breast tumors with corresponding normal tissues, sourced from the TCGA database. Results represent mean  $\pm$  standard error of the mean (SEM). (b) Evaluation of ZNF526 transcript expression patterns across distinct molecular classifications of breast cancer using TCGA data. Values are displayed as mean  $\pm$  SEM. (c-d) Quantification of ZNF526 protein by immunohistochemistry in samples from TNBC cases ( $n = 120$ ), non-TNBC breast cancers ( $n = 80$ ), and nearby non-malignant normal breast tissues ( $n = 35$ ). Data are reported as mean  $\pm$  SEM. (e-g) Examination of the relationship between ZNF526 expression and overall survival outcomes specifically in HER2-enriched, Luminal, and TNBC patient cohorts, based on the KM plotter online resource. \*\*\* marks statistical significance for  $P < 0.001$ .

**Table 1.** Correlation analysis of ZNF526 protein expression with invasion and histological grade in TNBC or non-TNBC breast cancer patients

| Characteristics | Non-TNBC<br>correlation<br>coefficient<br>( $n = 80$ ) | P     | TNBC<br>correlation<br>coefficient<br>( $n = 120$ ) | P        |
|-----------------|--------------------------------------------------------|-------|-----------------------------------------------------|----------|
|                 |                                                        |       |                                                     |          |
| Age             | 0.043                                                  | 0.707 | 0.172                                               | 0.061    |
| Left            | -0.012                                                 | 0.915 | 0.862                                               | < 0.0001 |

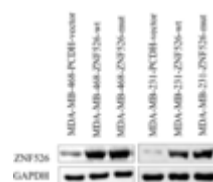
|                           |        |       |       |          |
|---------------------------|--------|-------|-------|----------|
| Invasive ductal carcinoma | -0.258 | 0.022 | 0.758 | < 0.0001 |
| Invasion                  | 0.149  | 0.232 | 0.778 | < 0.0001 |
| Histological grade        | 0.021  | 0.853 | 0.759 | < 0.0001 |

Note: Pairwise correlation analysis examined associations among age (continuous), laterality (binary), histologic grade (ordinal), and invasiveness (binary). Pearson point-biserial coefficients were applied for continuous-binary variable pairs, while Kendall's tau-b was used for ordinal-binary pairs. Significance was determined at  $P < 0.001$  following Bonferroni correction, with all computations performed in R version 4.3.2.

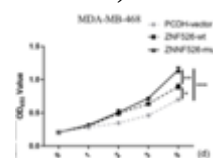
To more thoroughly explore the link between ZNF526 levels and clinical outcomes in breast cancer, TCGA data were examined. High ZNF526 expression was strongly associated with reduced overall survival. Still, this effect was restricted to TNBC patients (**Figure 1e**). In other subtypes, ZNF526 displayed either a weak positive trend (luminal) or lacked any meaningful prognostic association (HER2-positive) (**Figure 1f**). Collectively, these findings indicate that ZNF526 is markedly upregulated in breast tumors and is particularly associated with adverse prognosis in the TNBC subtype.

#### *ZNF526 promotes proliferation of triple-negative breast cancer cells*

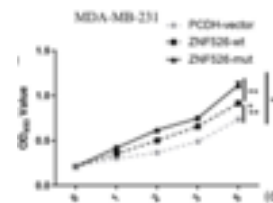
To determine the biological function of ZNF526 in TNBC, stable cell lines overexpressing ZNF526 were established in MDA-MB-231, MDA-MB-468, BT-20, and HCC1806 models (**Figures 2a and 2d**). Cell proliferation assays conducted in vitro revealed that forced ZNF526 expression substantially accelerated growth rates (**Figures 2b, 2c, 2e and 2f**). Interestingly, the cancer-associated mutant form of ZNF526 (Val94Ala) produced an even more pronounced enhancement of cell growth compared to the wild-type protein when overexpressed in these lines (**Figures 2b-2f**).



a)



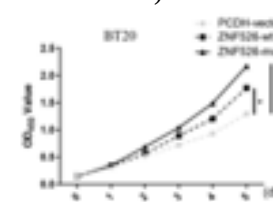
b)



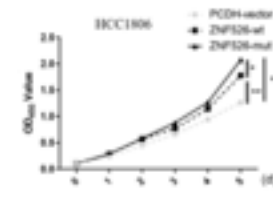
c)



d)



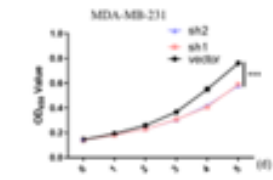
e)



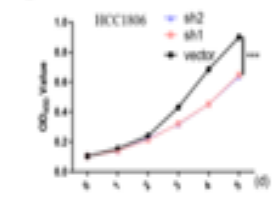
f)



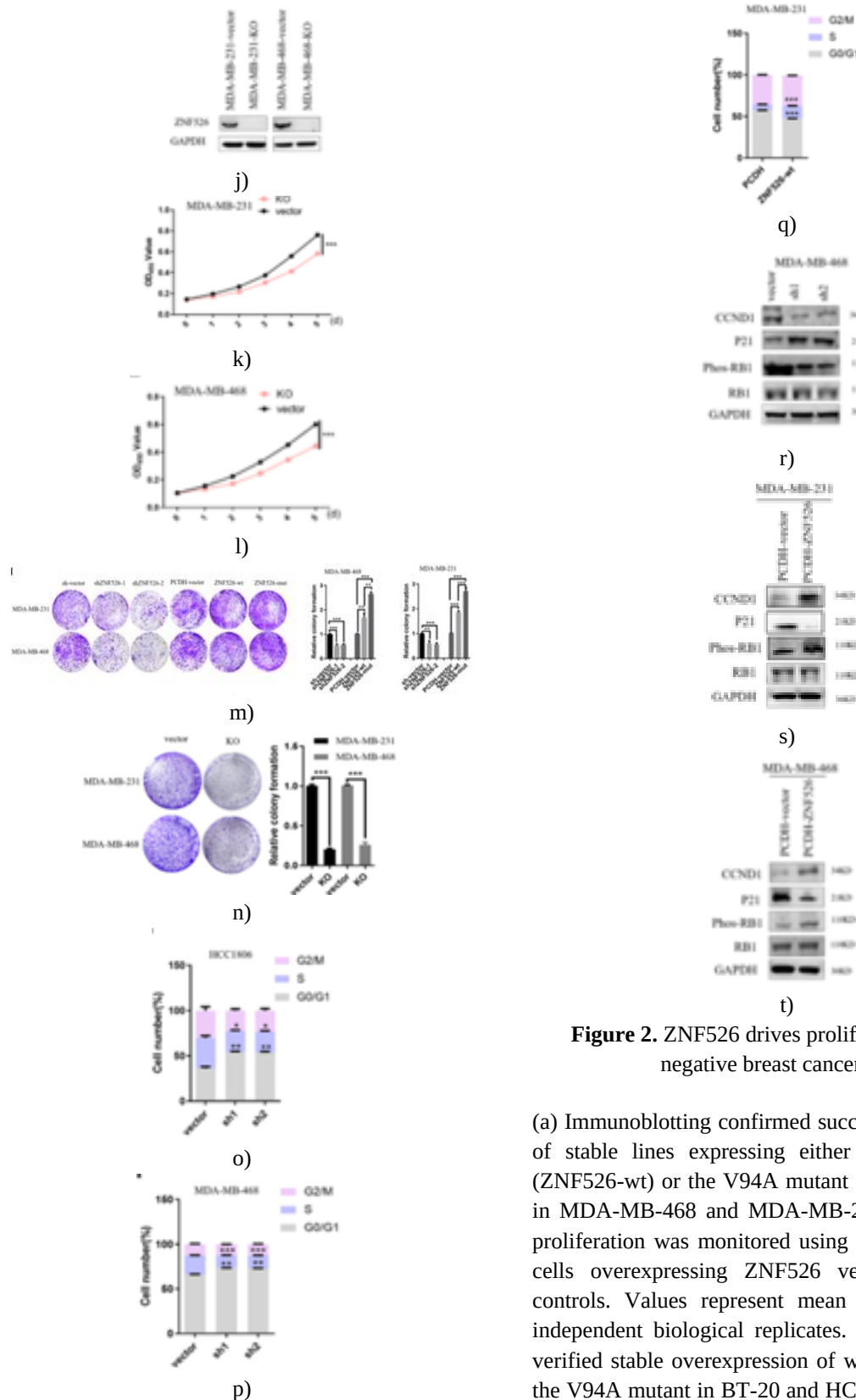
g)



h)



i)



**Figure 2.** ZNF526 drives proliferation in triple-negative breast cancer cells.

(a) Immunoblotting confirmed successful establishment of stable lines expressing either wild-type ZNF526 (ZNF526-wt) or the V94A mutant form (ZNF526-mut) in MDA-MB-468 and MDA-MB-231 cells. (b-c) Cell proliferation was monitored using the CCK-8 assay in cells overexpressing ZNF526 versus empty vector controls. Values represent mean  $\pm$  SEM from four independent biological replicates. (d) Immunoblotting verified stable overexpression of wild-type ZNF526 or the V94A mutant in BT-20 and HCC1806 cell lines. (e-f) Growth curves were generated by CCK-8 assay

comparing ZNF526-overexpressing cells to vector controls. Data are shown as mean  $\pm$  SEM ( $n = 4$  biological replicates). (g) Western blotting demonstrated efficient ZNF526 knockdown using two different shRNAs (sh1 and sh2) in MDA-MB-231 and HCC1806 cells, with an empty vector serving as the negative control. (h-i) CCK-8 assays measured proliferation in ZNF526-knockdown cells (sh1/sh2) relative to vector controls. Results are presented as mean  $\pm$  SEM ( $n = 4$  biological replicates). (j) Western blot analysis validated complete ZNF526 knockout in MDA-MB-468 and MDA-MB-231 cells. (k-l) Proliferation of ZNF526-knockout cells was evaluated using a CCK-8 assay compared with vector controls. Data shown as mean  $\pm$  SEM ( $n = 4$  biological replicates). (m-n) Clonogenic assays were performed on cells with ZNF526 knockdown, overexpression, or knockout, and colony numbers were quantified using ImageJ. Results expressed as mean  $\pm$  SEM ( $n = 4$  biological replicates). (o-q) Flow cytometry was used to determine cell cycle profiles, with data analysis performed in FlowJo. Values represent mean  $\pm$  SEM ( $n = 3$  biological replicates). (r-t) Immunoblotting was used to examine protein levels of Cyclin D1, phosphorylated RB, and P21 in cells with ZNF526 knockdown or overexpression. Statistical significance is indicated as \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

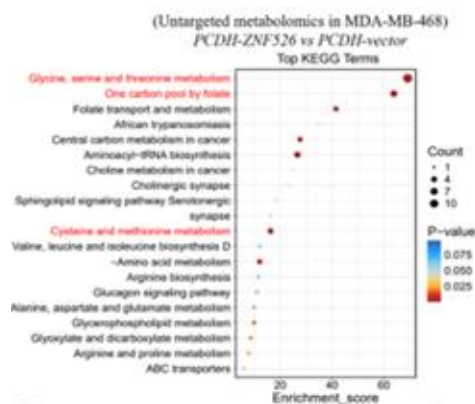
In addition, stable cell lines with ZNF526 depletion via shRNA or CRISPR knockout were created (**Figures 2g and 2j**). Growth assays in culture consistently showed that reducing or eliminating ZNF526 expression strongly inhibited cellular proliferation (**Figures 2h, 2i, 2k, and 2l**). Colony formation experiments reinforced these findings, revealing enhanced colony-forming ability upon ZNF526 overexpression and diminished clonogenicity following its depletion (**Figures 2m and 2n**).

Further experiments addressed how ZNF526 influences cell division. Cell cycle analysis by flow cytometry revealed that loss of ZNF526 caused pronounced G1-phase accumulation in HCC1806 and MDA-MB-468 cells (**Figures 2o and 2p**). In contrast, elevating ZNF526 levels in MDA-MB-231 cells led to a reduction in G1-phase cells accompanied by an increase in S-phase cells (**Figure 2q**). Molecular analysis by Western blotting aligned with these shifts: ZNF526 knockdown lowered Cyclin D1 expression, reduced Rb phosphorylation, and raised p21 protein (**Figure 2r**), whereas overexpression produced the opposite pattern — higher Cyclin D1 and

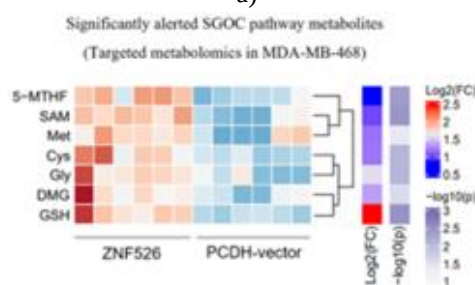
phospho-Rb with decreased p21 (**Figures 2s and 2t**). Importantly, modulation of ZNF526 expression had minimal impact on apoptotic rates in these TNBC models.

#### *ZNF526 controls serine-glycine-one-carbon metabolism and glutathione abundance*

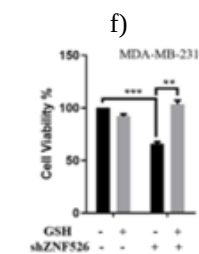
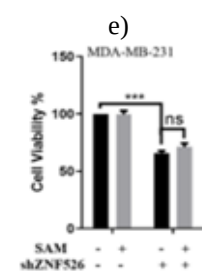
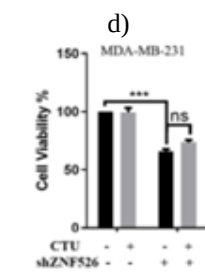
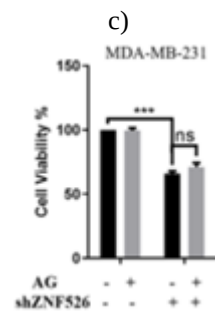
Prior work proposed that the ZNF526-V94A variant could affect metabolic regulation [7]. To uncover pathways underlying ZNF526-dependent growth promotion in TNBC, comprehensive untargeted metabolomics was performed on MDA-MB-468 and MDA-MB-231 cells engineered to overexpress ZNF526. Metabolite set enrichment analysis highlighted strong enrichment in pathways connected to serine-glycine-one-carbon (SGOC) metabolism, such as glycine-serine-threonine metabolism, cysteine-methionine metabolism, and folate-mediated one-carbon units (**Figures 3a and 3c**). Follow-up targeted metabolite quantification confirmed elevated concentrations of central SGOC intermediates, including glutathione (GSH), S-adenosylmethionine (SAM), cysteine, and 5-methyltetrahydrofolate (5-Methyl-THF), in ZNF526-overexpressing cells (**Figure 3b**). These observations establish ZNF526 as a significant modulator of SGOC metabolic activity in TNBC.



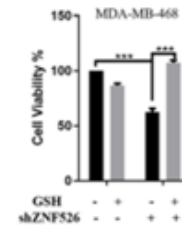
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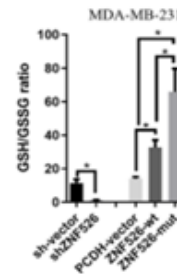
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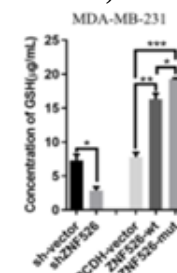
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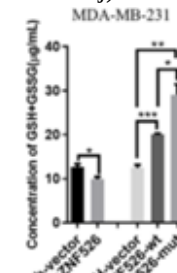
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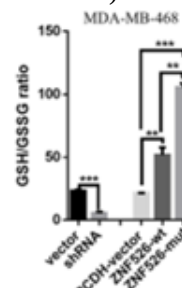
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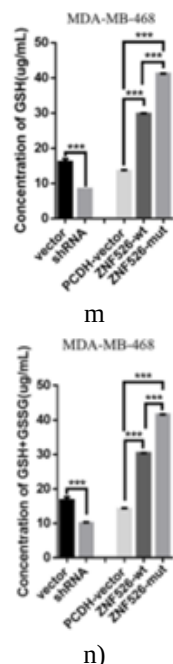
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**Figure 3.** ZNF526 controls SGOC metabolic activity and intracellular glutathione abundance.

(a) Bubble plot from metabolite enrichment analysis performed in MDA-MB-468 cells. The x-axis represents Enrichment Score (ranging from 0 to 0.2), while the y-axis displays various biological pathways. Individual bubbles vary in shape and color according to Count values (1–5) and P-value ranges (0.025–0.075), with darker colors indicating lower P-values. (b) Targeted metabolomics profiling of SGOC pathway intermediates in MDA-MB-468 cells upon ZNF526 overexpression. Volcano plot highlights significantly changed metabolites ( $P < 0.05$ ), with upregulated metabolites shown in red and downregulated metabolites in blue. (c) Overview diagram illustrating the SGOC metabolic network. Abbreviations: DMG (dimethylglycine), SAM (S-adenosyl methionine), SAH (S-adenosyl homocysteine), Met (methionine), hCys (homocysteine), Glc (glucose), Cys (cysteine). (d-f) CCK-8 assays tested the rescue effect of various SGOC-related metabolic intermediates on the growth inhibition caused by ZNF526 knockdown in MDA-MB-231 cells. AG: purine nucleosides; CTU: pyrimidine nucleosides; SAM: S-adenosylmethionine. Data shown as mean  $\pm$  SEM ( $n = 4$  biological replicates). (g-h) CCK-8 assays evaluated the impact of GSH supplementation on the proliferation defect induced by ZNF526 knockdown in MDA-MB-231 and MDA-MB-468 cells. Data presented as mean  $\pm$  SEM ( $n = 4$  biological replicates). (i-k) Measurements of

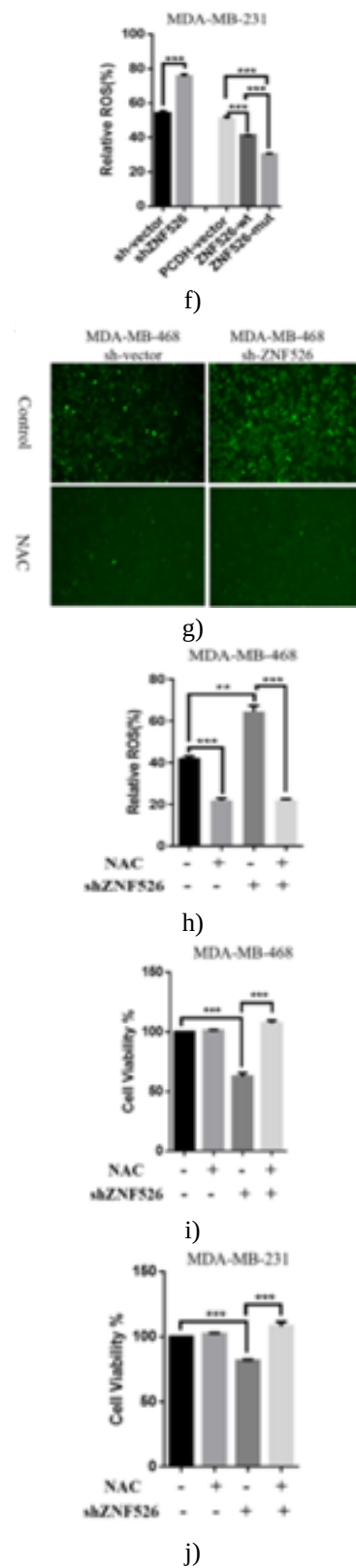
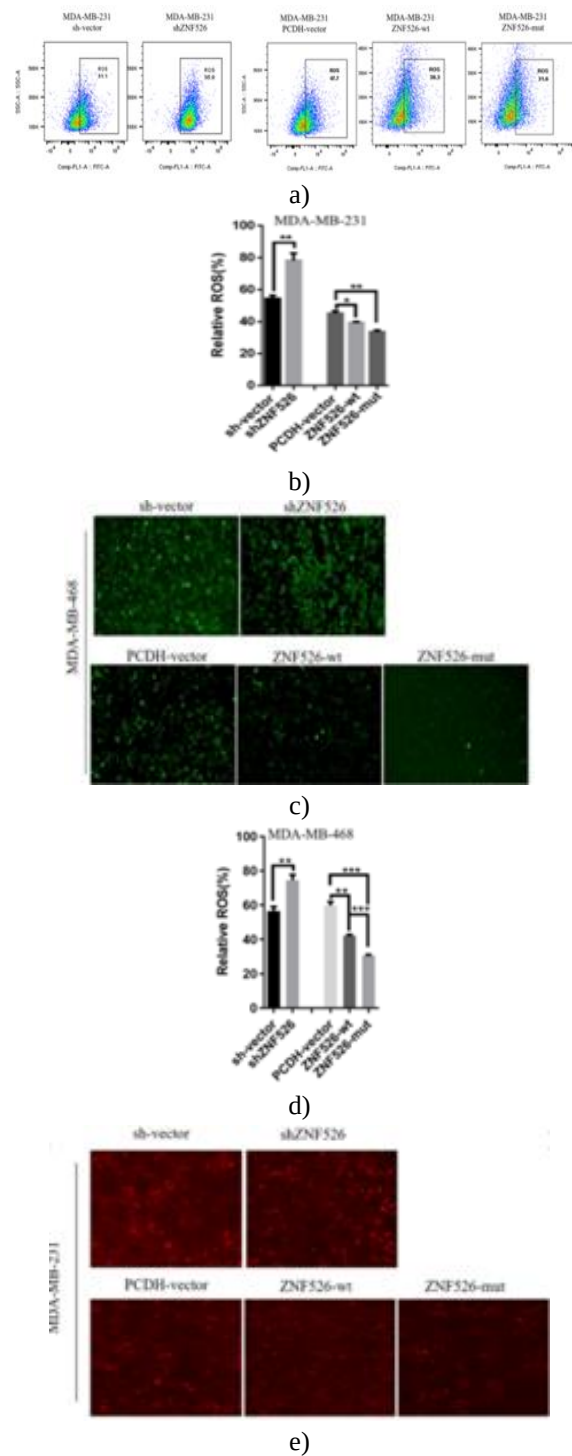
GSH/GSSG ratio (i), reduced GSH content (j), and total glutathione pool (GSH + GSSG) (k) in MDA-MB-231 cells with ZNF526 knockdown or overexpression. (l-n) Measurements of GSH/GSSG ratio (l), reduced GSH content (m), and total glutathione pool (GSH + GSSG) (n) in MDA-MB-468 cells with ZNF526 knockdown or overexpression. All experiments were repeated three times independently. Data are shown as mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

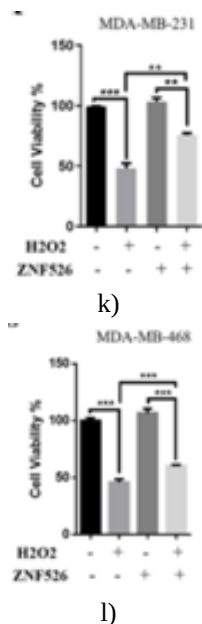
The SGOC metabolic network supports cancer cell expansion primarily by supplying precursors for nucleotide biosynthesis, enabling epigenetic modifications through methylation reactions, and maintaining redox balance via antioxidant production [21-23]. To identify which downstream products of SGOC metabolism are most critical for ZNF526-mediated growth in TNBC, rescue experiments were conducted using key metabolites to counteract the proliferation impairment caused by ZNF526 depletion. Metabolites representing major branches were examined, including nucleosides (for purine and pyrimidine synthesis), SAM (as a methyl donor), and reduced glutathione (GSH, as an antioxidant). Supplementation with SAM or nucleosides failed to reverse the growth suppression resulting from ZNF526 knockdown (**Figures 3d-3f**). In sharp contrast, addition of GSH substantially alleviated the proliferative defect induced by shZNF526 (**Figures 3g and 3h**). Because the SGOC pathway plays a central role in GSH production and is known to be important for TNBC cell survival and growth [20], we next examined whether ZNF526 directly influences cellular glutathione status. GSH and GSSG concentrations were quantified in both MDA-MB-231 and MDA-MB-468 cells after ZNF526 knockdown or overexpression. ZNF526 depletion led to significant decreases in the GSH/GSSG ratio, reduced GSH levels, and overall glutathione content. Conversely, ZNF526 overexpression produced marked increases in these parameters, with the V94A mutant form causing even greater elevations (**Figures 3i-3n**). These data collectively demonstrate that ZNF526 modulates SGOC metabolism and thereby regulates intracellular GSH abundance.

#### *ZNF526 strengthens cellular antioxidant defense*

Since GSH is vital for cancer cells to neutralize excess reactive oxygen species (ROS), intracellular ROS levels were quantified after manipulating ZNF526 expression. ZNF526 knockdown led to a substantial increase in ROS

accumulation in both MDA-MB-231 and MDA-MB-468 cells. In contrast, ZNF526 overexpression significantly lowered ROS levels, with the mutant ZNF526-V94A variant showing a stronger ROS-suppressing effect (Figure 4a).





**Figure 4.** ZNF526 strengthens cellular ability to counter oxidative stress.

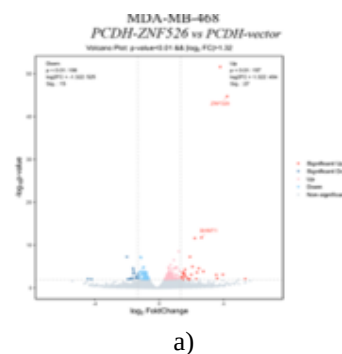
(a-b) Flow cytometry was used to measure and statistically evaluate intracellular ROS accumulation in MDA-MB-231 cells following either ZNF526 depletion or forced expression. All findings are derived from three biological repeats and are reported as mean  $\pm$  SEM. (c-d) Typical fluorescence microscope photographs displaying DCFH-DA staining (green signal corresponding to ROS) and subsequent ImageJ-based quantification of fluorescence intensity in MDA-MB-231 cells under conditions of ZNF526 overexpression or silencing. Data come from three independent experiments and are expressed as mean  $\pm$  SEM. (e-f) Representative images captured by fluorescence microscopy using DHE staining (red signal for ROS) together with ImageJ quantification of ROS in MDA-MB-231 cells after ZNF526 overexpression or knockdown. (g-h) ROS levels were examined in control and shZNF526-expressing MDA-MB-468 cells following a 6-hour incubation with 100  $\mu$ M N-acetylcysteine (NAC). Results are based on three independent experiments and shown as mean  $\pm$  SEM. (i-j) Cell proliferation was monitored via CCK-8 assay in MDA-MB-231 and MDA-MB-468 cells treated with 100  $\mu$ M NAC. Experiments were performed independently three times; data presented as mean  $\pm$  SEM. (k-l) Cell viability was determined by CCK-8 assay after exposing MDA-MB-231 and MDA-MB-468 cells to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 hours. Three independent experiments were

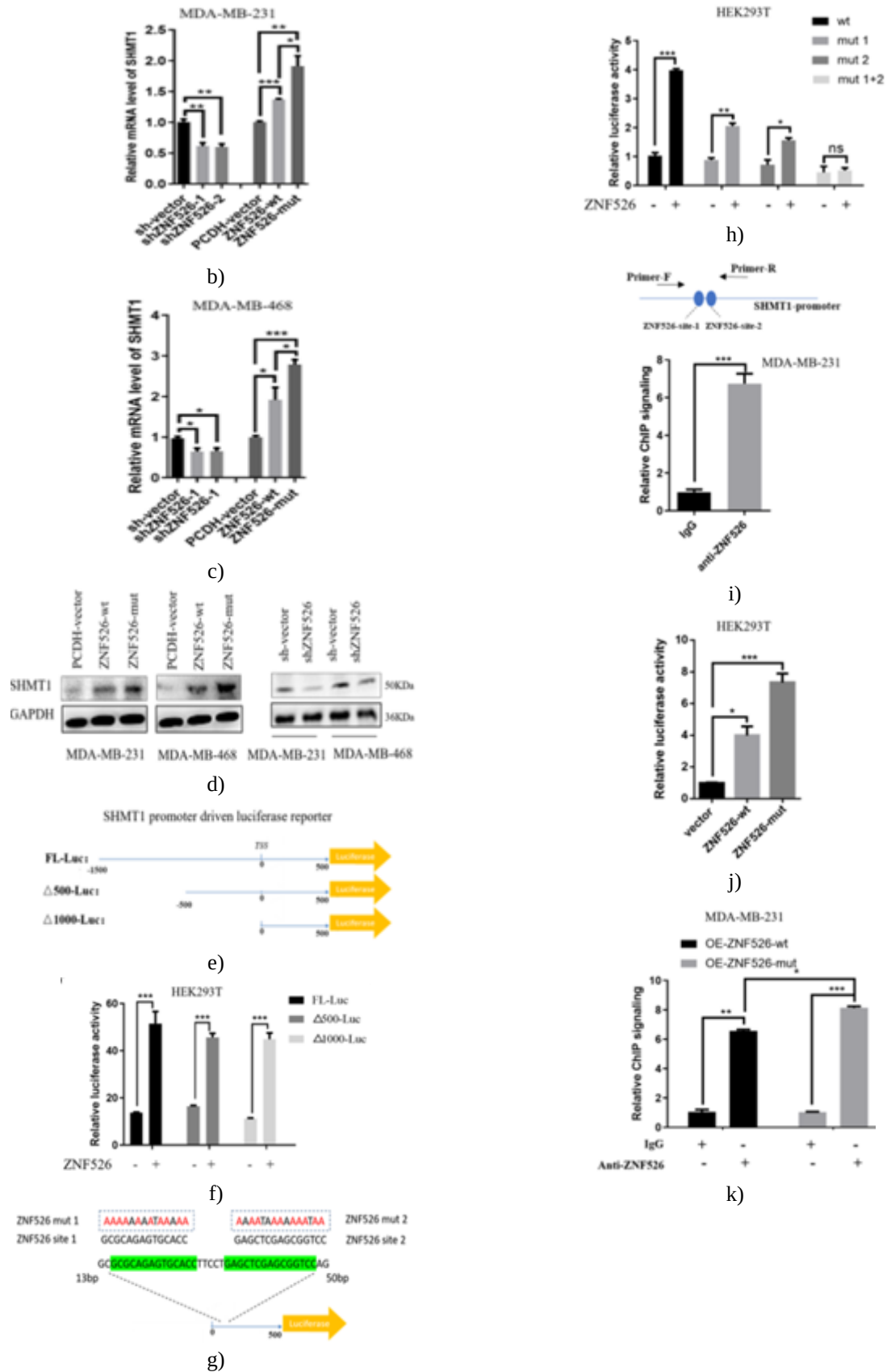
conducted, with results expressed as mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$ .

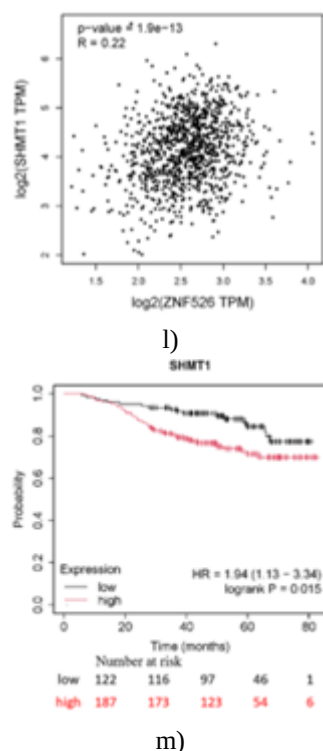
Rescue studies were carried out to test whether the observed changes in ROS abundance accounted for ZNF526's impact on cell proliferation. Treatment with the antioxidant N-acetylcysteine (NAC) successfully reduced ROS levels triggered by ZNF526 depletion and largely reversed the associated growth inhibition (**Figure 4g**). Separately, H<sub>2</sub>O<sub>2</sub> treatment caused a sharp decline in cell survival (**Figures 4k and 4l**). Overexpression of ZNF526 notably protected cells from this oxidative damage-induced loss of viability (**Figures 4k and 4l**). In summary, these experiments support the conclusion that ZNF526 drives TNBC cell expansion primarily by reinforcing the cell's antioxidant systems.

#### ZNF526 directly increases SHMT1 transcription

Additional RNA sequencing experiments were performed on MDA-MB-468 cells engineered to overexpress ZNF526, compared with control cells, to identify the downstream target responsible for altered SGOC metabolism and redox balance. Among the genes most prominently induced was SHMT1, a rate-limiting enzyme within the SGOC network (**Figure 5a**). Follow-up validation by quantitative RT-PCR demonstrated clear upregulation of SHMT1 mRNA in response to ZNF526 overexpression and downregulation upon its silencing (**Figure 5b**). The cancer-associated V94A mutant of ZNF526 triggered an even greater increase in SHMT1 transcript levels than the wild-type protein (**Figure 5b**). These gene expression changes were corroborated at the protein level through immunoblotting (**Figure 5d**).







**Figure 5.** ZNF526 drives transcriptional upregulation of SHMT1.

(a) Transcriptomic profiling via RNA-seq revealed gene expression changes in ZNF526-overexpressing MDA-MB-468 cells compared with control cells. Significantly altered genes ( $|\log_2$  fold change| > 1 and FDR < 0.01) are marked in red (upregulated), blue (downregulated), or gray (non-significant). (b-c) SHMT1 mRNA abundance was quantified by qPCR in MDA-MB-231 and MDA-MB-468 lines following ZNF526 silencing or forced expression. Results from three independent experiments are displayed as mean  $\pm$  SEM. (d) Immunoblotting detected SHMT1 protein abundance in MDA-MB-231 and MDA-MB-468 cells under ZNF526 knockdown or overexpression conditions. (e-f) Diagram illustrating progressive SHMT1 promoter luciferase reporter constructs (e) and corresponding reporter activity measured in HEK293T cells (f). Data from three independent experiments shown as mean  $\pm$  SEM. (g-h) Potential binding motifs for ZNF526 in the SHMT1 promoter were identified using the JASPAR database, based on its paralog ZNF574. Schematics depict wild-type and mutated sites (g). Luciferase assays testing these constructs were conducted in HEK293T cells (h). Three independent experiments, mean  $\pm$  SEM. (i) Chromatin immunoprecipitation (ChIP) was performed

in MDA-MB-231 cells using an anti-ZNF526 antibody or control IgG. Promoter enrichment was assessed by qPCR and normalized to IgG. Results from three independent experiments are shown as mean  $\pm$  SEM. (j) Luciferase reporter assays in HEK293T cells compared promoter activation by wild-type ZNF526 with that by the V94A mutant. Three independent experiments, mean  $\pm$  SEM. (k) ChIP-qPCR measured binding of wild-type versus V94A mutant ZNF526 to the SHMT1 promoter in MDA-MB-231 cells. Three independent experiments, mean  $\pm$  SEM. (l) Correlation plot of ZNF526 and SHMT1 mRNA expression in the TCGA breast cancer cohort. (m) Kaplan-Meier overall survival analysis of TNBC patients in the TCGA dataset stratified by high versus low SHMT1 expression. P-value determined by log-rank test. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

Promoter activity assays were carried out in HEK293T cells — chosen for their superior transfection efficiency — using multiple SHMT1 promoter fragments fused to luciferase. ZNF526 strongly stimulated promoter-driven luciferase signal (**Figure 5e**). Progressive deletion mapping pinpointed the responsive region to an area roughly 500 bp downstream of the transcription start site (**Figure 5e**). In silico screening identified two adjacent candidate binding sequences for ZNF526 (sites 1 and 2) in this interval (**Figure 5g**). Reporter plasmids carrying wild-type, single-mutant (mut1 or mut2), or double-mutant (mut1+mut2) versions of these sites were tested (**Figure 5g**). Disrupting either site individually reduced ZNF526 responsiveness, while mutating both sites fully prevented activation (**Figure 5h**).

Physical recruitment of ZNF526 to the endogenous SHMT1 promoter was confirmed by ChIP assays, which showed clear enrichment relative to the IgG control (**Figure 5i**).

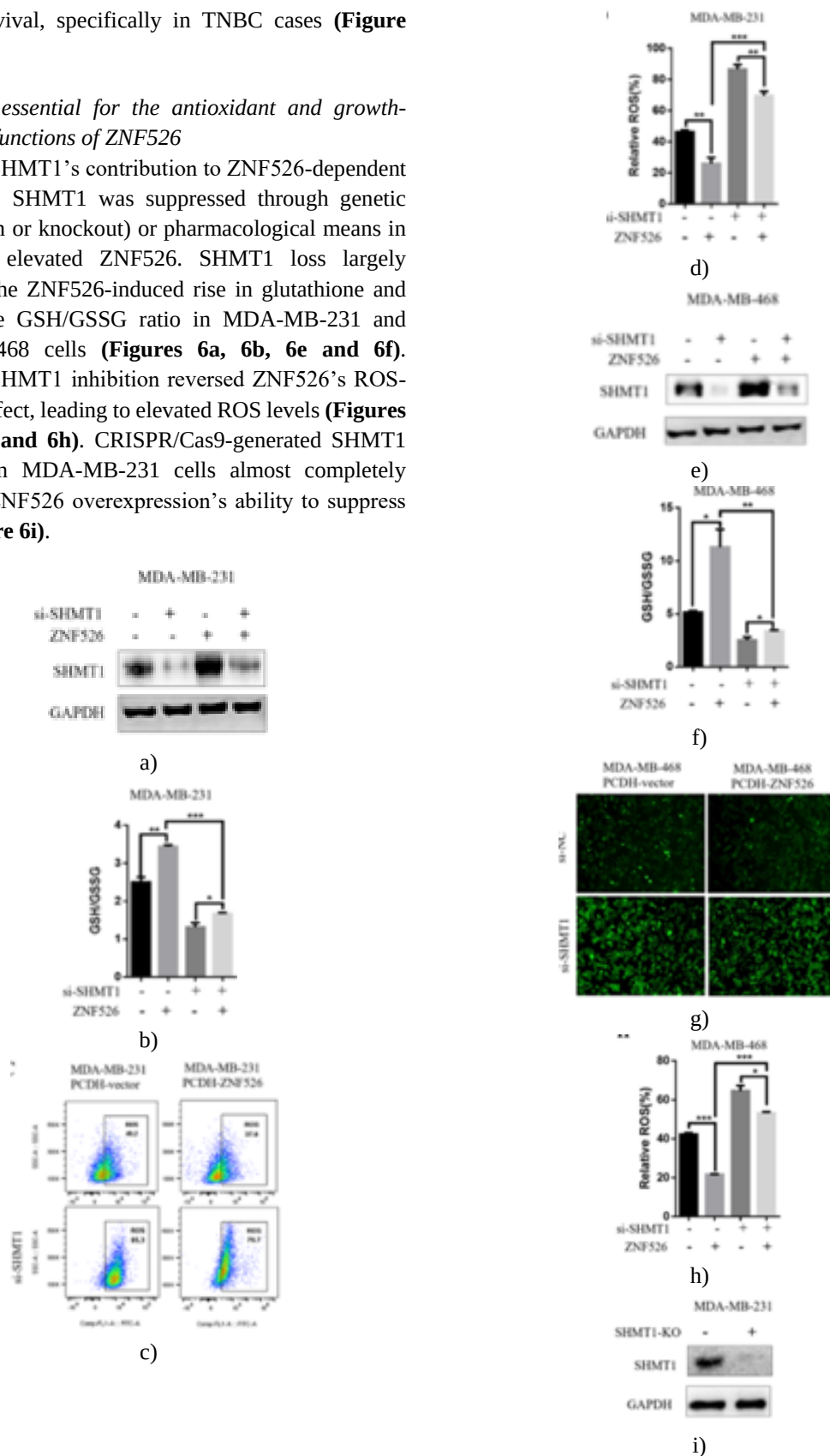
The V94A mutant version of ZNF526 exhibited substantially greater transcriptional activation of the SHMT1 promoter than wild-type ZNF526 in reporter assays (**Figure 5j**). Parallel ChIP experiments confirmed stronger promoter occupancy by the mutant protein (**Figure 5k**). Overall, these findings confirm that ZNF526 directly induces SHMT1 transcription, with the breast cancer-associated V94A mutation displaying enhanced potency.

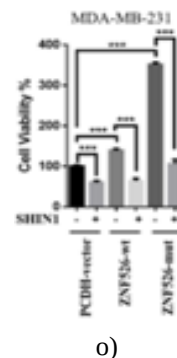
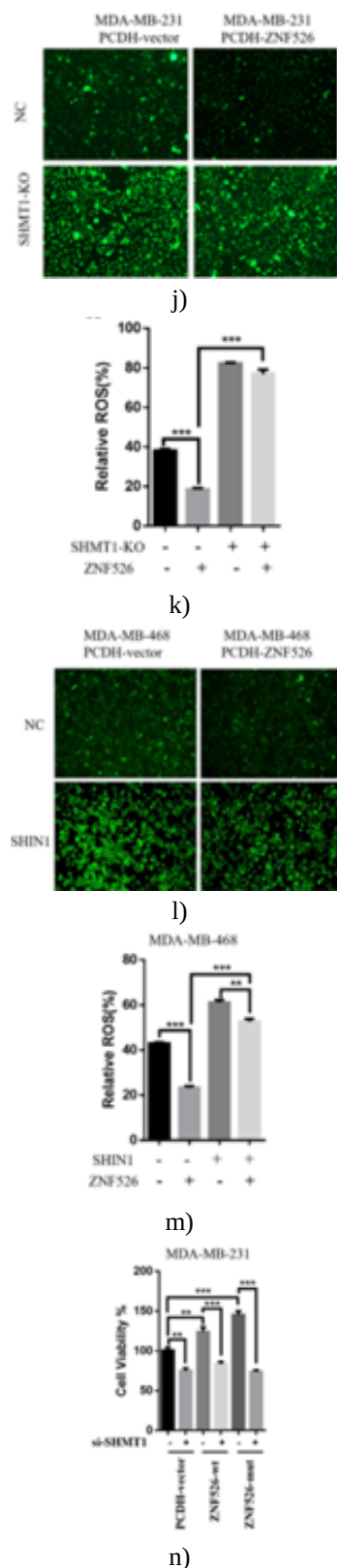
Analysis of TCGA patient data showed a clear positive association between ZNF526 and SHMT1 transcript levels in breast tumors (**Figure 5l**). High SHMT1 expression was also associated with significantly shorter

overall survival, specifically in TNBC cases (Figure 5m).

*SHMT1 is essential for the antioxidant and growth-promoting functions of ZNF526*

To clarify SHMT1's contribution to ZNF526-dependent phenotypes, SHMT1 was suppressed through genetic (knockdown or knockout) or pharmacological means in cells with elevated ZNF526. SHMT1 loss largely prevented the ZNF526-induced rise in glutathione and lowered the GSH/GSSG ratio in MDA-MB-231 and MDA-MB-468 cells (Figures 6a, 6b, 6e and 6f). Likewise, SHMT1 inhibition reversed ZNF526's ROS-lowering effect, leading to elevated ROS levels (Figures 6c, 6d, 6g and 6h). CRISPR/Cas9-generated SHMT1 knockout in MDA-MB-231 cells almost completely abolished ZNF526 overexpression's ability to suppress ROS (Figure 6i).





**Figure 6.** SHMT1 serves as a critical mediator of ZNF526's antioxidant and growth-promoting activities.

(a-b) Western blotting confirmed effective reduction of SHMT1 protein 48 hours after transfection with 20 nM si-SHMT1 using Lipofectamine RNAiMAX in ZNF526-overexpressing MDA-MB-231 stable cells (a). The GSH/GSSG ratio was subsequently measured following si-SHMT1 transfection (b). Three independent experiments were conducted; data shown as mean  $\pm$  SEM. (c-d) ROS levels were quantified by flow cytometry after 48-hour transfection with 20 nM si-SHMT1 (using Lipofectamine RNAiMAX) in ZNF526-overexpressing or control MDA-MB-231 cells. (e-f) Western blotting verified SHMT1 knockdown 48 hours post-transfection with 20 nM si-SHMT1 using Lipofectamine RNAiMAX in ZNF526-overexpressing MDA-MB-468 cells (e). GSH/GSSG ratio was determined afterward (f). Results from three independent experiments, mean  $\pm$  SEM. (g-h) ROS levels were assessed and quantified via DCFH-DA fluorescence (green signal) in ZNF526-overexpressing or control MDA-MB-468 cells following 48-hour siSHMT1 transfection (20 nM, Lipofectamine RNAiMAX). Data presented as mean  $\pm$  SEM. (i) Western blot analysis confirmed SHMT1 knockout in MDA-MB-231 cell lines. (j-k) Representative fluorescence microscopy images and quantitative analysis of ROS levels (DCFH-DA probe) in ZNF526-overexpressing or control MDA-MB-231 cells after SHMT1 knockout. Three independent experiments, mean  $\pm$  SEM. (l-m) ROS measurement and quantification using the DCFH-DA probe in MDA-MB-468 cells after ZNF526 overexpression combined with 10 nM SHIN1 treatment (HY-112066, MCE) for 48 hours. Three independent experiments, mean  $\pm$  SEM. (n-o) Cell viability was evaluated 72 hours after siSHMT1 transfection (20 nM,

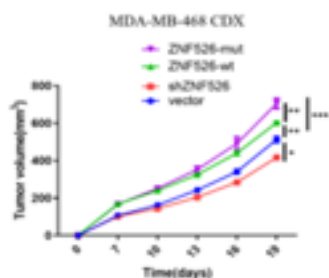
Lipofectamine RNAiMAX) or 10 nM SHIN1 treatment in MDA-MB-231 cells overexpressing wild-type ZNF526 or the V94A mutant. Three independent experiments, mean  $\pm$  SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

The SHMT1 inhibitor SHIN1 potently inhibited proliferation of MDA-MB-231 and MDA-MB-468 cells, yielding IC<sub>50</sub> values around 10 nM. Accordingly, 10 nM SHIN1 was selected for follow-up studies. ROS assays revealed that pharmacological blockade of SHMT1 with SHIN1 largely prevented ZNF526 from lowering ROS levels in MDA-MB-468 cells (**Figures 6l and 6m**).

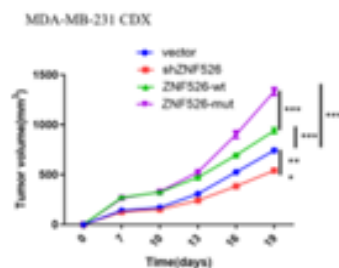
To assess whether SHMT1 is necessary for ZNF526-driven proliferation, both genetic silencing (si-SHMT1) and pharmacological inhibition (SHIN1) were applied. Both approaches strongly attenuated the growth advantage conferred by ZNF526 in MDA-MB-231 cells (**Figures 6n and 6o**). In conclusion, these results demonstrate that SHMT1 functions as an essential downstream effector through which ZNF526 maintains redox balance and drives tumor cell expansion, highlighting SHMT1 as a key mediator of ZNF526's oncogenic properties.

#### ZNF526 accelerates tumor growth in xenograft models

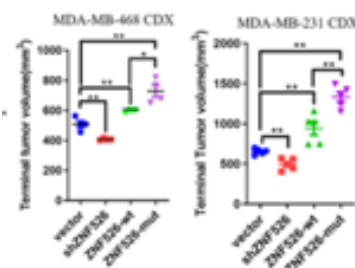
To confirm ZNF526's tumor-promoting role in vivo, subcutaneous xenograft models were generated using MDA-MB-231 and MDA-MB-468 cells in immunodeficient mice. In line with its pro-tumorigenic activity observed in vitro, ZNF526 knockdown significantly slowed tumor progression, while overexpression markedly enhanced tumor growth. Tumors carrying the ZNF526-V94A mutant displayed the most aggressive growth compared to those expressing wild-type ZNF526 (**Figure 7a**). Immunohistochemical staining for Ki67, a marker of cell proliferation, further supported that ZNF526 positively regulates tumor cell division in vivo (**Figure 7d**).



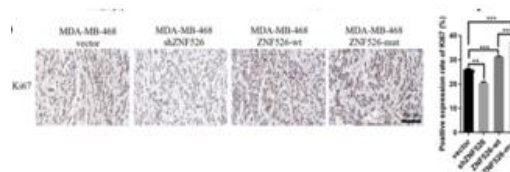
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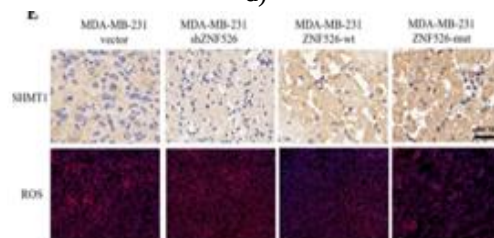
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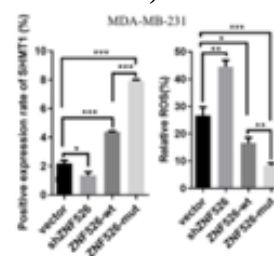
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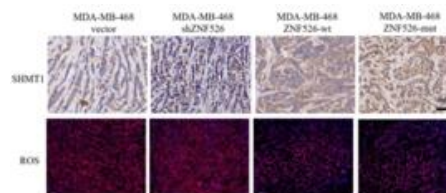
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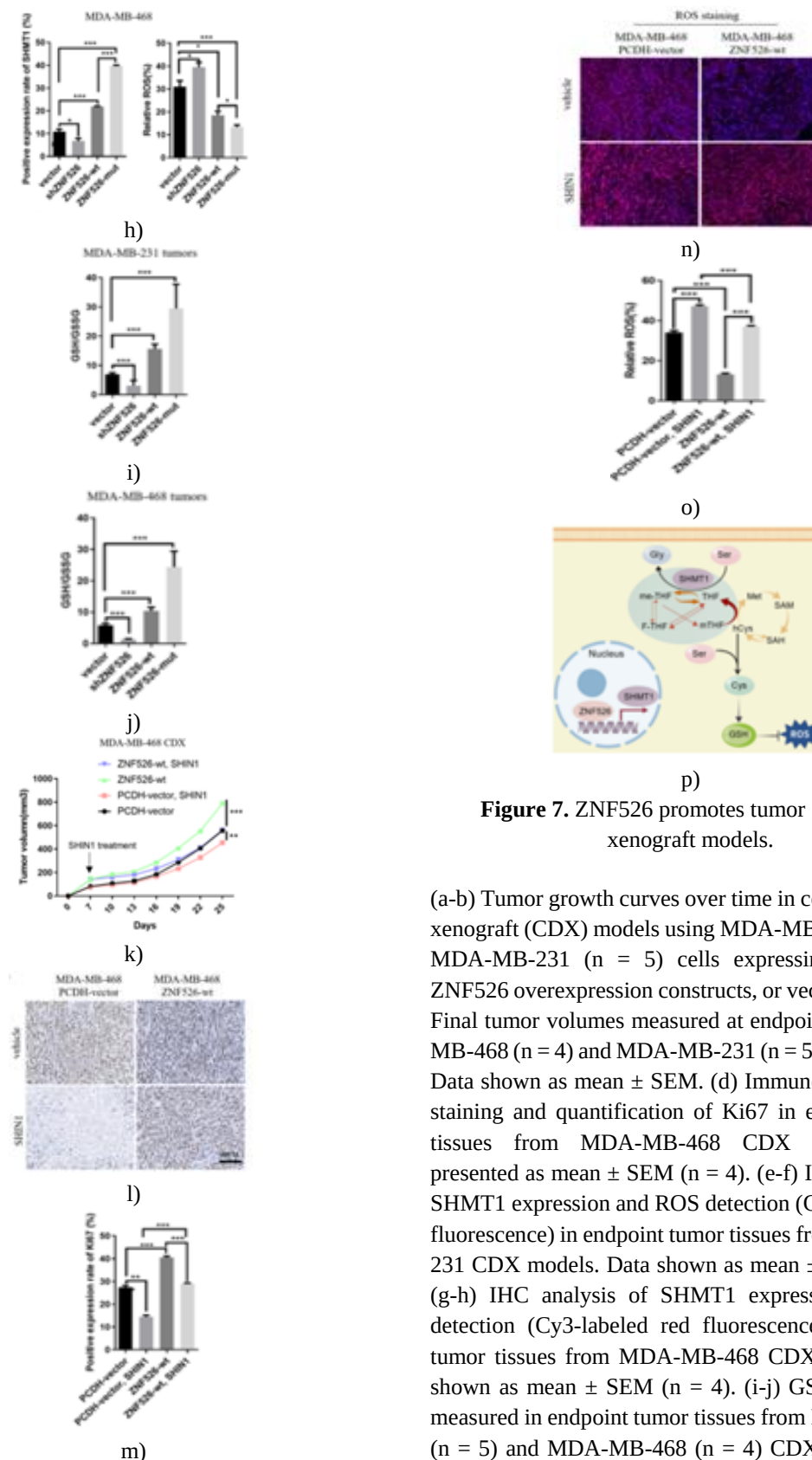
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f)



g)



**Figure 7.** ZNF526 promotes tumor growth in xenograft models.

(a-b) Tumor growth curves over time in cell line-derived xenograft (CDX) models using MDA-MB-468 ( $n = 4$ ) or MDA-MB-231 ( $n = 5$ ) cells expressing shZNF526, ZNF526 overexpression constructs, or vector control. (c) Final tumor volumes measured at endpoint from MDA-MB-468 ( $n = 4$ ) and MDA-MB-231 ( $n = 5$ ) CDX models. Data shown as mean  $\pm$  SEM. (d) Immunohistochemical staining and quantification of Ki67 in endpoint tumor tissues from MDA-MB-468 CDX models. Data presented as mean  $\pm$  SEM ( $n = 4$ ). (e-f) IHC analysis of SHMT1 expression and ROS detection (Cy3-labeled red fluorescence) in endpoint tumor tissues from MDA-MB-231 CDX models. Data shown as mean  $\pm$  SEM ( $n = 5$ ). (g-h) IHC analysis of SHMT1 expression and ROS detection (Cy3-labeled red fluorescence) in endpoint tumor tissues from MDA-MB-468 CDX models. Data shown as mean  $\pm$  SEM ( $n = 4$ ). (i-j) GSH/GSSG ratio measured in endpoint tumor tissues from MDA-MB-231 ( $n = 5$ ) and MDA-MB-468 ( $n = 4$ ) CDX models. Data shown as mean  $\pm$  SEM. (k) Tumor growth curves in

MDA-MB-231 CDX models bearing ZNF526-overexpressing or control cells treated with vehicle or 100 mg/kg SHIN1. Treatment began on day 7 after implantation and was administered three times weekly ( $n = 6$ ). (l-m) IHC staining and quantification of Ki67 in endpoint tumor tissues from MDA-MB-468 CDX models treated as described. Data shown as mean  $\pm$  SEM ( $n = 6$ ). (n-o) ROS detection in endpoint tumor tissues from MDA-MB-468 CDX models. Red fluorescence indicates Cy3-labeled ROS signal. Data shown as mean  $\pm$  SEM ( $n = 6$ ). (p) Schematic model illustrating that ZNF526 drives TNBC tumor progression and antioxidant defense by transcriptionally upregulating SHMT1, reprogramming SGOC metabolism, and increasing GSH levels. CDX: cell line-derived xenograft. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

Consistent with in vitro results, ZNF526 knockdown strongly decreased SHMT1 levels in tumors, whereas ZNF526 overexpression markedly elevated SHMT1 expression (**Figure 7e**). In addition, loss of ZNF526 caused notable ROS buildup in tumor tissues. In contrast, its overexpression effectively reduced ROS, reinforcing its importance in redox control (**Figure 7e**). The GSH/GSSG ratio was significantly decreased following ZNF526 knockdown but increased upon overexpression, with the V94A mutant producing the greatest increase (**Figures 7i and 7j**).

To examine the therapeutic potential of targeting the ZNF526-SHMT1 pathway in vivo, ZNF526-overexpressing MDA-MB-468 xenografts were treated with the SHMT1 inhibitor SHIN1. SHIN1 treatment potently blocked the accelerated tumor growth driven by ZNF526 overexpression, bringing tumor volumes to levels similar to those of control tumors (**Figure 7k**). This growth inhibition was accompanied by a clear decrease in Ki67-positive proliferating cells (**Figures 7l and 7m**). Furthermore, SHIN1 administration triggered ROS accumulation and largely reversed the ROS-suppressing effect of ZNF526 overexpression (**Figures 7n and 7o**).

Taken together, these results show that ZNF526 accelerates TNBC tumor development by inducing SHMT1 expression, which reprograms SGOC metabolism to boost GSH synthesis and strengthen cellular antioxidant capacity (**Figure 7p**).

Triple-negative breast cancer (TNBC) is the most aggressive form of breast malignancy, characterized by high metastatic potential, few targeted therapies, and dismal outcomes (median survival of approximately 18

months in metastatic disease) [24, 25]. Treatment currently relies almost entirely on conventional chemotherapy, underscoring a critical need for novel targeted therapies. The present work identifies ZNF526 as a key oncogenic factor in TNBC. We show that ZNF526 enhances tumor growth by transcriptionally activating SHMT1. This ZNF526-SHMT1 pathway augments SGOC metabolic activity, leading to increased GSH production, improved antioxidant defense, and sustained TNBC proliferation. These discoveries position ZNF526 as a promising therapeutic target in TNBC.

Although data are scarce, a germline variant in ZNF526 (rs144433879) has been linked to Hodgkin lymphoma susceptibility in genome-wide association studies [6]. Separately, the ZNF526 locus (particularly the rs3810151 p.Val94Ala variant) has been associated with breast cancer risk [7], yet its biological function in cancer development was previously unclear. Through comprehensive analysis of TCGA datasets and IHC validation, we identified TNBC-specific overexpression of ZNF526, which correlates with worse patient survival. Functional studies demonstrated ZNF526's essential contribution to TNBC cell proliferation and tumor progression. Remarkably, the Val94Ala variant exhibited significantly greater oncogenic activity than wild-type ZNF526, providing experimental confirmation of its gain-of-function properties.

The SGOC metabolic network constitutes a vital node in cancer metabolic rewiring, supplying one-carbon units for nucleotide synthesis, SAM-dependent methylation, and glutathione production — all essential for rapid proliferation and redox homeostasis [18, 26]. SHMT1, a core enzyme in this network, has emerged as an oncogenic driver in multiple tumor types, including breast cancer [27-29]. Untargeted metabolomics in our study showed that ZNF526 overexpression profoundly alters SGOC metabolism in TNBC cells. Mechanistic experiments established SHMT1 as a direct transcriptional target of ZNF526, with promoter luciferase assays confirming transactivation and ChIP-qPCR demonstrating physical binding. The Val94Ala mutation further amplified these effects, showing superior promoter binding and transcriptional activation relative to wild-type ZNF526 — clear evidence of gain-of-function. Future structural studies are warranted to elucidate how this mutation enhances ZNF526 activity. While SHMT1 was identified as a major effector, ZNF526 may influence additional targets or pathways in

TNBC, which should be explored in subsequent research.

Reactive oxygen species (ROS) exert context-dependent effects in cancer: moderate levels support signaling, whereas excessive ROS causes oxidative damage and cell death [30, 31]. Aggressive tumors such as TNBC often upregulate antioxidant systems, especially the GSH pathway, to counteract oxidative stress and maintain a favorable redox state for sustained growth [32]. Our data indicate that ZNF526 enhances TNBC fitness by upregulating SHMT1, thereby increasing SGOC flux, GSH synthesis, and the GSH/GSSG ratio to mitigate oxidative stress. Supplementation with antioxidants (NAC or GSH) rescued both proliferation and redox balance in ZNF526-deficient cells. Among major SGOC outputs (nucleotides, SAM, and GSH), only GSH effectively reversed the growth defect caused by ZNF526 loss. These findings firmly establish GSH as the primary metabolic mediator of the ZNF526-SHMT1 axis in TNBC. However, this pathway may also contribute to other malignant features such as metastasis, therapy resistance, or cancer stemness via additional SGOC branches, warranting further investigation.

In summary, this study uncovers a novel oncogenic signaling cascade in TNBC centered on the ZNF526-SHMT1-GSH axis. ZNF526, especially its Val94Ala cancer-associated variant, acts as a metabolic regulator, transcriptionally activating SHMT1, reprogramming SGOC metabolism to elevate GSH levels and create a supportive redox environment for tumor expansion. These insights not only highlight ZNF526 as an important previously unrecognized driver in TNBC but also identify the downstream SHMT1/GSH pathway as a potential therapeutic vulnerability.

## Conclusion

This work establishes ZNF526 as a central regulator of redox balance in TNBC. The gain-of-function Val94Ala variant (rs3810151) amplifies its oncogenic potential through enhanced transcriptional activity. Mechanistically, ZNF526 upregulates SHMT1 to increase glutathione biosynthesis, thereby conferring resistance to oxidative stress and promoting tumor growth. These discoveries position ZNF526 as a key metabolic controller in TNBC and suggest therapeutic strategies targeting ZNF526 itself or its downstream SHMT1/GSH axis.

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