

Article

Chromatin Engagement and Transcriptional Activity of the ZNF217 Exon 4–Skipping Isoform Are Associated with Breast Cancer Aggressiveness and Bone Metastasis

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Simple Summary

Breast cancer remains a significant global health issue, particularly due to the detrimental effects of bone metastases on patient outcome. Generation of aberrant splicing variants with oncogenic potential contribute to tumor development and progression. While the ZNF217 wild-type oncogenic transcription factor has been well studied, the unique role of the ZNF217 exon 4-splice isoform in breast cancer remains elusive. The aim of this study is to elucidate the chromatin engagement and transcriptional activity retained by this isoform, as well as its role in breast cancer aggressiveness and its contribution to the development of bone metastases. Our study highlights this ZNF217 isoform as an emerging oncogenic player, opening new avenues for its potential as a therapeutic target and biomarker for breast cancers prone to developing bone metastases.



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Abstract

Background: Breast cancer remains a major health issue, with bone metastases negatively impacting patient outcomes. The biochemical and biological functions of the exon 4-splice isoform (ZNF217-ΔE4) of the oncogenic transcription factor ZNF217 have been poorly investigated. **Methods/Results:** This study, for the first time, elucidates through advanced live-cell single-molecule tracking microscopy that the C-terminus of ZNF217 influences chromatin engagement and binding stability. ZNF217-ΔE4 retains its ability to be recruited and to promote positive transcriptional activity. CRISPR/Cas9-mediated silencing of the ZNF217 gene in MDA-MB-231 breast cancer cells impairs cell aggressiveness, while reintroduction of the ZNF217-ΔE4 isoform is sufficient to restore increased cell proliferation,

migration, invasion, and stemness features. In vivo, ZNF217 Δ E4—although less potent than the wild-type isoform—accelerates the formation of bone marrow micrometastases. A retrospective analysis of primary breast tumors revealed that patients with high ZNF217- Δ E4 mRNA levels had a higher risk of developing bone metastases. Conclusions: Overall, this study identifies ZNF217- Δ E4 as a novel functional isoform that mediates breast cancer cell aggressiveness and bone marrow homing. It also highlights this isoform as a promising biomarker and potential therapeutic target for breast cancers at elevated risk of bone metastasis.

Keywords: ZNF217; oncogene; isoform; splice variant; breast cancer; bone metastasis; biomarker

1. Introduction

Breast cancer remains the most diagnosed type of cancer and the most common cause of cancer-related death in women. It is the second leading cause of global cancer incidence in 2022, with an estimated 2.3 million new cases [1]. The zinc-finger protein 217 (ZNF217) orchestrates tumor progression at both early and late stages, is a powerful biomarker of poor prognosis, and a candidate target for future anti-cancer therapies [2,3]. ZNF217 wild-type (ZNF217-WT) has been shown to play deleterious functions in various human cancers, and more particularly in breast cancer [4–8]. This oncogene cooperates with several intracellular signaling networks to reprogram integrated circuits governing hallmark capabilities within cancer cells and impacts one of the most fundamental traits of cancer cells involving sustained chronic proliferation, resistance to chemotherapy, invasion, and migration [2,3]. In vivo experiments showed that ZNF217-WT overexpression confers highly aggressive properties to breast cancer cells, resulting in the development of metastases [4,5,8]. In primary breast tumors, high ZNF217 expression levels are of poor prognosis and predictive for several anti-cancer therapies response [5,6,8–11]. More than 70% of breast cancer patients will develop bone metastases [12]. Breast cancer-associated bone metastasis is essentially incurable with current therapies, the mechanisms driving the preferential spread of breast cancer to the bone remain poorly understood, and few drivers of bone metastasis development have been formally identified [13]. We previously discovered that the ZNF217-WT oncogene is one of the few demonstrated key drivers of bone metastases in breast cancer and that ZNF217 expression is an early indicator of bone metastases development in breast cancer [4,14].

The ZNF217 protein (1048 amino acids, aa) is a member of the Kruppel-like family of transcription factors and contains eight predicted C2H2 zinc finger motifs and a Proline-rich region [15]. ZNF217 was first described to mainly act as a transcriptional repressor complex [16]; however, additional research has shown that it positively regulates the expression of specific target genes [8,17–19], making it a complex and double-faceted transcriptional regulator. ZNF217's C-terminus region (750–1048 aa) is of utmost interest, as it possesses the transcriptional repressor domain [20] and a Proline-rich (757–1005 aa) domain [15] that might be involved in the ZNF217's transcriptional activator properties. The *ErbB3* gene encoding for a key growth factor receptor in breast cancer was identified as a direct target for the ZNF217 transcription factor and was the first gene shown to be positively regulated by the recruitment of ZNF217 to its promoter [18].

Alternative splicing in breast cancer has emerged as a novel hallmark, participating in the complexity of this pathology. Generation of aberrant splicing variants with oncogenic potential are involved in the development and progression of tumors, in apoptosis, cell proliferation, invasion, tumor metastasis, and drug resistance [21]. Splice variants thus

represent new therapeutic targets, and specific alternative splicing signatures represent new prognostic biomarkers in cancers [22,23]. In the first study discovering the human *ZNF217* gene, an alternative splicing of the exon 4 was predicted, resulting in a *ZNF217* mRNA variant (*ZNF217-ΔE4*) encoding a protein isoform with a unique C-terminal sequence (amino acids 1013–1061), distinct from *ZNF217*-WT protein sequence (amino acids 1013–1048) [15]. Only one study from our group has explored the significance of the exon 4-skipping isoform of the *ZNF217* oncogene in breast cancer cells [24]. This work reported for the first time the existence of *ZNF217-ΔE4* transcripts in primary breast tumors, and a positive correlation between *ZNF217-ΔE4* mRNA levels and those of *ZNF217*-WT. Ectopic expression of *ZNF217-ΔE4* in MDA-MB-231 breast cancer cells promoted an aggressive phenotype, potentially due to the concomitant increase in endogenous *ZNF217*-WT expression levels. Despite being pioneering, our previous work did not conclusively determine any *ZNF217*-WT independent impact of the *ZNF217-ΔE4* isoform, given the complex interplay with *ZNF217*-WT molecules. Finally, our pilot retrospective analysis explored, using RTq-PCR, the prognostic value of *ZNF217-ΔE4* mRNA levels with specific primers pairs and emphasized the need to assess both *ZNF217*-WT and *ZNF217-ΔE4* expression levels to obtain the most powerful poor prognosis biomarker value in primary breast tumors, at least in the tested cohort [24]. Considering the small size of the cohort used in this study, and taking into account that most of previous studies aiming at deciphering *ZNF217*'s prognostic potential investigated in reality either the expression of *ZNF217*-WT alone or the expression of both *ZNF217*-WT and that of its possible isoforms [4–6,8–11], there is an urgent need to get full insights into the biomarker value of *ZNF217-ΔE4* expression levels alone as well as the biological functions driven by the *ZNF217-ΔE4* protein in breast cancer cells.

The first aim of this study was to newly investigate how the *ZNF217* oncogene's C-terminus regulates genomic binding dynamics and transcriptional activity. To date, nobody has ever studied whether *ZNF217-ΔE4* retains any transcriptional activity or whether this latter is different from that of *ZNF217*-WT. The second aim was to decipher whether the *ZNF217-ΔE4* isoform is a mediator of breast cancer cells' aggressiveness, independently from *ZNF217*-WT. The *in vivo* impact of *ZNF217-ΔE4* splice variant and its role in the development of bone metastases being unknown, the third aim of this study was to decipher whether the expression of *ZNF217-ΔE4* splice variant in breast cancer cells is associated with bone metastases development.

Here, we provide evidence that *ZNF217-ΔE4*, independently from *ZNF217*-WT, is a new mediator of breast cancer cell aggressiveness and promotes *in vivo* breast cancer cell dissemination to the bone marrow. Using sophisticated live-cell Single Molecule Tracking, we demonstrated that *ZNF217-ΔE4*'s C-terminus alters chromatin engagement and binding stability. *In vitro* experiments also demonstrated that the *ZNF217-ΔE4* isoform retains *ZNF217*-WT's direct transcriptional activity. Our study reports for the first time the poor prognostic value of *ZNF217-ΔE4* mRNA levels in a large breast cancer cohort, and a pilot study highlighted that breast cancers with high *ZNF217-ΔE4* mRNA levels are prone to metastasize to the bone.

2. Materials and Methods

2.1. Analysis of RNA-seq Data

A total of 1031 RNA-seq aligned BAM files of breast cancer samples were acquired from the TCGA repository (Cancer Genome Atlas 2012). Gene expression was determined using RNA-seq and was DESeq normalized. Filtering was applied to retain only primary tumor samples from female subjects. Tumor purity information was not available for the

analyzed datasets and therefore was not included as a covariate in the DESeq normalization, which was performed using the standard DESeq2 workflow.

The calculation of the *ZNF217* gene's exon isoforms in each sample involved assessing the number of reads with spliced alignments from exon 4 to exon 5 junction (*ZNF217-WT*), and all reads mapped from the exon 3 to exon 5 junction (*ZNF217-ΔE4* isoform). Expression for exon 3 was computed to assess the expression of *ZNF217-all*. The expression for each exon was determined using the formula: $\text{Exon_DESeq} = (\text{Exon_reads} / \text{Total_reads}) * \text{Gene_DESeq}$. The correlation of expression was tested using the Pearson correlation coefficient with statistical significance set at $p < 0.05$. Cox proportional hazards regression was computed for each available cut-off value between the lower and upper quartiles, and false discovery rate was computed to correct for multiple hypothesis testing. Kaplan–Meier survival plots with hazard rate and 95% confidence intervals were used to visualize survival differences.

2.2. *ZNF217* Constructs

Taking benefit of the *ZNF217-WT* and *ZNF217-ΔE4* sequences we previously cloned into the pcDNA6 plasmid (Invitrogen, Cergy Pontoise, Paris, France) [7,24], we constructed and cloned into the pHTN HaloTag[®] CMV-neo vector (pHTN-Halo-CMV) (Promega, Madison, WI, USA) three *ZNF217*-derived constructs, all in frames at their respective N-terminus with the HaloTag sequence: (i) the *ZNF217-WT* isoform; (ii) the *ZNF217-ΔE4* isoform; and (iii) the truncated *ZNF217-G1013* variant, encoding the first 1013 aa sequence present on both *ZNF217-WT* and *ZNF217-ΔE4* proteins. The artificial *ZNF217-G1013* protein thus does not possess the C-terminus sequence that differs between the *ZNF217-WT* and *ZNF217-ΔE4* isoforms, as a result of *ZNF217* alternative splicing. The in-fusion cloning technology (Phusion[™] High-Fidelity DNA polymerase, Thermo Fisher Scientific, Waltham, MA, USA) was used for generating the resulting recombinant vectors pHTN-Halo-CMV-*ZNF217-WT*, pHTN-Halo-CMV-*ZNF217-ΔE4*, and pHTN-Halo-CMV-*ZNF217-G1013* vectors. The DNA constructs were validated by full sequencing.

2.3. RT-qPCR

Total RNA extraction, reverse transcription, and RT-qPCR measurements were performed as described previously [7,8]. CFX96 equipment with the SsoAdvanced Universal SYBR green supermix (BioRad, Hercules, CA, USA) was used for RT-qPCR measurements, according to the manufacturer's recommendations. Previously validated specific primers were used to amplify the DNA sequence for *ZNF217* variants [24]. Briefly, the 5'-AGTCCAAATCCCTGCCATCT-3' and 5'-GGGGAAACACTGGTTTTAGG-3' primers amplify a region within *exon 3* (E3), common to the two variants. The 5'-CTCGACGTTAGAAGGAAAAAG-3' and the 5'-TGGTCGATAATGTGCATTCC-3' primers were used to specifically explore *ZNF217-WT* expression: the forward primer hybridized onto the *exon 3–exon 4* junction of *ZNF217-WT* (E3-E4) isoform and the reverse primer to *exon 4*. The 5'-GTGGCTGACTGTTCAGAAGCCC-3' and 5'-GACATCCACCAAGACCTTCTA-3' primers are specific for the *ZNF217-ΔE4* isoform: the reverse primer hybridized onto the *exon 3–exon 5* junction of *ZNF217-ΔE4* (E3-E5) isoform and the forward primer to *exon 3*. The 5'-ATGGGCAAATCCGACAAACC-3' and 5'-CCAGTCGTGAATGACCAGGA-3' were used to amplify the DNA sequence for the Halo-Tag region. Normalization of all measurements was carried out with respect to the ribosomal 28S gene expression, using the 5'-CGATCCATCATCCGCAATG-3' and 5'-AGCCAAGCTCAGCGCAAC-3' primers. Relative gene expression was calculated using the ΔCt method, where $\Delta\text{Ct} = \text{Ct}(\text{gene of interest}) - \text{Ct}(28\text{S gene})$. When comparing experimental conditions, relative expression levels were determined using the $2^{-\Delta\Delta\text{Ct}}$ method.

2.4. Western Blotting

Western blot experiments were performed as previously described [8]. The antibodies used were: anti-ZNF217 (HPA051857, Sigma-Aldrich, Sigma, Saint Louis, MO, USA) targeting the 525–616 aa ZNF217's sequence; anti-ZNF217 antibody (#720352, Thermo Fisher Scientific) obtained after immunization against the 844–862 aa and 1029–1048 aa within ZNF217's peptide sequence; anti-ZNF217 antibody generated using a peptide located within the amino acid sequence of ZNF217-WT (1000–1048 aa) (Ab48133; Abcam, Paris, France); anti-HaloTag polyclonal antibody (G9281, Promega) and anti- α -tubulin antibody (#T5168; Sigma).

2.5. Cell Lines

HEK293 and HEK293T human embryonic kidney cell lines were purchased from the American Type Culture Collection (ATCC) and cultured according to recommendations in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (10,000 units/mL)-streptomycin (10,000 μ g/mL) antibiotics (Thermo Fisher Scientific). MDA-MB-231 breast cancer cells were purchased from ATCC and grown according to recommendations.

The MDA-MB-231-derived ZNF217-knock-out cell line was engineered using CRISPR/Cas9 technology by Creative Biogen company (Shirley, NY, USA). In brief, single-guide RNA (sgRNA) was designed according to ZNF217 human sequence, after which Cas9 and sgRNA vectors were co-transfected into MDA-MB-231 cells. Three days after transfection, pool cells were collected to extract genomic DNA to confirm a successful knockdown of ZNF217. The cell pool went through monoclonal growth after which PCR and Sanger sequencing were done for further validation of ZNF217 knockdown. A specific clone with 156 bp and 112 bp deletion alleles (MDA-MB-231-ZNF217-KO cell line) was selected and used in our study (Supplementary Figure S1). MDA-MB-231 and MDA-MB-231-ZNF217-KO cell lines were grown in DMEM F-12 medium (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin–streptomycin antibiotics. ZNF217 silencing was validated by PCR (using the 5'-CAGCTCTCTTGGCAGTCCGA-3' and 5'-GGCCGGTGTTCATTAAGAC-3' primers) and by Western blotting. This study focuses on the rescue of the knockout phenotype through the reintroduction of the ZNF217 WT, ZNF217 Δ E4, or ZNF217 G1013 isoforms in the MDA-MB-231-ZNF217-KO cell line (see Section 2.6). As the study was specifically designed to compare the phenotypes driven by the three isoforms within the same knockout background (MDA-MB-231-ZNF217-KO cell line), the likelihood of off target-related artifacts was minimized.

A model of breast cancer progression involving mammary epithelial cells (MCF10A, mimicking the benign state) and three further-derived cell lines mimicking the pre-cancerous state (early-transformed human mammary MCF10AT1 cells), the ductal carcinoma in situ state (MCF10DCIS), and the malignant state (MCF10CA1) were purchased from Karmanos Institute (Detroit, USA). Cells were maintained in complete DMEM/Ham's F12 medium with 5% horse serum (Thermo Fisher Scientific) and additional supplements: 100 ng/mL cholera enterotoxin, 10 mg/mL insulin, 0.5 mg/mL hydrocortisol, 20 ng/mL epidermal growth factor (Sigma) and 1% penicillin–streptomycin antibiotics.

2.6. Establishment of ZNF217 Isoforms Stable Transfectants

Cell lines were stably transfected with pHTN-Halo-CMV empty vector, pHTN-Halo-CMV-ZNF217-WT, pHTN-Halo-CMV-ZNF217- Δ E4 or pHTN-Halo-CMV-ZNF217-G1013 plasmids, then selected in the presence of 1 mg/mL geneticin/G418 (Thermo Fisher Scientific).

2.7. Live-Cell Single Molecule Imaging of ZNF217 Isoforms

Low passage number HEK293 cell lines stably expressing Halo-tagged ZNF217-WT, ZNF217-G1013, or ZNF217-ΔE4 isoforms were plated on a 35 mm Mattek imaging dish and allowed to attach for 72 h at 37 °C. After attachment, cells were labeled with 0.5 nM of JF549-HTL (Promega) for 15 min. Media containing the JF549-HTL was removed, and L-15 (Gibco, Waltham, MA, USA) was added to support cells in conditions lacking CO₂. To minimize microscope drift and cell movement, imaging experiments were performed at room temperature. Cells were imaged on a customized inverted Nikon Eclipse Ti microscope with a 100X oil-immersion objective lens (Nikon, Melville, NY, U.S.A, 1.49 NA) and further magnified 1.69X post-objective. Cells were continuously illuminated using a 532 nm (13 W/cm², Coherent) and time-lapse two-dimensional (2D) images of single molecules were acquired for ~10 min at ~2 Hz (effectively 0.532 s/frame) using a Prime 95B sCMOS camera (Teledyne) with a 1200 × 1200 pixel field of view (final pixel size of 65 nm).

2.8. Image Processing and Single Molecule Tracking

ImageJ was used to subtract background noise using a rolling ball radius of 50 pixels. Multi-Target Tracking (MTT) [25] using SLIMfast [26] was used to determine the X,Y position of discrete single spots via fitting Point Spread Functions (PSFs) with a 2D gaussian function. Binding trajectories of individual chromatin binding events were determined by connecting ZNF217 X,Y localizations between consecutive frames based upon a maximum expected diffusion constant of 0.05 microns²/s allowing for 1.5 s gaps in trajectories due to blinking or missed localizations. X, Y positions over time from individual chromatin binding trajectories were averaged to generate a 2D projection map of ZNF217 binding events over 10 min of imaging. 2D projection maps of ZNF217 binding events showed clear enrichment of trajectories in a pattern equivalent to the expected size and shape of a nucleus. ZNF217 tracks that fell outside of the nucleus were excluded. Photobleach rates were then determined for each movie based upon exponential decay of the global fluorescence of chromatin bound ZNF217-WT, ZNF217-G1013, or ZNF217-ΔE4.

2.9. Analysis of ZNF217 Isoforms Chromatin Binding Residence Times

Chromatin binding residence time was determined using GRID analysis [27] performed on residence times aggregated from multiple cells using a regularization parameter of 0.005 and kmin/kmax values of −3 and 1, respectively, for all conditions. Fits were resampled 100 times containing 80% of the data to obtain mean and error values. Statistical significance was determined by performing an unpaired two sample *t*-test. An event spectrum analysis was used to better define two closely related populations that contained elevated residence times.

2.10. Analysis of ZNF217 Isoforms Clustering in Hubs

2D projection maps of ZNF217 binding events lasting at least one second were expanded 10-fold in the X and Y directions yielding a final pixel size of 6.5 nm. A ZNF217 binding density map was determined by counting the number of binding events within an octagon window (diameter 130 nm) as it was raster scanned across the nucleus of the expanded 2D projection map. Contiguous octagon windows centered on an individual pixel containing at least 3 ZNF217 binding events lasting longer than 1.5 s were defined and labeled as hubs. A previous study has shown that there is a strict linear relationship between the number of hubs detected and the number of chromatin binding events in a cell [28]. To normalize the number of ZNF217 hubs within cells containing different numbers of chromatin binding events, we divided the total number of hubs within a cell

by the total number of chromatin binding events and then multiplied by 10,000 (e.g., Total number of hubs/10,000 binding events).

2.11. Chromatin Immunoprecipitation (ChIP) Experiments

HEK293T cells were transfected with pHTN-Halo-CMV empty vector, pHTN-Halo-CMV-ZNF217-WT, pHTN-Halo-CMV-ZNF217-ΔE4 or pHTN-Halo-CMV-ZNF217-G1013 plasmids, then grown for 24 h. ChIP experiments were performed as previously described [8] using the following antibodies: anti-ZNF217 antibody (#720352, Thermo Fisher Scientific), anti-acetyl-Histone H3 (Lys9) antibody (AcH3K9) (#07-352, Sigma-Aldrich), anti-trimethyl-Histone H3 (Lys4) Antibody (3meH3K4) (#07-473, Sigma-Aldrich), anti-trimethyl-histone H3 (Lys9) antibody (3meH3K9) (#07-442, Sigma-Aldrich), anti-trimethyl-histone H3 (Lys27) antibody (3meH3K27) (#07-449, Sigma-Aldrich) or anti-rabbit IgG as negative control (Ab171870, Abcam). Immunoprecipitated DNA was quantified by q-PCR using the sense 5'-TTATGGGGTTTCGAGTCTGG-3' and the anti-sense 5'-GGTTGGTTCACCTTGGTGGAT-3' primers that hybridize onto the endogenous *ERBB3* proximal promoter (−458 to −200), as previously described [18]. The background signal obtained with anti-rabbit IgG was subtracted, and the results were presented as a percentage of the input DNA quantified through q-PCR.

2.12. Luciferase Reporter Assay

HEK293T cells were transfected with either 150 ng *ERBB3*_pGL4 Luciferase Reporter construct generously provided by Dr. Sweeney [18], 10 ng Renilla luciferase plasmid (pTK-RL) and with either pHTN-Halo-CMV empty vector, or ZNF217 variants expressing vectors (pHTN-Halo-CMV-ZNF217-WT, pHTN-Halo-CMV-ZNF217-ΔE4 or pHTN-Halo-CMV-ZNF217-G1013 plasmids). After transfection, cells were grown for 24 h and luciferase activities were then assessed using the Dual Luciferase Kit (Promega) [8].

2.13. Transcriptomic Analysis

RNA isolation was performed, as previously described [7,8], from three independent cell culture replicates of each tested cell line (HEK293 cells stably expressing Halo-tagged ZNF217-WT, ZNF217-ΔE4 or ZNF217-G1013 isoforms; control cells were HEK293 cells stably transfected with pHTN-Halo-CMV empty vector). Library preparation and RNA sequencing were performed at the ProfileXpert platform (UCBL, Lyon, France). Quality of samples was checked by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and RNA was quantified by Quantifluor RNA kit (Promega). First, mRNA was enriched from 200 ng of total RNA, then library preparation was realized with the MGIEasy RNA Directional Library Prep Set (MGI). Quality of libraries were checked by Fragment Analyzer (Agilent) and quantified by Qubit 1X dsDNA HS Assay Kit. Samples were pooled and after circularization in ssDNA, DNB (DNA NanoBalls) were prepared, following the manufacturer protocol. Then sequencing was performed on the MGI DNBSEQ-G400, run Single Read 100 bp on a Flow Cell FCL SE100 (MGI). Data were demultiplexed with BasecallLite v1.5.0.323 producing fastQ file. Data analysis were performed using Partek suite software (version 8.0). Reads trimming was performed using cutadapt v4.2 software. Then the reads were mapped using STAR aligner (ver2.7.8a) with default parameters on the human genome GRCh38. Data normalization was realized using median ratio calculation and differential expression was performed by DEseq algorithm. Genes were considered as differentially expressed for a fold change of 1.5 and *p*-value < 0.05.

Pathways significantly deregulated were characterized using Gene set enrichment (Partek suite) using KEGG data base (Homo sapiens hsa_v1_22_08_24) and Panther pathway software (pantherdb.org/tools/compareToRefList.jsp) version 16 [29]. In the latest version, Panther pathway introduced reactome resources. *p*-value is the probability or

chance of seeing at least x number of genes out of the total n genes in the list annotated to a particular GO term, given the proportion of genes in the whole genome that are annotated to that GO Term. That is, the GO terms shared by the genes in the user's list are compared to the background distribution of annotation. The closer the p -value is to zero, the more significant the particular GO term associated with the group of genes is (i.e., the less likely the observed annotation of the particular GO term to a group of genes occurs by chance). A Gene Set Enrichment Analysis (GSEA) has been performed with fast preranked gene set enrichment analysis software (Galaxy version 1.8) using mouse collection gene set (MSigDB).

2.14. Cell Proliferation Assay

Cells (8000 cells per well) were plated into a 96-well plate then grown up to 96 h. The medium was changed every 2 days. Proliferating cells were analyzed as previously described using a Cell Proliferation ELISA 5-bromodeoxyuridine (BrdU) Kit (Roche, Meylan, France) [6].

2.15. Cell Migration and Cell Invasion

For the transwell migration assay, we used BD Falcon™ Cell Culture Inserts with an 8 μ m pore size from BD Biosciences (Bedford, MA, USA). For the invasion assay, the same inserts were employed, but they were pre-coated with 10% growth factor-reduced matrigel (Corning, Bedford, MA, USA) at a final concentration of 0.3 mg/mL. In both assays, 50,000 cells were seeded onto the top chamber in 300 μ L of serum-free medium, while 700 μ L of complete medium containing 10% FBS was added to the bottom chamber in a 24-well plate. The migration assays were stopped by fixing the cells after 24 h, and invasion assays after 48 h. Subsequently, the inserts were stained with hematoxylin and eosin (H&E), mounted, and photographed according to reference [30]. The counting of migrated or invaded cells was conducted using ImageJ® software (version 1.54d).

2.16. Mammosphere Formation Efficiency (MFE)

Single-cell suspensions were seeded using non-adherent mammosphere culture conditions [31]. After 7 days, primary mammospheres (first generation) were counted, collected, trypsinized, and re-plated for 10 days in non-adherent culture conditions to generate second-generation mammospheres. The culture media were replenished every 2–3 days. The mammospheres from passages 1 and 2 were counted, and the size of mammospheres was measured for passage 2 using ImageJ® software (ImageJ 1.54d).

2.17. Animal Studies

All protocols, activities involving animals, including their housing, care, euthanasia method, and experimental protocols, were carried out conforming with the guidelines established by the local ethical committee (Comité d'Expérimentation Animale de l'Université Claude Bernard Lyon 1, CEEA-55) under project license MESRI Number: APAFIS #46312-2024011214359545. Four-week-old female immunodeficient BALB/c *nude* mice were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France). Low passage number MDA-MB-231-ZNF217-KO cell lines stably transfected with the pHTN-Halo-CMV, the pHTN-Halo-CMV-ZNF217-WT or the pHTN-Halo-CMV-ZNF217- Δ E4 plasmids were inoculated into the tail artery (5×10^5 cells in 100 μ L of PBS) of anesthetized mice at day 0. Each group included 7 mice. After one week from tumor cell introduction [32–34], mice under anesthesia were sacrificed via cervical dislocation, and collection of bone marrow was performed for tumor cell colony assays (bone micrometastasis) following previously established protocols [33]. The intra-arterial injection model is preferable to intracardiac injection because it results in a higher proportion of mice developing metastases and avoids the

significant mortality risk associated with intracardiac delivery [32,35,36]. This intra-arterial approach is therefore well suited to studying the ability of cancer cells to disseminate and colonize the lungs and bones. More details are provided in the Supplementary Material and Methods. This study was conducted without any conflicts of interest.

2.18. Primary Breast Tumor Cohort

Women with primary breast tumors ($n = 107$) and known clinical follow-up who had not received any therapy before surgery and who relapsed, or not, while receiving endocrine therapy and/or chemotherapy were recruited from the BB-0033-00050, Biological Resources Center (CRB) Centre Léon Bérard, Lyon France (Supplementary Table S1). This study has been approved by the local ethics committee (CRB Centre Léon Bérard, France). The CRB Centre Léon Bérard is quality certified according to NFS96-900 French standard and ISO 9001 for clinical trials, ensuring scientific rigor for sample conservation, traceability, and quality, as well as ethical rules observance and defined rules for transferring samples for research purposes (Ministry of Health for activities authorization n° AC-2019-3426 and DC-2008-99). The localization of all metastases was known. Patients who relapsed were classified as having either “bone-only” metastases or other distant metastatic sites. Patients presenting both bone and visceral metastases were very uncommon in our cohort and were therefore included in the “Other” category, in accordance with previously published analyses [4]. The material used in the study has been collected in agreement with all applicable laws, rules, and requests of French and European government authorities, including the patients’ informed written consents. Extraction of total RNA from frozen tumor samples and RT-qPCR measurements were performed as previously described [8]. Univariate (log-rank) analysis and all statistical analyses were performed using the SPSSTM Software (version 16.0) (IBM, Armonk, NY, USA). For univariate analyses, the data were divided at the median value of *ZNF217*-WT (*E3–E4*), or *ZNF217-ΔE4* (*E3–E5*) mRNA expression into two groups with either high or low expression levels. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Retrospective RNA-seq Analysis of 1031 Primary Breast Tumor Samples Reveals That High *ZNF217-ΔE4* mRNA Expression Levels Are Associated with Poor Prognosis

This study investigated for the first time the prognostic significance of the *ZNF217-ΔE4* isoform expression levels in a large cohort of breast cancer patients. In 1031 primary breast tumors, we retrospectively analyzed spliced alignments from exon 3 to exon 5 (*ZNF217-ΔE4* isoform), reads mapped to the exon 3 to exon 4 junction (*ZNF217*-WT isoform), and exon 3 reads (assessing exon 3 present on both *ZNF217*-WT and *ZNF217-ΔE4* isoform, and named *ZNF217-all*). In accordance with previous literature [4–6,8,10], Figure 1A,B illustrated that both high *ZNF217-all* mRNA levels or high *ZNF217*-WT mRNA levels were significantly associated with shorter overall survival (OS) (p value of 0.045 and of 0.01, respectively). Remarkably, high *ZNF217-ΔE4* mRNA levels were also significantly associated with poor prognosis (p value of 0.041, Figure 1C), suggesting that aberrant splicing of *ZNF217*’s exon 4 is a new biomarker of poor prognosis. When included together in a multivariate Cox proportional hazards regression model, neither *ZNF217*-WT nor *ZNF217-ΔE4* remained statistically significant, indicating that these two biomarkers are not independent prognostic factors.

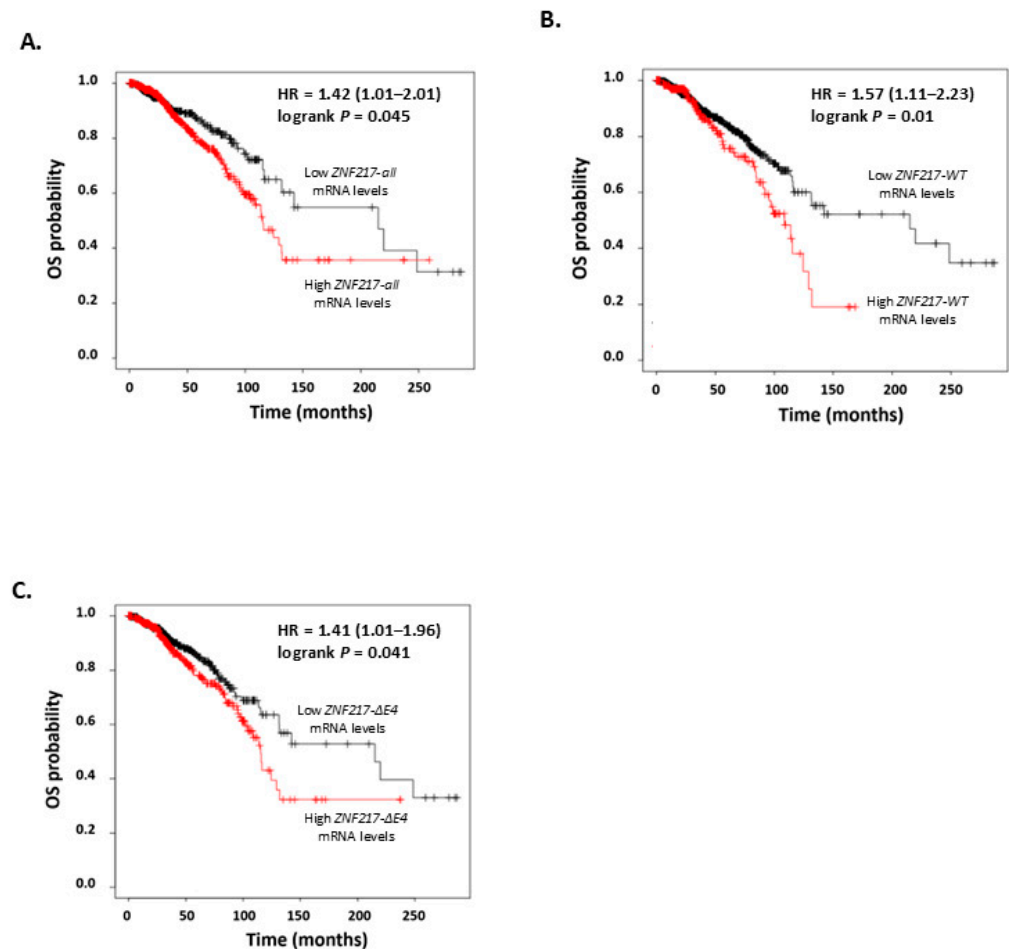


Figure 1. Primary breast cancers ($n = 1031$) with high ZNF217- $\Delta E4$ mRNA expression levels are associated with shorter OS. Kaplan–Meier analysis (univariate analysis) of (A) ZNF217-*all* mRNA expression levels, (B) ZNF217-WT mRNA expression levels, and (C) ZNF217- $\Delta E4$ mRNA expression levels. HR, hazard ratio; the 95% confidence interval (CI) is indicated in brackets.

3.2. Establishment and Validation of ZNF217 Variants Expression Plasmids

The ZNF217- $\Delta E4$ protein isoform possesses a C-terminus sequence [1013–1061 amino acids (aa)] distinct from that of ZNF217-WT protein (1013–1048 aa sequence) [15]. More precisely, the C-terminus sequence is different between the two isoforms, starting from aa1014, as aa1013 is a glycine (G) in the sequence of both isoforms. In order to decipher ZNF217- $\Delta E4$'s biological function and the role played by the ZNF217's C-terminus in both isoforms, we engineered three N-terminus Halo-tagged constructs: (i) the ZNF217-WT isoform; (ii) the ZNF217- $\Delta E4$ isoform; and (iii) the truncated ZNF217-G1013 variant, which contains the sequence shared both by ZNF217-WT and ZNF217- $\Delta E4$ proteins (1013 aa). We confirmed the expression of the different ZNF217 constructs in transfected HEK293T cells (which express very weak/no endogenous ZNF217 levels) at both the mRNA (Supplementary Figure S2A–C) and protein (Supplementary Figure S2D–G) levels. The three isoforms are predicted to possess the DNA binding domain [15,16].

3.3. The ZNF217- $\Delta E4$'s C-Terminus Alters Chromatin Engagement and Binding Stability

Live-cell imaging studies have shown that several transcription factors and chromatin remodelers, including Sox2, RNA Pol II, and PBAF, dynamically access their genomic sites [28,37–43]. Many of these imaging studies also showed that these factors formed discrete clusters or hubs of binding of approximately ~200–300 nm in diameter within the nucleus [28,37–42]. To better define how ZNF217 variants engage chro-

matin, we performed live-cell Motion Blur HILO microscopy combined with 2D Single Molecule Tracking (SMT) using N-terminally Halo-tagged ZNF217-WT, ZNF217-G1013 and ZNF217-ΔE4 variants. Under our imaging conditions (~0.5 s/frame), fast diffusing Halo-ZNF217 molecules are blurred out while chromatin bound Halo-tagged ZNF217 molecules appear as bright discrete spots that are spatially and temporally resolved (Supplementary Figure S3A and Movies S1–S3).

Many transcription factors and chromatin remodelers rapidly cycle on and off of their target sites on chromatin in select nuclear regions or hubs [28,37–42]. To determine if Halo-tagged ZNF217-WT also displayed clustered dynamic binding on chromatin, we closely examined two-dimensional projection maps of the average X,Y positions of Halo-tagged ZNF217-WT binding trajectories (Supplementary Figure S3B). Halo-tagged ZNF217-WT clearly bound to select regions of the nucleus (e.g., hubs) while binding was not present in other nuclear areas (Supplementary Figure S3B). To better quantify Halo-tagged ZNF217-WT chromatin binding hubs, we generated dynamic binding frequency heat maps [28], which identified discrete chromatin binding hubs (~200–400 nm in diameter) scattered throughout the nucleus (Supplementary Figure S3C). These chromatin binding hubs likely represent repeated dynamic cycling of Halo-tagged ZNF217-WT on target genomic sites within a highly confined region of the nucleus. We further assessed the dynamic binding on chromatin displayed by Halo-tagged ZNF217-G1013 and ZNF217-ΔE4 molecules. Quantification of the normalized number of hubs in a cell revealed no significant differences in the number of target sites accessed by the Halo-tagged ZNF217-WT, ZNF217-G1013, and ZNF217-ΔE4 molecules, suggesting that the ZNF217's C-terminus is unlikely involved in genomic target site selection (Supplementary Figure S3D). Control experiments, where we computationally randomized X,Y positions of binding events in a nucleus, showed significantly reduced clustering of Halo-tagged ZNF217 molecules (Supplementary Figure S3D, “Cont.”).

SMT has been previously used to determine the residence time or dissociation rate of various factors bound to chromatin [28,43]. To kinetically define multiple populations (~4–5) of chromatin bound factors, we performed GRID analysis on residence times of the Halo-tagged ZNF217-WT, ZNF217-G1013 and ZNF217-ΔE4 molecules [27,28]. GRID analysis revealed three well defined chromatin binding populations (P1, P2 & P3) displaying average residence times ranging from ~0.5–12 s, which are not significantly affected by modification of ZNF217's C-terminus (Figure 2A,B). This suggests that short-lived (~3–12 s) chromatin interactions are not regulated by ZNF217's C-terminus.

For the Halo-tagged ZNF217-WT and ZNF217-G1013 proteins, the fourth and fifth chromatin binding populations (P4 & P5) were more diffuse but still discernable (Figure 2A). In stark contrast, the Halo-tagged ZNF217-ΔE4 protein showed much more clearly defined fourth and fifth chromatin binding populations (P4 & P5). In addition, Halo-tagged ZNF217-ΔE4 molecules had significantly reduced residence times compared with Halo-tagged ZNF217-WT and ZNF217-G1013 molecules (e.g., ~27 and ~79 s for t4 and t5, respectively, of Halo-tagged ZNF217-ΔE4 versus ~42–43 and ~100–105 s for t4 and t5, respectively, and of Halo-tagged ZNF217-WT/ZNF217-G1013, $p < 0.001$) (Figure 2B). This suggests that alteration of ZNF217's C-terminus in the ZNF217-ΔE4 natural variant selectively destabilizes ZNF217's ability to bind chromatin for long periods of time (e.g., >~40 s) relative to ZNF217-WT and the ZNF217-G1013 variant, which behave similarly.

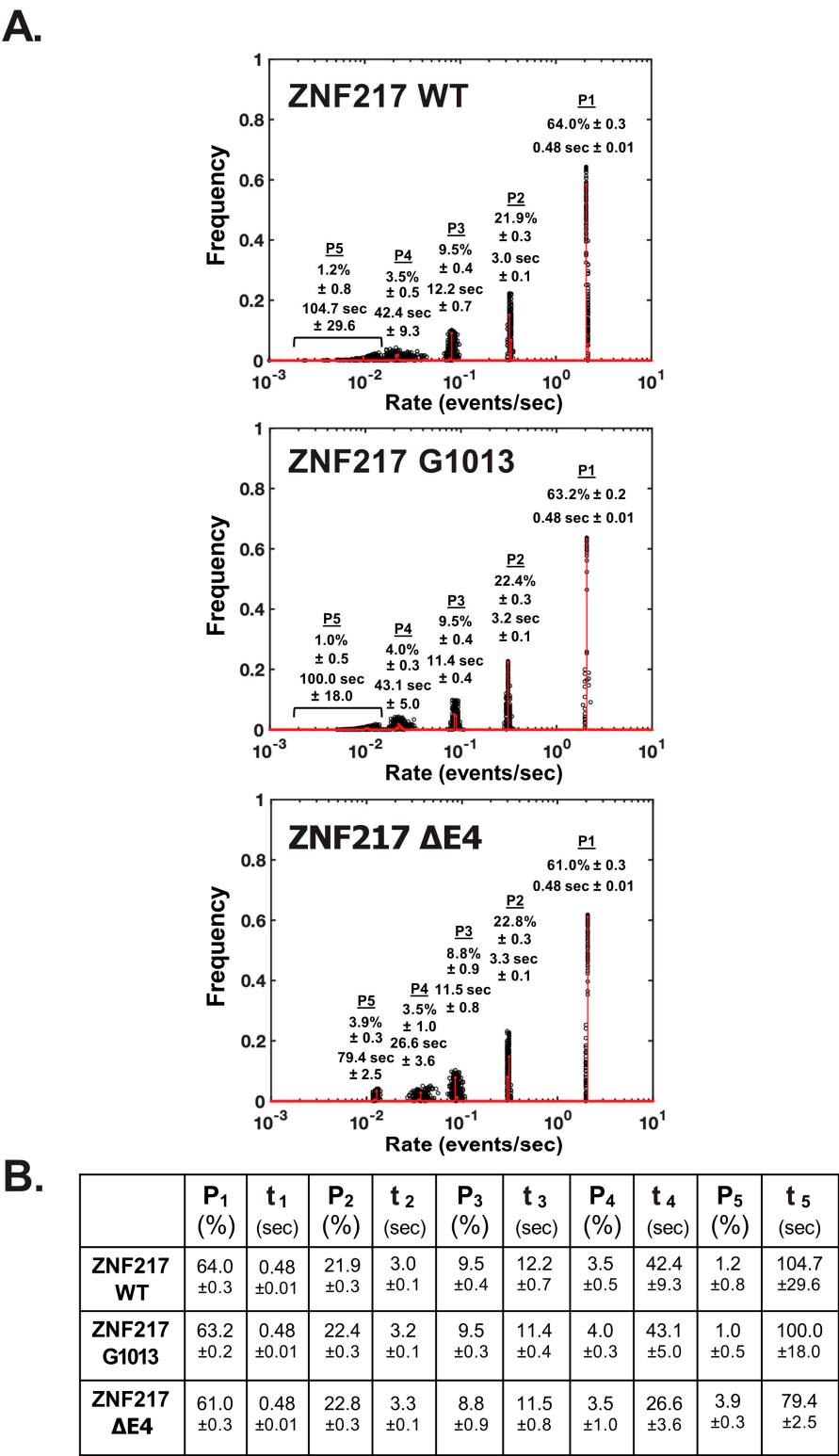


Figure 2. Residence Time Analysis of the stability of Halo-tagged ZNF217 variants on chromatin. **(A)** GRID analysis of Halo-tagged ZNF217’s chromatin binding residence time inside cells. **(B)** Table of GRID analysis of Halo-tagged ZNF217-WT, ZNF217 G1013 and ZNF217-ΔE4’s chromatin binding residence time aggregated across all cells ($n = 163,297$ Halo-tagged ZNF217-WT in 18 cells, $n = 364,197$ Halo-tagged ZNF217-G1013 binding events in 22 cells, and $n = 284,288$ and Halo-tagged ZNF217-ΔE4 binding events in 21 cells). Populations (P) in percentage (%) and time (t) in seconds are illustrated for each ZNF217 variant.

Many eukaryotic proteins contain a predominant binding population (e.g., >60% of binding events) lasting less than 1 s when visualized using Motion Blur Microscopy [27,28,43]. These extremely short-lived binding events are thought to represent factors that are searching for their targets and engaging non-specifically with chromatin. Interestingly, the ZNF217-ΔE4 natural spliced variant has a significantly lower percentage of binding events in this “target search mode” (e.g., P1) compared with ZNF217-WT or ZNF217-G1013 (Figure 2B, 61% for ZNF217-ΔE4 versus 64% for ZNF217-WT, $p < 0.001$). Correspondingly, ZNF217-ΔE4 has an increased percentage of binding events in its longest lasting population (P5) compared to ZNF217-WT or ZNF217-G1013 (Figure 2B, 3.9% for ZNF217-ΔE4 versus 1.2% for ZNF217-WT $p < 0.001$). This suggests that ZNF217-ΔE4 finds and engages its long-lived chromatin targets more efficiently yet is less stable on these chromatin targets compared to ZNF217-WT or ZNF217-G1013. Overall, our results suggest that the ZNF217-ΔE4 variant may contain select key residues within its C-terminus that enhance its rapid association with its chromatin targets. This rapid association of the ZNF217-ΔE4 variant with chromatin may help potentiate transcription of select target genes. Alternatively, additional residues with the ZNF217-ΔE4 variant may also bind a negative regulator which now destabilizes ZNF217 on select target genes to prematurely shut down transcription.

3.4. The ZNF217-ΔE4 Isoform Binds and Positively Regulates the ERBB3 Proximal Promoter in a Greater Manner than ZNF217-WT

Previous ChIP-chip array investigations have shown that the *ERBB3* proximal promoter (−458 to −200) contains ZNF217 binding sites [17], and that ZNF217-WT occupancy at *ERBB3* proximal promoter is associated with transcription activation [18]. Having analyzed by SMT the global behavior of ZNF217 variants at the genomic level, we sought to investigate, as a proof of concept, the binding of ZNF217-ΔE4 natural splice isoform and that of ZNF217-G1013 truncated variant at the *ERBB3* proximal promoter. Chromatin immunoprecipitation (ChIP) experiments were conducted in transiently transfected HEK293T cells using an anti-ZNF217 antibody recognizing a specific epitope present on both ZNF217-WT, ZNF217-G1013, and ZNF217-ΔE4 molecules. Figure 3A shows a high ZNF217-WT enrichment at the *ERBB3* proximal promoter, supporting previous findings [18]. Our study newly highlights that both the ZNF217-ΔE4 isoform and the ZNF217-G1013 truncated variant can bind to the *ERBB3* proximal promoter (Figure 3A), leading us to further explore the chromatin state of the latter. ChIP experiments were conducted with antibodies for histone activation marks (AcH3K9 and 3meH3K4) and for histone repression marks (3meH3K9 and 3meH3K27). We confirmed that ZNF217-WT was associated with a significant enrichment of AcH3K9 and 3meH3K4 histone activation marks ($p < 0.001$, respectively, Figure 3B). Conversely, histone repression marks (3meH3K9 and 3meH3K27), usually found associated with promoter repression [44], were absent from *ERBB3* proximal promoter (Figure 3B). The presence of the ZNF217-ΔE4 protein led to a dramatic enrichment of AcH3K9 and 3meH3K4 marks ($p < 0.001$ Figure 3B), even significantly greater than those observed with the ZNF217-WT isoform ($p < 0.001$, Figure 3B), while there was an absence of 3meH3K9 and 3meH3K27 marks (Figure 3B). In the presence of the ZNF217-G1013 truncated variant, histone marks present at the *ERBB3* proximal promoter displayed a pattern similar to that observed with ZNF217-WT (Figure 3B), with a lower enrichment of AcH3K9 and 3meH3K4 marks compared to that of ZNF217-ΔE4 ($p < 0.001$ Figure 3B). To validate our findings at the transcriptional level, HEK293T cells were co-transfected with an *ERBB3*-promoter driven luciferase reporter plasmid [18] along with ZNF217 variants expression plasmids. Ectopic expression of ZNF217-WT, ZNF217-ΔE4 and ZNF217-G1013 molecules significantly increased the relative luciferase activity in a dose-dependent manner (Supplementary Figure S4A–C). At a steady concentration of transfected ZNF217 expression vectors, ZNF217-ΔE4 was associated with enhanced transcriptional activity ($p < 0.001$)

compared to ZNF217-WT or ZNF217-G1013 (Figure 3C). No difference was observed between ZNF217-WT or ZNF217-G1013 transcriptional activity (Figure 3C) supporting Figure 3B's data. Finally, in silico RNAseq analysis on 1031 primary breast tumors revealed that *ZNF217-ΔE4* mRNA levels are significantly positively correlated with *ERBB3* mRNA levels (Pearson coefficient_{ZNF217-ΔE4} = 0.26 $p < 10^{-17}$, respectively), as well as *ZNF217-WT* mRNA levels (Pearson coefficient_{ZNF217-WT} = 0.37, $p < 10^{-33}$) (Supplementary Figure S5).

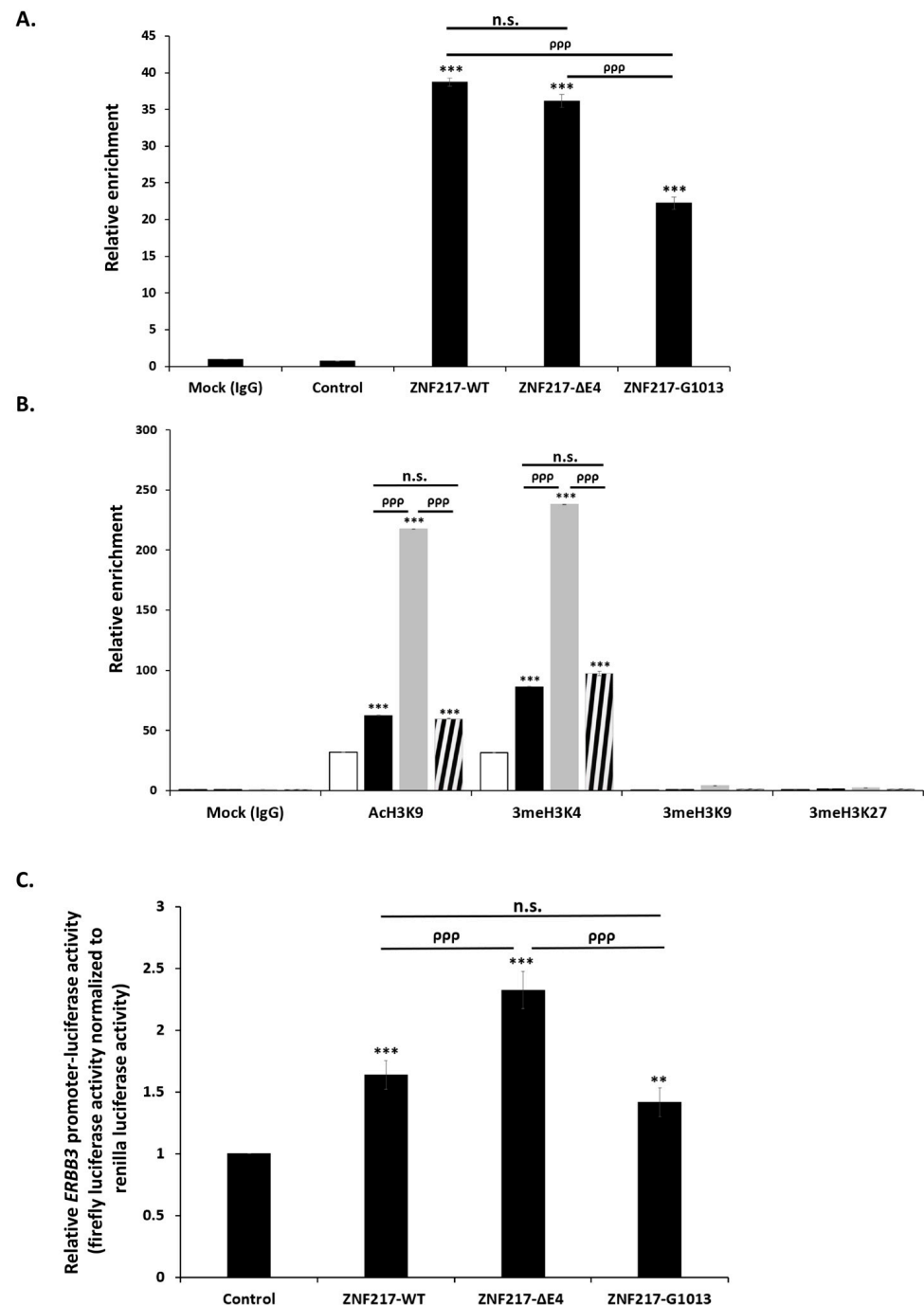


Figure 3. ZNF217-ΔE4 binds and positively regulates the *ERBB3* promoter in a higher manner compared to the ZNF217-WT. (A) ChIP assays using anti-ZNF217 antibody or human IgG as a control on the endogenous *ERBB3* promoter in HEK293T cells transiently transfected for 24 h with pHTN-Halo-CMV (control), pHTN-Halo-ZNF217-WT, pHTN-Halo-ZNF217-ΔE4 or pHTN-Halo-ZNF217-G1013 plasmids. The amount of immunoprecipitated DNA were quantified using RT-qPCR. Values, expressed as a % of the input DNA, are representative of three independent ChIP experiments.

*** $p < 0.001$ (Student's t -test). (B) ChIP assays performed as in (A) using antibodies for histone activation marks (AcH3K9 and 3meH3K4) and repression marks (3meH3K9 and 3meH3K27) or human IgG as a control on the endogenous *ERBB3* promoter in HEK293T cells transiently transfected for 24 h with pHTN-Halo-CMV (control) (white bar), pHTN-Halo-ZNF217-WT (black bar), pHTN-Halo-ZNF217-ΔE4 (grey bar) or pHTN-Halo-ZNF217-G1013 (dashed bar) plasmids. Representative of three independent ChIP experiments. For significance, n.s. = not significant, *** $p < 0.001$ versus control and ρρρ $p < 0.001$ versus ZNF217-ΔE4 (Student's t -test). (C) Relative luciferase activity of HEK293T cells co-transfected with an *ERBB3* promoter-driven luciferase reporter along with pHTN-Halo-CMV (control), pHTN-Halo-ZNF217-WT, pHTN-Halo-ZNF217-ΔE4 or pHTN-Halo-ZNF217-G1013 plasmids for 24 h. Histogram represents the ratio of the *firefly* value normalized to the *renilla* value (mean ± SD of at least three independent experiments). For significance, n.s. = not significant, *** $p < 0.001$ and ** $p < 0.01$ versus control and ρρρ $p < 0.001$ versus ZNF217-ΔE4 (Student's t -test).

Taken together, our study robustly demonstrates that the ZNF217-ΔE4 isoform retains recruitment and positive transcriptional activity at the *ERBB3* promoter. Most importantly, the ZNF217-ΔE4 isoform seems to be more efficient than the ZNF217-WT isoform, suggesting that the specific 1013–1061 aa C-terminal sequence present on the spliced variant is crucial for powerful positive transcriptional activity, at least at the *ERBB3* proximal promoter. Surprisingly, both ChIP and reporter assays indicated that the ZNF217-G1013 variant behaves similarly to the ZNF217-WT isoform, supporting the hypothesis that positive transcriptional activity of ZNF217 molecules at the *ERBB3* promoter is not fully held by the C-terminal region. However, the presence of the ZNF217-ΔE4's 1013–1061 aa sequence was associated with greater transcriptional activity and a significant enrichment of positive histone marks.

3.5. Gene Expression Profiling of ZNF217 Isoforms-Overexpressing Cells

To provide new mechanistic insights into how ZNF217 variants drive expression profiles, we performed transcriptomic analysis in HEK293 cells stably transfected with ZNF217-WT, ZNF217-G1013, and ZNF217-ΔE4 variants (Supplementary Figure S6A). Control cells were stably transfected with the pHTN-Halo-CMV plasmid. Statistical analysis identified 590 deregulated genes (DEGs) in the ZNF217-WT gene signature, 545 DEGs in the ZNF217-ΔE4 gene signature, and 393 DEGs in the ZNF217-G1013 gene signature (Supplementary Figure S6B and Table S2). Interestingly, 57.6% and 62.4% of the ZNF217-WT and ZNF217-ΔE4 gene signatures, respectively, shared in common the same DEGs. On the other hand, only 34.2% and 42.9% of the ZNF217-WT and ZNF217-ΔE4 gene signatures, respectively, were found jointly deregulated with the ZNF217-G1013 gene signature. Focusing on the significantly deregulated genes, we identified the top positively enriched biological pathways (FDR < 0.05) using PANTHER analysis (Supplementary Figure S6C). Notably, the ZNF217-WT and ZNF217-ΔE4 gene signatures displayed highly similar enrichment profiles, including the cadherin-signaling pathway, two glutamate receptor pathways, and the Wnt signaling pathway. The ZNF217-ΔE4 signature additionally showed positive enrichment for the integrin-signaling pathway. In contrast, the ZNF217-G1013 gene signature exhibited enrichment only for the glutamate receptor and cadherin-signaling pathways, and with lower FDR values than those observed for ZNF217-WT and ZNF217-ΔE4. Complementary GSEA analysis supported the PANTHER results, highlighting epithelial-mesenchymal transition (EMT) as one of the most enriched pathways associated with the two natural isoforms (Supplementary Figure S6D). This analysis also confirmed that global differences in enriched pathways between ZNF217-WT and ZNF217-ΔE4 remain modest, whereas the ZNF217-G1013 signature is markedly distinct (Supplementary Figure S6D).

3.6. CRISPR/Cas9 Silencing of the ZNF217 Gene in MDA-MB-231 Breast Cancer Cells Impairs Breast Cancer Cell Aggressiveness

Our previous work [24] did not conclusively determine any ZNF217-WT independent impact of the ZNF217-ΔE4 isoform. We thus used CRISPR/Cas9 technology to establish a specific clone (MDA-MB231-ZNF217-KO) where *ZNF217* gene was fully silenced (validation by genomic DNA sequencing, Supplementary Figure S1), resulting in abrogation of ZNF217-WT both at the mRNA and protein levels (Supplementary Figure S7). Compared to the parental MDA-MB-231 cells, MDA-MB-231-ZNF217-KO cells displayed a significant and dramatic decrease in cell proliferation (measurement of the proportion of cells entering S phase) (Figure 4A, $p < 0.001$). CRISPR/Cas9 silencing of *ZNF217* in MDA-MB-231 cells also resulted in a spectacular inhibition of cell migration (Figure 4B, $p < 0.001$) and cell invasion (Figure 4C, $p < 0.001$). Finally, we investigated the growth and size of cancer stem cell populations upon *ZNF217* silencing. Figure 4D illustrates that silencing *ZNF217* does not affect mammosphere formation, as no significant change in the number of mammospheres was observed between MDA-MB-231 and MDA-MB-231-ZNF217-KO-derived mammospheres. Conversely, we found that silencing *ZNF217* greatly decreased the size of the formed mammospheres, indicating that ZNF217 silencing impacts mammosphere growth (Figure 4E, $p < 0.001$). Representative cell images corresponding to Figure 4B,C,E are provided in Supplementary Figure S8. Altogether, constitutive silencing of *ZNF217* in breast cancer cells dramatically decreases features of cell aggressiveness, making ZNF217 an attractive therapeutic strategy.

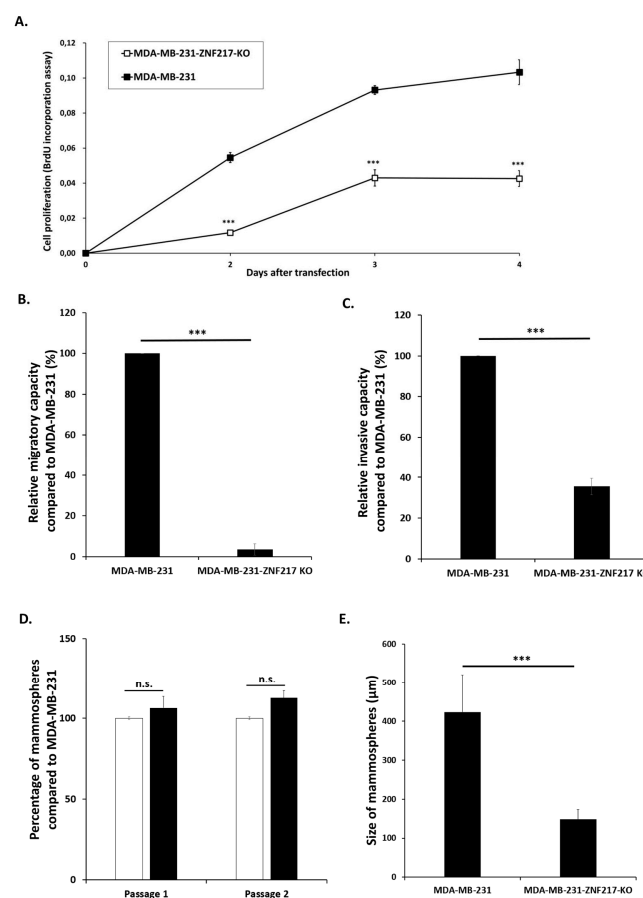


Figure 4. CRISPR/Cas9 silencing of the *ZNF217* gene in MDA-MB-231 cells impairs cell proliferation, migration, invasion and mammosphere size. (A) Cell proliferation was assessed at different time points by BrdU labeling (values are representative of three independent experiments). *** $p < 0.001$ (Student's *t*-test). (B) Bar charts showing the percentage of MDA-MB-231 and MDA-MB-231-ZNF217-KO migrated

cells (mean $\pm$ SD of three independent experiments) *** $p < 0.001$ (Student's t -test). (C) Bar charts representing the percentage of MDA-MB-231 and MDA-MB-231-ZNF217-KO invading cells (mean $\pm$ SD of three independent experiments) *** $p < 0.001$ (Student's t -test). (D) MDA-MB-231 (white bars) and MDA-MB-231-ZNF217-KO (black bars) cells were grown in non-adherent culture conditions and mammospheres were counted. (E) Mammospheres size measurement at passage 2 (mean $\pm$ SD of 3 independent experiments). For significance, n.s. = not significant, *** $p < 0.001$ (Student t -test).

3.7. Ectopic Expression of the ZNF217- Δ E4 Isoform in MDA-MB-231-ZNF217-KO Cells Restored an Aggressive Phenotype

We sought to decipher whether reintroducing the ZNF217- Δ E4 isoform in MDA-MB-231-ZNF217-KO cells was sufficient to recover cell aggressiveness by investigating cell proliferation (cells entering S phase), cell migration, cell invasion and stemness properties. Our previous work did not allow to state the precise impact of ZNF217- Δ E4, as ZNF217- Δ E4 over-expression in MDA-MB-231 cells was associated with upregulated expression of endogenous ZNF217-WT [24]. Having carefully checked that reintroducing ZNF217- Δ E4 in the MDA-MB231-ZNF217-KO cells did not lead, as expected, to any increase in endogenous ZNF217-WT expression levels (Supplementary Figure S9), we were thus able in this study to investigate the biological functions displayed by the ZNF217- Δ E4 molecule, independently from that of ZNF217-WT.

Previous studies from our group have shown that ectopic expression of ZNF217-WT in breast cancer cells induced several features of aggressiveness [7,8]. In accordance with those observations, overexpression of ZNF217-WT in MDA-MB231-ZNF217-KO cells (Figure 5A) was associated with greater cell proliferation (Figure 5B, $p < 0.001$), increased cell migration (Figure 5C, $p < 0.001$), increased cell invasion (Figure 5D, $p < 0.01$), increased first and second mammosphere formation efficiency (Figure 5E, $p < 0.001$ and $p < 0.01$, respectively) and increased mammosphere size (Figure 5F, $p < 0.001$). Strikingly, our study discovered that the reintroduction of ZNF217- Δ E4 in MDA-MB-231-ZNF217-KO cells was sufficient to restore an aggressive phenotype, thus recapitulating ZNF217-WT biological activity (Figure 5A–F). Unexpectedly, the ZNF217- Δ E4's stimulating impact on cell proliferation, cell migration, cell invasion and mammosphere formation efficiency was even greater than that observed after introducing ZNF217-WT (Figure 5B–E). Surprisingly, introducing the ZNF217-G1013 truncated variant in MDA-MB-231-ZNF217-KO cells had no significant impact in terms of cell proliferation, cell migration, cell invasion, mammosphere formation efficiency and mammosphere size (Figure 5A–F), emphasizing that the C-terminus regions of both the natural ZNF217-WT isoform and ZNF217- Δ E4 splice variant are crucial in mediating functional deleterious impact in breast cancer cells. Representative cell images corresponding to Figure 5C,D,F are provided in Supplementary Figure S9.

We next explored by RT-qPCR whether deregulation of endogenous ZNF217-WT or ZNF217- Δ E4 expression could be detected in a breast cancer cell model of tumor progression. The MCF10 cells system represents a unique system for examining the molecular alterations that occur during breast cancer progression [45–49]. This system includes: (i) the spontaneously immortalized non-transformed and non-tumorigenic human mammary epithelial MCF10A cell line; (ii) the premalignant MCF10AT1 cells, derived from xenograft-passaged MCF10A cells transfected with T24 Ha-ras, which generate carcinomas in ~25% of xenografts; (iii) The MCF10DCIS cells which represent a cellular model of ductal carcinoma in situ, i.e., non-invasive cancer cells, generating xenografts in mice in 100% of cases; and (iv) the fully malignant MCF10CA1 cells which generate in vivo invasive tumors. Figure 5G,H illustrated that MCF10AT1, MCFDCIS, and MCF10CA1 cells showed weak but significantly higher endogenous expression levels of both ZNF217-WT and ZNF217- Δ E4 isoforms compared to the MCF10A cells, with pre-malignant MCF10AT1 cells showing the highest level of expression of both isoforms.

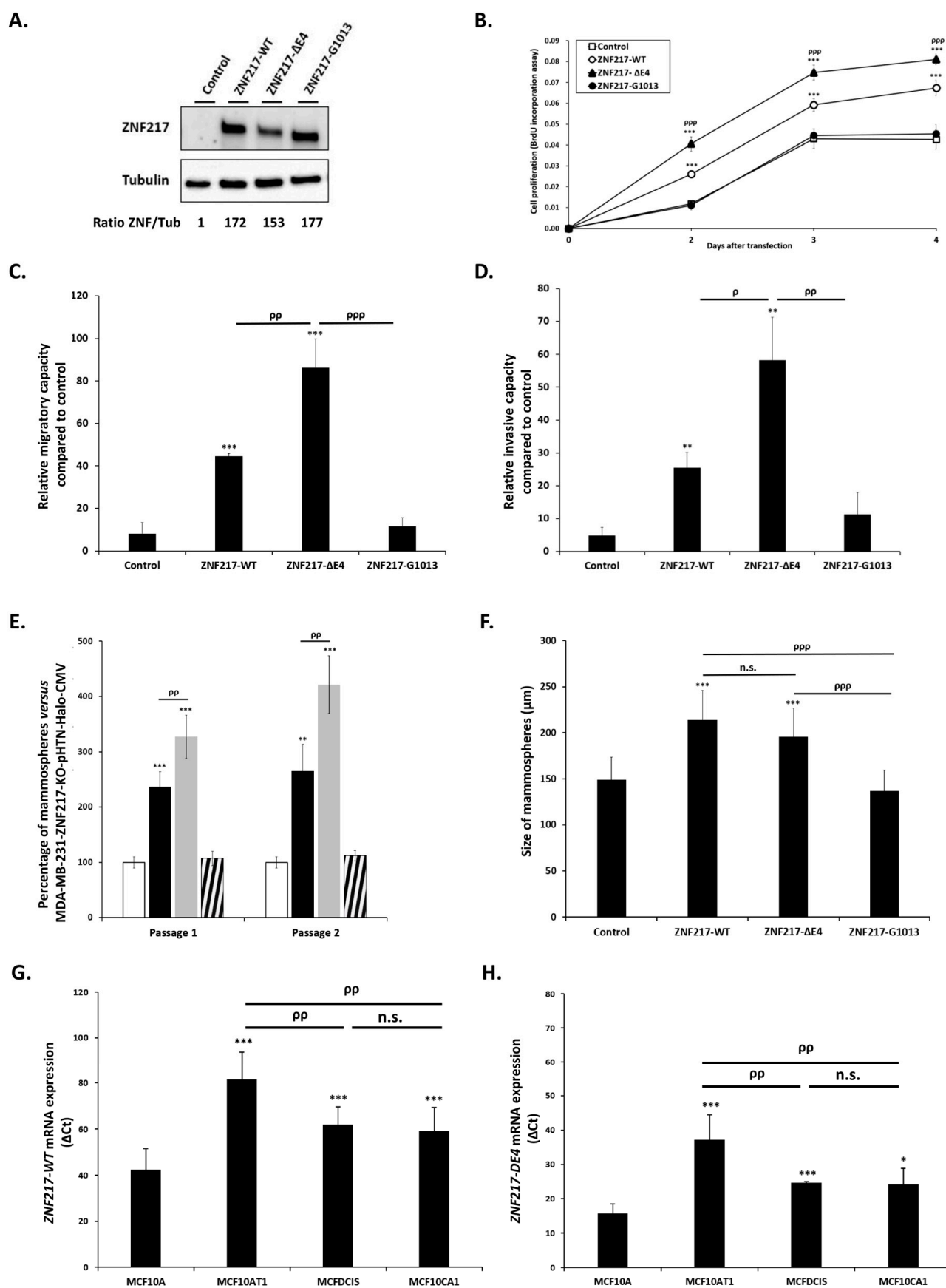


Figure 5. The introduction of ZNF217-ΔE4 in MDA-MB-231-ZNF217-KO cells restores an aggressive phenotype. The experiments were conducted after transient transfection of MDA-MB-231-ZNF217-KO

cells with either the pHTN-Halo-CMV (control), pHTN-Halo-ZNF217-WT, pHTN-Halo-ZNF217-ΔE4 or pHTN-Halo-ZNF217-G1013 plasmids. (A) Western Blot of ZNF217 variants expression (HPA051857 Ab). (B) Cell proliferation was assessed at different time points by BrdU labeling (values are representative of three independent experiments). *** $p < 0.001$ versus control; $\rho\rho\rho p < 0.001$, ZNF217-ΔE4 versus ZNF217-WT (Student's *t*-test). (C) Bar charts showing the percentage of migrated cells (mean $\pm$ SD of three independent experiments). *** $p < 0.001$ versus control; $\rho\rho\rho p < 0.001$ and $\rho\rho p < 0.01$, versus ZNF217-ΔE4 (Student's *t*-test). (D) Bar charts representing the percentage of invading cells (mean $\pm$ SD of three independent experiments). ** $p < 0.01$ versus control; $\rho\rho p < 0.01$ and $\rho p < 0.05$ versus ZNF217-ΔE4 (Student's *t*-test). (E) Mammosphere formation assay performed in MDA-MB-231-ZNF217-KO cells transiently transfected with the pHTN-Halo-CMV (white bar), the pHTN-Halo-ZNF217-WT (black bar), pHTN-Halo-ZNF217-ΔE4 (grey bar) or pHTN-Halo-ZNF217-G1013 plasmids (dashed bar) (mean $\pm$ SD of three independent experiments). *** $p < 0.001$ and ** $p < 0.01$ versus control; $\rho\rho p < 0.01$ ZNF217-ΔE4 versus ZNF217-WT (Student's *t*-test). (F) Bar graphs representing the size of mammosphere (values are representative of three independent experiments after passage 2). For significance, n.s. = not significant, *** $p < 0.001$ versus control and $\rho\rho\rho p < 0.001$ versus ZNF217-G1013. (G,H) RT-qPCR measurement of endogenous ZNF217-WT (G) and ZNF217-ΔE4 (H) mRNA levels in MCF10A, MCF10AT1, MCFDCIS, and MCF10CA1 cells (means $\pm$ SD of three independent experiments). *** $p < 0.001$, * $p < 0.05$ versus MCF10A cells; $\rho\rho p < 0.01$ versus MCF10AT1; n.s. = not significant (Student's *t*-test).

Altogether, our data highlighted that: (i) ZNF217-ΔE4 promotes breast cancer cell aggressiveness, independently of ZNF217-WT; (ii) the ZNF217-ΔE4 deleterious biological impact seems to be greater than that developed by ZNF217-WT, at least in vitro, suggesting an important role of its specific C-terminus sequence; and (iii) ZNF217-G1013 variant had no impact in mediating cell aggressiveness in the tested functional assays, suggesting that the C-terminus regions of ZNF217-WT or ZNF217-ΔE4 isoforms are crucial in mediating cell aggressiveness.

3.8. ZNF217-ΔE4 Promotes In Vivo Breast Cancer Cell Dissemination to the Bone Marrow

To investigate whether ZNF217-ΔE4 could drive tumor cell anchorage in bone marrow, control MDA-MB-231-ZNF217-KO stably transfected with the pHTN-Halo-CMV, the pHTN-Halo-CMV-ZNF217-WT or the pHTN-Halo-CMV-ZNF217-ΔE4 plasmids (Figure 6A) were injected into the tail artery of immunodeficient mice ($n = 7$ mice per group). One week after tumor cell inoculation, these animals were culled and the number of micrometastases in bone marrow was quantified 6 weeks later (count colonies under geneticin selection) (Figure 6B). Our in vivo data demonstrated that MDA-MB-231-ZNF217-KO-pHTN-Halo-CMV cells did not lead to any bone marrow micrometastasis formation, while reintroducing ZNF217-WT led to a significant extent of bone micrometastases in six out of seven mice (Figure 6C,D, $p < 0.001$). Those new data are in accordance with our previous in vivo study that reported that ZNF217-WT overexpression confers highly aggressive properties to MDA-MB-231 breast cancer cells, resulting in the development of severe bone metastases after intracardiac injection to the mice [4]. Strikingly, reintroduction of ZNF217-ΔE4 in MDA-MB-231-ZNF217-KO cells was associated with a significant increase in bone marrow micrometastasis formation in five out of seven mice (Figure 6C,D, $p < 0.001$), yet lower than that developed by the ZNF217-WT isoform (Figure 6D, $p < 0.001$). Overall, the ZNF217-ΔE4 splice variant was a new mediator of breast cancer cells homing in the bone marrow, while less efficient than the ZNF217-WT isoform.

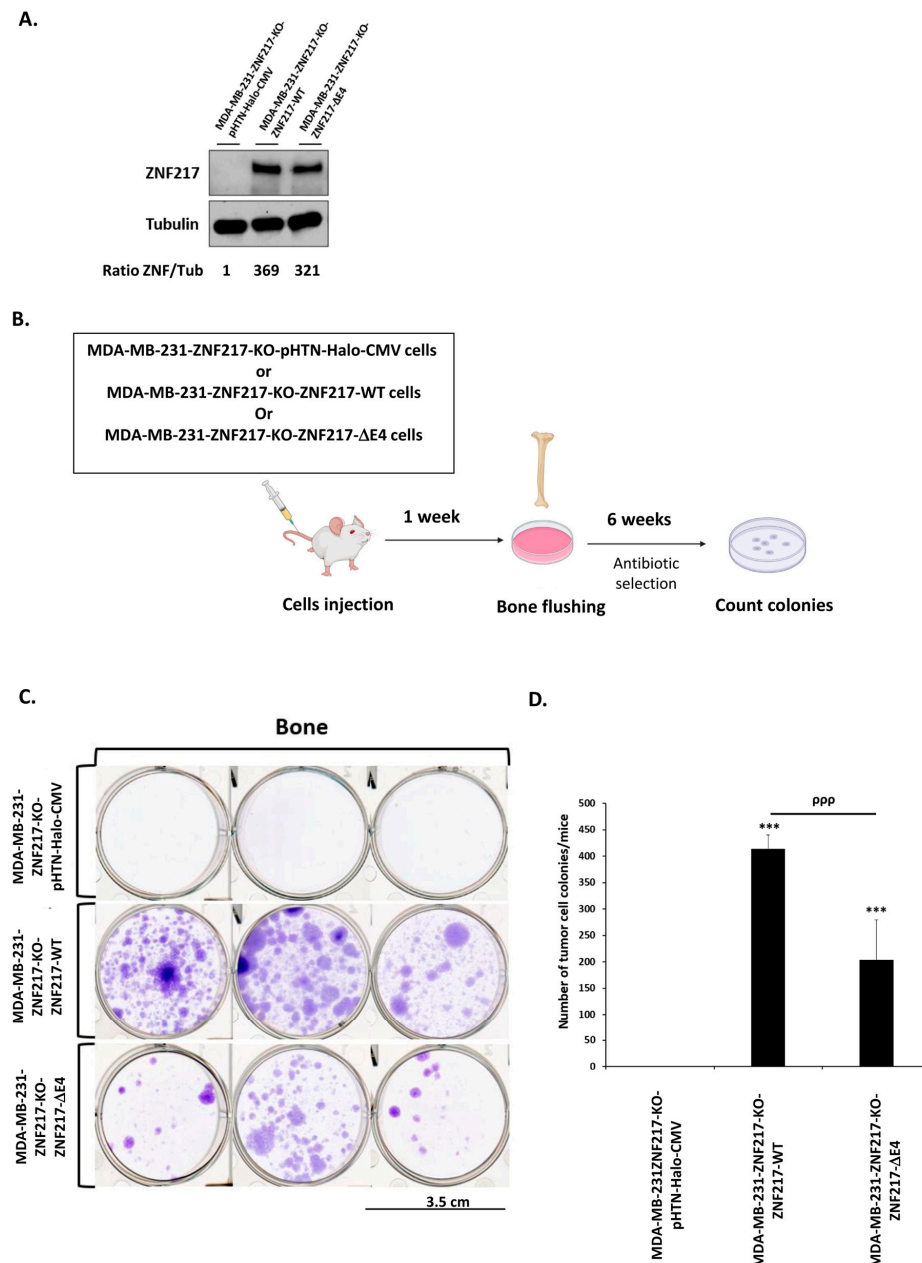


Figure 6. ZNF217-WT and ZNF217-ΔE4 in MDA-MB-231-ZNF217-KO cells promote the burden of micrometastatic disease in the bone marrow in vivo. **(A)** Western blot analysis (HPA051857 Ab) of ZNF217 isoforms expression in MDA-MB-231-ZNF217-KO cells stably transfected with the pHTN-Halo-CMV (control cells), the pHTN-Halo-CMV-ZNF217-WT or the pHTN-Halo-CMV-ZNF217-ΔE4 plasmids. **(B)** Schematic representation of the experimental protocol. Control MDA-MB-231-ZNF217-KO stably transfected with the pHTN-Halo-CMV, the pHTN-Halo-CMV-ZNF217-WT or the pHTN-Halo-CMV-ZNF217-ΔE4 plasmids were inoculated intra-arterially to Balb/c nude mice ($n = 7$ per group). One week after tumor cell inoculation, animals were culled, and the bone marrow was collected for tumor cell colony assays. **(C)** representative images of tumor cell colonies in the bone marrow are shown for each cell line. **(D)** Bar graphs showing the average number of tumor cell colonies formed in the bone marrow for each cell line. Data are expressed as the mean $\pm$ SD. *** $p < 0.001$ versus control cells; ppp $p < 0.001$ ZNF217-ΔE4 versus ZNF217-WT (Student's t -test).

3.9. ZNF217-ΔE4 mRNA Levels Are Prognostic Factor for Breast Cancer Bone Metastasis

We performed a retrospective analysis by quantifying by RT-qPCR ZNF217-WT (E3–E4 primers pair) and ZNF217-ΔE4 (E3–E5 primer pairs) mRNA levels in 107 human primary breast cancers that did or did not metastasize, with the location of metastases known. We

observed that the occurrence of bone-only metastases was associated with: (i) the *ZNF217*-WT-high group (100%) rather than with the *ZNF217*-WT-low group (0%) ($p = 0.011$; Table 1); and (ii) the *ZNF217*- $\Delta E4$ -high group (89%) rather than with the *ZNF217*- $\Delta E4$ -low group (11%) ($p = 0.041$; Table 1). Kaplan–Meier analyses showed that patients in the *ZNF217*-WT-high group had a higher risk of developing bone-only metastases ($p = 0.024$; Figure 7A), whereas *ZNF217*-WT mRNA levels were not significantly associated with other distant metastases ($p = 0.617$; Figure 7B). Patients in the *ZNF217*- $\Delta E4$ -high group had also a higher risk of developing bone-only metastases ($p = 0.023$; Figure 7C), whereas *ZNF217*- $\Delta E4$ mRNA levels were not significantly associated with other distant metastases ($p = 0.356$; Figure 7D). Overall, we newly report that both patients with high *ZNF217*-WT mRNA expression levels or with high *ZNF217*- $\Delta E4$ mRNA expression levels in primary breast tumors had a higher risk of developing bone metastases.

Table 1. *ZNF217*-WT and *ZNF217*- $\Delta E4$ mRNA levels in primary breast tumors of patients with metastatic disease; patients who developed metastases ($n = 27$) are compared according to whether they had high or low *ZNF217* expression, and whether they had bone-only metastases or metastases in other distant sites.

	<i>ZNF217</i> -WT mRNA Expression Levels			<i>ZNF217</i> - $\Delta E4$ mRNA Expression Levels		
	Low	High	p^a	Low	High	p^a
Bone-only metastases ($n = 9$)	0 (0%)	9 (100%)	0.011	1 (11%)	8 (89%)	0.041
Other distant metastases ($n = 18$)	9 (50%)	9 (50%)		10 (56%)	8 (44%)	

p^a (Fisher's exact test) was considered significant when $p < 0.05$.

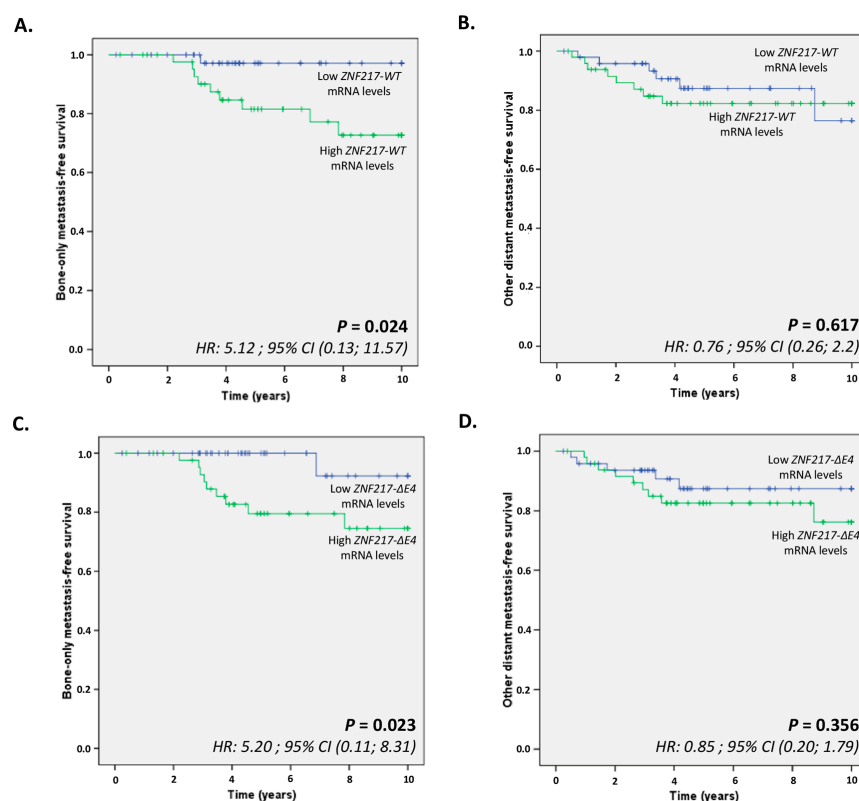


Figure 7. Breast cancers with high *ZNF217*-WT or high *ZNF217*- $\Delta E4$ mRNA expression levels are prone to metastasize to the bone. (A,B) Kaplan–Meier analyses of *ZNF217*-WT mRNA expression levels for bone-only metastasis-free survival (A) or other distant metastasis-free survival (B). (C,D) Kaplan–Meier analyses of *ZNF217*- $\Delta E4$ mRNA expression levels for bone-only metastasis-free survival (C) or other distant metastasis-free survival (D). CI, 95% confidence interval; HR, hazard ratio.

4. Discussion

An increasing amount of research indicates that alternative splicing events (ASE) play a significant role in tumor progression, particularly in breast cancer. Multiple studies have shown that ASE in breast cancer can lead to the production of proteins with novel functions [22,50]. The molecular mechanisms that govern ASE in specific biological contexts remain largely unclear, and the regulatory processes leading to exon 4 alternative splicing in the *ZNF217* gene are unknown. However, previous studies have emphasized the importance of N6-methyladenosine (m6A) modifications of RNA as a crucial marker that promotes the recruitment of splicing factors, thereby affecting ASE [51]. A comprehensive investigation has shown that the transcription factor ZFP217 (the mouse homolog of human ZNF217) actively facilitates m6A methylation on certain transcripts, including its own RNA at exons 3 and 5 [52]. This observation leads to the hypothesis that processes driven by ZNF217-WT may potentially influence splicing events that result in the formation of the *ZNF217-DE4* isoform. Future work is, however, needed to clarify the specific mechanisms involved.

In the present study, the data presented provide compelling evidence for the role of the ZNF217-ΔE4 isoform in breast cancer cells, and suggest that the deleterious functions displayed by the ZNF217-WT oncogene may also be exerted by its exon 4 spliced form. Moreover, our work highlights that the small C-terminal sequence—35 aa in the WT and 48 aa in the splice variant (ΔE4)—has a significant impact on both the genomic function of the protein and its biological activity.

The first key finding of the study was demonstrating that the splice ZNF217-ΔE4 protein isoform retains genomic engagement and transcriptional activity. Many transcription factors and chromatin remodelers rapidly cycle on and off of their target sites on chromatin in select nuclear regions or hubs [28,37–42]. Our findings extend this knowledge by detailing the differential binding dynamics of ZNF217 isoforms to chromatin, as demonstrated through sophisticated live-cell microscopy and ChIP assays. Overall, our data highlighted that ZNF217-WT, ZNF217-G1013, and ZNF217-ΔE4 molecules—all possessing the DNA binding domain—bind to a similar number of genomic target sites, indicating that the C-terminus of ZNF217 is likely not involved in genomic target site selection. ZNF217-WT and ZNF217-G1013 variants behave similarly kinetically, suggesting that the 1013–1048 C-terminus sequence present on the WT isoform has a minimal impact on chromatin engagement.

Conversely, the presence of the 1013–1061 C-terminal sequence in the ZNF217-ΔE4 natural splice variant leads to different kinetics, resulting in more efficient engagement of the ZNF217 molecule with long-lived chromatin targets. However, once the protein bound to chromatin, the C-terminus sequence resulted in a weaker binding of ZNF217-ΔE4 relative to the ZNF217-WT and ZNF217-G1013 variants on long-lived chromatin. Prior live-cell imaging studies involving chromatin remodeling and DNA replication factors indicated that their residence time on chromatin was directly proportional to the remodeling efficiency of chromatin or inversely proportional to the enzymatic activity of chromatin remodeling complexes or DNA Polymerase [28,42,53]. In simple terms, the amount of time spent binding to chromatin reflected the amount of time that it took for factors to complete their job at long-lived times scales (e.g., >3–4 s). In this context, transcription/epigenetic factors are often involved in recruiting many different transcription complexes (e.g., TFIID, RNA Polymerase II, and Mediator) to the promoters of the genes that they regulate. During a transcriptional burst, many different RNA Polymerase II molecules are recruited in succession to form a convoy on the gene [54]. Faster dissociation of these transcription/epigenetic cofactors from chromatin after their cargo assembles on the gene allows faster recruitment of the next cofactor/RNA Polymerase II complex thereby increasing

the total RNA Polymerase II density on the gene allowing for higher levels of transcription. Overall, these findings suggest that specific residues within the C-terminal region of ZNF217-ΔE4 may facilitate a faster association with chromatin targets, although the binding stability appears reduced. This could lead to heightened transcriptional activity for certain target genes. As a proof of concept, our data show that the inclusion of the 1013–1061 aa sequence in the ZNF217-ΔE4 isoform correlates with increased positive transcriptional activity and a notable greater activation of histone positive marks at the *ERBB3* promoter, compared to ZNF217-WT isoform. Notably, ZNF217-WT and ZNF217-G1013 behaved similarly, supporting the idea that the 1013–1048 C-terminal sequence in the wild-type isoform has little to no significant impact on positive transcriptional activity at the *ERBB3* promoter. Of utmost interest for a future study would be to explore the differences between the C-terminal regions of ZNF217-WT and ZNF217-ΔE4, for example through crosslinking assays to identify potential interaction partners within the transcriptional complex.

The second key finding of this study was demonstrating that the 1013–1048 aa sequence present on ZNF217-WT and the 1013–1061 aa sequence on the ZNF217-ΔE4 protein, although small relative to the entire protein, play major roles in the cellular functions of the two ZNF217 isoforms. This is the first demonstration that ZNF217-ΔE4 retains deleterious biological functions, regardless of the presence of ZNF217-WT, a point not addressed in our previous work [24]. Both our in vitro and in vivo experiments support the aggressive phenotype conferred by ZNF217-ΔE4. Silencing *ZNF217* in breast cancer cells reduced proliferation and migration, while reintroducing *ZNF217-ΔE4* restored these traits and increased mammosphere formation efficiency. Interestingly, ZNF217-ΔE4 was more effective than ZNF217-WT in restoring these phenotypes, suggesting that residues within its C-terminus are critical for modulating these properties. This is further supported by the observation that the ZNF217-G1013 variant, lacking the extended C-terminal sequence, failed to produce similar effects, highlighting the importance of the C-terminal domain in ZNF217's biological functions. While these differences between ZNF217 variants were not observed in our genomic or luciferase reporter assays, this may be explained by the fact that the C-terminus present on ZNF217-WT molecules—absent in the ZNF217-G1013 variant—has previously been described as being involved in protein–protein interactions with specific chromatin remodelers, transcriptional co-factors, or other protein partners [6,20] that could contribute to ZNF217-driven deleterious functions. This would also suggest that the C-terminus present on ZNF217-ΔE4 molecules (and absent from ZNF217-G1013) may participate in protein–protein interactions that remain to be identified. Additionally, the cellular phenotype observed with the ZNF217-G1013 variant may reflect a drastic conformational change that compromises its biological functions, notably by affecting protein stability, interactions, or overall activity.

Our comprehensive transcriptomic analysis revealed that cells overexpressing ZNF217 isoforms exhibit both unique and overlapping gene expression profiles, underscoring potential functional similarity and differences. Interestingly, global differences in enriched pathways between ZNF217-WT and ZNF217-ΔE4 remain modest, whereas the ZNF217-G1013 signature is markedly distinct and the top-ranked significantly enriched signaling pathways were less significant than those associated with ZNF217-WT or ZNF217-ΔE4 signatures. Significant enriched pathways common to ZNF217-WT and ZNF217-ΔE4 included cadherin, glutamate receptor, Wnt, EMT, and Alzheimer's disease–presenilin pathways—findings that are novel for ZNF217-ΔE4. ZNF217-WT has previously been associated with EMT and enriched cadherin and integrin pathways [8]. The additional enrichment of the integrin signaling pathway in the ZNF217-ΔE4 signature supports our data indicating that this isoform may regulate cell adhesion and migration, processes critical for metastasis. Interestingly, intricate interplays between ZNF217 and epigenetic processes

have been recently highlighted [55], and links between ZNF217 and Alzheimer's disease have been proposed [23,56–59]. Metabolic reprogramming is a hallmark of cancer, and recent studies pinpoint glutamate receptor signaling in cancer progression [60,61]. Further work is however needed to elucidate potential interactions between ZNF217 isoforms and this pathway. The Wnt signaling pathway is well known for its roles in carcinogenesis, embryonic development, EMT, cell proliferation, cell migration, and metastasis formation, making it an attractive therapeutic target [62–66]. Dysregulated expression of members of the Wnt signaling pathway has been identified in previous transcriptomic signatures following ectopic overexpression of ZNF217-WT [5,8], although a functional link between ZNF217 and activation of the Wnt pathway remains to be formally established in cancer cells. In this study, the Wnt pathway is one of the top-ranked significantly deregulated pathways associated with both ZNF217-WT and ZNF217-ΔE4 transcriptomic signatures, while it is absent in the ZNF217-G1013 gene signature. Overall, the shared pathways between ZNF217-WT and ZNF217-ΔE4, such as cadherin and Wnt signaling, highlight common mechanisms through which ZNF217 may influence tumor aggressiveness.

The regulation of organ-specific metastasis, such as to bone, remains poorly understood. It is believed that metastatic breast cancer cells need gene signatures that confer bone-homing capabilities. Identifying driver genes involved in this process could reveal therapeutic targets for bone metastatic breast cancers. Our previous work demonstrated that ZNF217-WT is a key mediator of the development of bone metastases in breast cancer [4]. This study provides novel evidence that both ZNF217-ΔE4 and ZNF217-WT actively facilitate the dissemination of breast cancer cells to the bone marrow, while ZNF217-ΔE4 is, however, less powerful than ZNF217-WT. This implies that the two isoforms may regulate common pathways involved in bone colonization. Notably, the Wnt pathway—deregulated in both ZNF217-WT and ZNF217-ΔE4 gene signatures—is a well-known key pathway in the “seed and soil” interaction between breast cancer cells and the bone microenvironment, thus representing an attractive candidate [12,63,65–69]. Finally, beyond ZNF217-WT [4], our study indicates that ZNF217-ΔE4 also functions as an early marker of bone metastasis development in breast cancer. Specifically, primary human breast tumors with elevated ZNF217-ΔE4 mRNA levels are more likely to develop bone metastases. The association of high ZNF217-ΔE4 expression with poor prognosis and bone-only metastases supports the idea that primary breast tumor molecular profiles may predict metastatic site preferences. This study presents some limitations. Although ZNF217-ΔE4 outperforms the WT isoform in migration, invasion, and proliferation assays in vitro, it appears less effective in mouse experiments. While our transcriptomic analyses did not reveal prominent signaling pathways that clearly discriminate between ZNF217-WT and ZNF217-ΔE4, we hypothesize that pathways involved in tumor cell adhesion, interaction with the bone microenvironment, and anchorage may be more efficiently activated in breast tumor cells expressing ZNF217-WT. We also cannot exclude the possibility that, in humans, breast tumor cells expressing ZNF217-ΔE4 may be more susceptible to immune-mediated clearance.

In summary, this study provides evidence for the functional characterization of the ZNF217-ΔE4 isoform both in vitro and in vivo in breast cancer cells. Further research is needed to elucidate the precise molecular mechanisms by which ZNF217-ΔE4 influences transcriptional regulation and chromatin dynamics. The interactions between ZNF217-ΔE4 and other signaling pathways in breast cancer should be explored to better understand its role within the tumor microenvironment. Moreover, the implications of our findings extend beyond basic research, as they highlight potential opportunities for therapeutic intervention. The development of specific inhibitors targeting ZNF217-ΔE4 may offer a valuable therapeutic approach, particularly for high-risk patients identified through biomarker screening. However, our current work, along with previous studies, indicates

that both ZNF217-WT and ZNF217-ΔE4 are viable therapeutic targets and should be addressed accordingly. Presently, there are no small molecule inhibitors explicitly designed to block ZNF217 isoforms activity. Alternative strategies could include targeting downstream survival signaling pathways activated by ZNF217 isoforms; however, this approach is complicated by the multitude of pathways involved—such as EMT, PI3K/Akt, TGFβ, and BMP—that are referenced in the literature [2,3]. Other promising strategies involve targeting transcription factors directly, such as using antisense oligonucleotides to reduce mRNA expression [70] or employing molecules that induce specific degradation of transcription factors, like proteolysis-targeting chimeras (PROTACs) [71–73]. Interestingly, ZNF217 has been identified as a key component of core transcriptional regulatory circuitry (CRC) in several cancers, including breast cancer [74–77]. The recent model of core CRC highlights that a small set of core transcription factors predominantly regulate gene expression programs within specific cell types or biological/pathological contexts. Considering these circuits from a therapeutic perspective opens new avenues for intervention, such as exploiting vulnerabilities within the CRC network by identifying synthetic lethal interactions or by targeting combinations of factors that can synergistically disrupt the CRC. Overall, therapeutic strategies targeting ZNF217—WT and/or the ΔE4 variant—represent promising approaches for the treatment of ZNF217-positive breast cancers.

5. Conclusions

In summary, our findings unveil ZNF217-ΔE4 as a critical player in breast cancer aggressiveness and bone homing, reinforcing the importance of alternative splicing in cancer biology. The identification of ZNF217-ΔE4 as a prognostic marker and potential therapeutic target opens new avenues for improving outcomes in breast cancer patients, particularly those at high risk for bone metastases. Our research suggests that splicing variants like ZNF217-ΔE4 not only serve as biomarkers but may also actively promote cancer aggressiveness and metastasis, a notion that warrants further exploration within the context of therapeutic development and personalized medicine.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers18040664/s1>, Figure S1: Validation of ZNF217 knock-out in MDA-MB-231 cells by genomic DNA sequencing; Figure S2: Validation of the ectopic expression of ZNF217 isoforms in HEK293T cells; Figure S3: Spatial analysis of ZNF217 variants chromatin binding events using SMT to define chromatin binding hubs; Figure S4: ZNF217 variants activate transcription at the *ERBB3* promoter in a dose-dependent manner; Figure S5: RNAseq expression of ZNF217 isoforms and *ERBB3*; Figure S6: Transcriptomic analysis of ZNF217 isoforms-overexpressing HEK293 cells; Figure S7: Validation of CRISPR/Cas9 silencing of the ZNF217 gene in MDA-MB-231 cells; Figure S8: Representative cell images corresponding to Figure 4B,C,E; Figure S9: ZNF217-WT remains undetectable after ZNF217-ΔE4 transfection in MDA-MB-231-ZNF217-KO cells. Figure S10: Representative cell images corresponding to Figure 5C,D,F; Table S1: Characteristics of the 107 patients with primary breast cancers; Table S2: Significant deregulated genes (DEG) ($FC > 1.5$ et $p < 0.05$) upon ZNF217 isoforms overexpression; Movie S1: SMT of Halo-tagged ZNF217-WT; Movie S2: SMT of Halo-tagged ZNF217-G1013; Movie S3: SMT of Halo-tagged ZNF217-ΔE4; Supplementary Material and Methods. Original Western blots.

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Institutional Review Board Statement: The in vivo study was conducted in accordance with the Declaration of Helsinki. The animal study protocol was approved by the local ethical committee (Comité d’Expérimentation Animale de l’Université Claude Bernard Lyon 1, CEEA-55) under project license MESRI Number: APAFIS #46312-2024011214359545, on the 21 February 2024. The study conducted on breast cancer tumor samples has been approved by the local ethics committee (CRB Centre Léon Bérard, France). The CRB Centre Léon Bérard is quality certified according to NFS96-900 French standard and ISO 9001 for clinical trials, ensuring scientific rigor for sample conservation, traceability, and quality, as well as ethical rules observance and defined rules for transferring samples for research purposes (Ministry of Health for activities authorization n° AC-2019-3426 and DC-2008-99). The material used in the study has been collected in agreement with all applicable laws, rules, and requests of French and European government authorities.

Informed Consent Statement: All subjects gave written informed consent. The patients/participants provided their written informed consent to participate in this study.

Data Availability Statement: The microarray data have been deposited in the Gene Expression Omnibus (GEO) Database at <http://www.ncbi.nlm.nih.gov/geo> (GEO accession no. GSE269986). The data and materials used in this study are available once published and upon request.

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References

1. Bray, F.; Laversanne, M.; Sung, H.; Ferlay, J.; Siegel, R.L.; Soerjomataram, I.; Jemal, A. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2024**, *74*, 229–263. [CrossRef]
2. Cohen, P.A.; Donini, C.F.; Nguyen, N.T.; Lincet, H.; Vendrell, J.A. The dark side of ZNF217, a key regulator of tumorigenesis with powerful biomarker value. *Oncotarget* **2015**, *6*, 41566–41581. [CrossRef]
3. Li, Y.; Wu, H.; Wang, Q.; Xu, S. ZNF217: The cerberus who fails to guard the gateway to lethal malignancy. *Am. J. Cancer Res.* **2021**, *11*, 3378–3405.
4. Bellanger, A.; Donini, C.F.; Vendrell, J.A.; Lavaud, J.; Machuca-Gayet, I.; Ruel, M.; Vollaie, J.; Grisard, E.; Gyorffy, B.; Bieche, I.; et al. The critical role of the ZNF217 oncogene in promoting breast cancer metastasis to the bone. *J. Pathol.* **2017**, *242*, 73–89. [CrossRef]
5. Littlepage, L.E.; Adler, A.S.; Kouros-Mehr, H.; Huang, G.; Chou, J.; Krig, S.R.; Griffith, O.L.; Korkola, J.E.; Qu, K.; Lawson, D.A.; et al. The transcription factor ZNF217 is a prognostic biomarker and therapeutic target during breast cancer progression. *Cancer Discov.* **2012**, *2*, 638–651. [CrossRef]
6. Nguyen, N.T.; Vendrell, J.A.; Poulard, C.; Gyorffy, B.; Goddard-Leon, S.; Bieche, I.; Corbo, L.; Le Romancer, M.; Bachelot, T.; Treilleux, I.; et al. A functional interplay between ZNF217 and estrogen receptor alpha exists in luminal breast cancers. *Mol. Oncol.* **2014**, *8*, 1441–1457. [CrossRef]
7. Thollet, A.; Vendrell, J.A.; Payen, L.; Ghayad, S.E.; Ben Larbi, S.; Grisard, E.; Collins, C.; Villedieu, M.; Cohen, P.A. ZNF217 confers resistance to the pro-apoptotic signals of paclitaxel and aberrant expression of Aurora-A in breast cancer cells. *Mol. Cancer* **2010**, *9*, 291. [CrossRef] [PubMed]
8. Vendrell, J.A.; Thollet, A.; Nguyen, N.T.; Ghayad, S.E.; Vinot, S.; Bieche, I.; Grisard, E.; Josserand, V.; Coll, J.L.; Roux, P.; et al. ZNF217 is a marker of poor prognosis in breast cancer that drives epithelial-mesenchymal transition and invasion. *Cancer Res.* **2012**, *72*, 3593–3606. [CrossRef] [PubMed]

9. Cohen, P.A.; Loudig, O.; Liu, C.; Albanese, J.; Fineberg, S. The ZNF217 Biomarker Predicts Low- and High-Risk Oncotype DX((R)) Recurrence Score in ER-Positive Invasive Breast Cancers. *Front. Pharmacol.* **2019**, *10*, 524. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Frietze, S.; O'Geen, H.; Littlepage, L.E.; Simion, C.; Sweeney, C.A.; Farnham, P.J.; Krig, S.R. Global analysis of ZNF217 chromatin occupancy in the breast cancer cell genome reveals an association with ERalpha. *BMC Genom.* **2014**, *15*, 520. [\[CrossRef\]](#)
11. Vendrell, J.A.; Solassol, J.; Gyorffy, B.; Vilquin, P.; Jarlier, M.; Donini, C.F.; Gamba, L.; Maudelonde, T.; Rouanet, P.; Cohen, P.A. Evaluating ZNF217 mRNA Expression Levels as a Predictor of Response to Endocrine Therapy in ER+ Breast Cancer. *Front. Pharmacol.* **2018**, *9*, 1581. [\[CrossRef\]](#)
12. Clezardin, P.; Coleman, R.; Puppo, M.; Ottewell, P.; Bonnelye, E.; Paycha, F.; Confavreux, C.B.; Holen, I. Bone metastasis: Mechanisms, therapies, and biomarkers. *Physiol. Rev.* **2021**, *101*, 797–855. [\[CrossRef\]](#)
13. Coleman, R.E.; Croucher, P.I.; Padhani, A.R.; Clezardin, P.; Chow, E.; Fallon, M.; Guise, T.; Colangeli, S.; Capanna, R.; Costa, L. Bone metastases. *Nat. Rev. Dis. Primers* **2020**, *6*, 83. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Vollaie, J.; Machuca-Gayet, I.; Lavaud, J.; Bellanger, A.; Bouazza, L.; El Moghrabi, S.; Treilleux, I.; Coll, J.L.; Peyruchaud, O.; Josserand, V.; et al. The Bone Morphogenetic Protein Signaling Inhibitor LDN-193189 Enhances Metastasis Development in Mice. *Front. Pharmacol.* **2019**, *10*, 667. [\[CrossRef\]](#)
15. Collins, C.; Rommens, J.M.; Kowbel, D.; Godfrey, T.; Tanner, M.; Hwang, S.I.; Polikoff, D.; Nonet, G.; Cochran, J.; Myambo, K.; et al. Positional cloning of ZNF217 and NABC1: Genes amplified at 20q13.2 and overexpressed in breast carcinoma. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 8703–8708. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Quinlan, K.G.; Verger, A.; Yaswen, P.; Crossley, M. Amplification of zinc finger gene 217 (ZNF217) and cancer: When good fingers go bad. *Biochim. Biophys. Acta* **2007**, *1775*, 333–340. [\[CrossRef\]](#)
17. Krig, S.R.; Jin, V.X.; Bieda, M.C.; O'Geen, H.; Yaswen, P.; Green, R.; Farnham, P.J. Identification of genes directly regulated by the oncogene ZNF217 using chromatin immunoprecipitation (ChIP)-chip assays. *J. Biol. Chem.* **2007**, *282*, 9703–9712. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Krig, S.R.; Miller, J.K.; Frietze, S.; Beckett, L.A.; Neve, R.M.; Farnham, P.J.; Yaswen, P.I.; Sweeney, C.A. ZNF217, a candidate breast cancer oncogene amplified at 20q13, regulates expression of the ErbB3 receptor tyrosine kinase in breast cancer cells. *Oncogene* **2010**, *29*, 5500–5510. [\[CrossRef\]](#)
19. Thillainadesan, G.; Iovic, M.; Loney, E.; Andrews, J.; Tini, M.; Torchia, J. Genome analysis identifies the p15ink4b tumor suppressor as a direct target of the ZNF217/CoREST complex. *Mol. Cell Biol.* **2008**, *28*, 6066–6077. [\[CrossRef\]](#)
20. Cowger, J.J.; Zhao, Q.; Iovic, M.; Torchia, J. Biochemical characterization of the zinc-finger protein 217 transcriptional repressor complex: Identification of a ZNF217 consensus recognition sequence. *Oncogene* **2007**, *26*, 3378–3386. [\[CrossRef\]](#)
21. Gahete, M.D.; Herman-Sanchez, N.; Fuentes-Fayos, A.C.; Lopez-Canovas, J.L.; Luque, R.M. Dysregulation of splicing variants and spliceosome components in breast cancer. *Endocr. Relat. Cancer* **2022**, *29*, R123–R142. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Martinez-Montiel, N.; Anaya-Ruiz, M.; Perez-Santos, M.; Martinez-Contreras, R.D. Alternative Splicing in Breast Cancer and the Potential Development of Therapeutic Tools. *Genes* **2017**, *8*, 217. [\[CrossRef\]](#)
23. Zhu, J.; Zhang, J.; Hu, P.; Fan, M.; Song, D.; Yin, H.; Yan, P.; Xian, S.; Li, Z.; Guo, J.; et al. Identification of Bone Metastatic and Prognostic Alternative Splicing Signatures in Prostate Adenocarcinoma. *Biochem. Genet.* **2023**, *61*, 2242–2259. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Bellanger, A.; Le, D.T.; Vendrell, J.; Wierinckx, A.; Pongor, L.S.; Solassol, J.; Lachuer, J.; Clezardin, P.; Gyorffy, B.; Cohen, P.A. Exploring the Significance of the Exon 4-Skipping Isoform of the ZNF217 Oncogene in Breast Cancer. *Front. Oncol.* **2021**, *11*, 647269. [\[CrossRef\]](#)
25. Serge, A.; Bertaux, N.; Rigneault, H.; Marguet, D. Dynamic multiple-target tracing to probe spatiotemporal cartography of cell membranes. *Nat. Methods* **2008**, *5*, 687–694. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Normanno, D.; Boudarene, L.; Dugast-Darzacq, C.; Chen, J.; Richter, C.; Proux, F.; Benichou, O.; Voituriez, R.; Darzacq, X.; Dahan, M. Probing the target search of DNA-binding proteins in mammalian cells using TetR as model searcher. *Nat. Commun.* **2015**, *6*, 7357. [\[CrossRef\]](#)
27. Reisser, M.; Hettich, J.; Kuhn, T.; Popp, A.P.; Grosse-Berkenbusch, A.; Gebhardt, J.C.M. Inferring quantity and qualities of superimposed reaction rates from single molecule survival time distributions. *Sci. Rep.* **2020**, *10*, 1758. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Kenworthy, C.A.; Haque, N.; Liou, S.H.; Chandris, P.; Wong, V.; Dziuba, P.; Lavis, L.D.; Liu, W.L.; Singer, R.H.; Coleman, R.A. Bromodomains regulate dynamic targeting of the PBAF chromatin-remodeling complex to chromatin hubs. *Biophys. J.* **2022**, *121*, 1738–1752. [\[CrossRef\]](#)
29. Mi, H.; Thomas, P. PANTHER pathway: An ontology-based pathway database coupled with data analysis tools. *Methods Mol. Biol.* **2009**, *563*, 123–140. [\[CrossRef\]](#)
30. Fahs, A.; Hussein, N.; Zalzal, H.; Ramadan, F.; Ghamloush, F.; Tamim, H.; El Homs, M.; Badran, B.; Boulos, F.; Tawil, A.; et al. CD147 Promotes Tumorigenesis via Exosome-Mediated Signaling in Rhabdomyosarcoma. *Cells* **2022**, *11*, 2267. [\[CrossRef\]](#)
31. Dontu, G.; Abdallah, W.M.; Foley, J.M.; Jackson, K.W.; Clarke, M.F.; Kawamura, M.J.; Wicha, M.S. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.* **2003**, *17*, 1253–1270. [\[CrossRef\]](#) [\[PubMed\]](#)

32. Leblanc, R.; Lee, S.C.; David, M.; Bordet, J.C.; Norman, D.D.; Patil, R.; Miller, D.; Sahay, D.; Ribeiro, J.; Clezardin, P.; et al. Interaction of platelet-derived autotaxin with tumor integrin α V β 3 controls metastasis of breast cancer cells to bone. *Blood* **2014**, *124*, 3141–3150. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Pantano, F.; Croset, M.; Driouch, K.; Bednarz-Knoll, N.; Iuliani, M.; Ribelli, G.; Bonnelye, E.; Wikman, H.; Geraci, S.; Bonin, F.; et al. Integrin α 5 in human breast cancer is a mediator of bone metastasis and a therapeutic target for the treatment of osteolytic lesions. *Oncogene* **2021**, *40*, 1284–1299. [\[CrossRef\]](#)
34. Reynaud, C.; Ferreras, L.; Di Mauro, P.; Kan, C.; Croset, M.; Bonnelye, E.; Pez, F.; Thomas, C.; Aimond, G.; Karnoub, A.E.; et al. Lysyl Oxidase Is a Strong Determinant of Tumor Cell Colonization in Bone. *Cancer Res.* **2017**, *77*, 268–278. [\[CrossRef\]](#)
35. Croset, M.; Pantano, F.; Kan, C.W.S.; Bonnelye, E.; Descotes, F.; Alix-Panabieres, C.; Lecellier, C.H.; Bachelier, R.; Alloli, N.; Hong, S.S.; et al. miRNA-30 Family Members Inhibit Breast Cancer Invasion, Osteomimicry, and Bone Destruction by Directly Targeting Multiple Bone Metastasis-Associated Genes. *Cancer Res.* **2018**, *78*, 5259–5273. [\[CrossRef\]](#)
36. Matsubara, T.; Kida, K.; Yamaguchi, A.; Hata, K.; Ichida, F.; Meguro, H.; Aburatani, H.; Nishimura, R.; Yoneda, T. BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J. Biol. Chem.* **2008**, *283*, 29119–29125. [\[CrossRef\]](#)
37. Cho, W.K.; Jayanth, N.; English, B.P.; Inoue, T.; Andrews, J.O.; Conway, W.; Grimm, J.B.; Spille, J.H.; Lavis, L.D.; Lionnet, T.; et al. RNA Polymerase II cluster dynamics predict mRNA output in living cells. *Elife* **2016**, *5*, e13617. [\[CrossRef\]](#)
38. Cisse, I.I.; Izeddin, I.; Causse, S.Z.; Boudarene, L.; Senecal, A.; Muresan, L.; Dugast-Darzacq, C.; Hajj, B.; Dahan, M.; Darzacq, X. Real-time dynamics of RNA polymerase II clustering in live human cells. *Science* **2013**, *341*, 664–667. [\[CrossRef\]](#)
39. Liu, Z.; Legant, W.R.; Chen, B.C.; Li, L.; Grimm, J.B.; Lavis, L.D.; Betzig, E.; Tjian, R. 3D imaging of Sox2 enhancer clusters in embryonic stem cells. *Elife* **2014**, *3*, e04236. [\[CrossRef\]](#)
40. Chong, S.; Dugast-Darzacq, C.; Liu, Z.; Dong, P.; Dailey, G.M.; Cattoglio, C.; Heckert, A.; Banala, S.; Lavis, L.; Darzacq, X.; et al. Imaging dynamic and selective low-complexity domain interactions that control gene transcription. *Science* **2018**, *361*. [\[CrossRef\]](#) [\[PubMed\]](#)
41. Mir, M.; Stadler, M.R.; Ortiz, S.A.; Hannon, C.E.; Harrison, M.M.; Darzacq, X.; Eisen, M.B. Dynamic multifactor hubs interact transiently with sites of active transcription in *Drosophila* embryos. *Elife* **2018**, *7*, e40497. [\[CrossRef\]](#)
42. Kim, J.M.; Visanpattanasin, P.; Jou, V.; Liu, S.; Tang, X.; Zheng, Q.; Li, K.Y.; Snedeker, J.; Lavis, L.D.; Lionnet, T.; et al. Single-molecule imaging of chromatin remodelers reveals role of ATPase in promoting fast kinetics of target search and dissociation from chromatin. *Elife* **2021**, *10*, e69387. [\[CrossRef\]](#)
43. Chen, J.; Zhang, Z.; Li, L.; Chen, B.C.; Revyakin, A.; Hajj, B.; Legant, W.; Dahan, M.; Lionnet, T.; Betzig, E.; et al. Single-molecule dynamics of enhanceosome assembly in embryonic stem cells. *Cell* **2014**, *156*, 1274–1285. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Martin, C.; Zhang, Y. The diverse functions of histone lysine methylation. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 838–849. [\[CrossRef\]](#)
45. Basolo, F.; Elliott, J.; Tait, L.; Chen, X.Q.; Maloney, T.; Russo, I.H.; Pauley, R.; Momiki, S.; Caamano, J.; Klein-Szanto, A.J.; et al. Transformation of human breast epithelial cells by c-Ha-ras oncogene. *Mol. Carcinog.* **1991**, *4*, 25–35. [\[CrossRef\]](#)
46. Dawson, P.J.; Wolman, S.R.; Tait, L.; Heppner, G.H.; Miller, F.R. MCF10AT: A model for the evolution of cancer from proliferative breast disease. *Am. J. Pathol.* **1996**, *148*, 313–319.
47. Miller, F.R. Xenograft models of premalignant breast disease. *J. Mammary Gland Biol. Neoplasia* **2000**, *5*, 379–391. [\[CrossRef\]](#)
48. Miller, F.R.; Soule, H.D.; Tait, L.; Pauley, R.J.; Wolman, S.R.; Dawson, P.J.; Heppner, G.H. Xenograft model of progressive human proliferative breast disease. *J. Natl. Cancer Inst.* **1993**, *85*, 1725–1732. [\[CrossRef\]](#)
49. Santner, S.J.; Dawson, P.J.; Tait, L.; Soule, H.D.; Eliason, J.; Mohamed, A.N.; Wolman, S.R.; Heppner, G.H.; Miller, F.R. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res. Treat.* **2001**, *65*, 101–110. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Read, A.; Natrajan, R. Splicing dysregulation as a driver of breast cancer. *Endocr. Relat. Cancer* **2018**, *25*, R467–R478. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Adhikari, S.; Xiao, W.; Zhao, Y.L.; Yang, Y.G. m(6)A: Signaling for mRNA splicing. *RNA Biol.* **2016**, *13*, 756–759. [\[CrossRef\]](#)
52. Aguilo, F.; Zhang, F.; Sancho, A.; Fidalgo, M.; Di Cecilia, S.; Vashisht, A.; Lee, D.F.; Chen, C.H.; Rengasamy, M.; Andino, B.; et al. Coordination of m(6)A mRNA Methylation and Gene Transcription by ZFP217 Regulates Pluripotency and Reprogramming. *Cell Stem Cell* **2015**, *17*, 689–704. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Drosopoulos, W.C.; Vierra, D.A.; Kenworthy, C.A.; Coleman, R.A.; Schildkraut, C.L. Dynamic Assembly and Disassembly of the Human DNA Polymerase delta Holoenzyme on the Genome In Vivo. *Cell Rep.* **2020**, *30*, 1329–1341.e5. [\[CrossRef\]](#)
54. Tantale, K.; Mueller, F.; Kozulic-Pirher, A.; Lesne, A.; Victor, J.M.; Robert, M.C.; Capozzi, S.; Chouaib, R.; Backer, V.; Mateos-Langerak, J.; et al. A single-molecule view of transcription reveals convoys of RNA polymerases and multi-scale bursting. *Nat. Commun.* **2016**, *7*, 12248. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Fahme, P.; Ramadan, F.; Le, D.T.; Thi, K.N.; Ghayad, S.E.; Hussein, N.; Diaz, C.; Croset, M.; Clezardin, P.; Cohen, P.A. The Intricate Interplay between the ZNF217 Oncogene and Epigenetic Processes Shapes Tumor Progression. *Cancers* **2022**, *14*, 6043. [\[CrossRef\]](#)
56. Gao, Y.; Zhang, N.; Lv, C.; Li, N.; Li, X.; Li, W. lncRNA SNHG1 Knockdown Alleviates Amyloid-beta-Induced Neuronal Injury by Regulating ZNF217 via Sponging miR-361-3p in Alzheimer's Disease. *J. Alzheimers Dis.* **2020**, *77*, 85–98. [\[CrossRef\]](#)

57. Wang, J.; Zhou, T.; Wang, T.; Wang, B. Suppression of lncRNA-ATB prevents amyloid-beta-induced neurotoxicity in PC12 cells via regulating miR-200/ZNF217 axis. *Biomed. Pharmacother.* **2018**, *108*, 707–715. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Wu, L.; Du, Q.; Wu, C. CircLPAR1/miR-212-3p/ZNF217 feedback loop promotes amyloid beta-induced neuronal injury in Alzheimer's Disease. *Brain Res.* **2021**, *1770*, 147622. [\[CrossRef\]](#) [\[PubMed\]](#)
59. Xie, Y.; Xie, D.; Chen, C. Hsa_circ_0049472 contributed to amyloid-beta peptide-induced neurotoxicity, apoptosis and inflammation via regulating PI3K-AKT signaling pathway by interacting with miR-22-3p/ZNF217 axis. *Brain Res. Bull.* **2024**, *215*, 111004. [\[CrossRef\]](#)
60. Eddy, K.; Eddin, M.N.; Fateeva, A.; Pompili, S.V.B.; Shah, R.; Doshi, S.; Chen, S. Implications of a Neuronal Receptor Family, Metabotropic Glutamate Receptors, in Cancer Development and Progression. *Cells* **2022**, *11*, 2857. [\[CrossRef\]](#)
61. Yu, L.J.; Wall, B.A.; Wangari-Talbot, J.; Chen, S. Metabotropic glutamate receptors in cancer. *Neuropharmacology* **2017**, *115*, 193–202. [\[CrossRef\]](#)
62. Ben-Ghedalia-Peled, N.; Vago, R. Wnt Signaling in the Development of Bone Metastasis. *Cells* **2022**, *11*, 3934. [\[CrossRef\]](#)
63. Cui, J.; Chen, H.; Zhang, K.; Li, X. Targeting the Wnt signaling pathway for breast cancer bone metastasis therapy. *J. Mol. Med.* **2022**, *100*, 373–384. [\[CrossRef\]](#)
64. Raut, D.; Vora, A.; Bhatt, L.K. The Wnt/beta-catenin pathway in breast cancer therapy: A pre-clinical perspective of its targeting for clinical translation. *Expert Rev. Anticancer Ther.* **2022**, *22*, 97–114. [\[CrossRef\]](#)
65. Thiele, S.; Rachner, T.D.; Rauner, M.; Hofbauer, L.C. WNT5A and Its Receptors in the Bone-Cancer Dialogue. *J. Bone Miner. Res.* **2016**, *31*, 1488–1496. [\[CrossRef\]](#)
66. Xu, X.; Zhang, M.; Xu, F.; Jiang, S. Wnt signaling in breast cancer: Biological mechanisms, challenges and opportunities. *Mol. Cancer* **2020**, *19*, 165. [\[CrossRef\]](#) [\[PubMed\]](#)
67. Elshimy, Y.; Alkhatib, A.R.; Atassi, B.; Mohammad, K.S. Biomarker-Driven Approaches to Bone Metastases: From Molecular Mechanisms to Clinical Applications. *Biomedicines* **2025**, *13*, 1160. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Fan, G.; Zuo, S.; Wang, Z.; Zhang, S.; Liu, L.; Luo, H.; Xie, Y.; Zhang, Y.; Li, D. Targeting of the IL-33/Wnt axis restricts breast cancer stemness and metastasis. *Sci. Rep.* **2025**, *15*, 18172. [\[CrossRef\]](#)
69. Mariz, K.; Ingolf, J.B.; Daniel, H.; Teresa, N.J.; Erich-Franz, S. The Wnt inhibitor dickkopf-1: A link between breast cancer and bone metastases. *Clin. Exp. Metastasis* **2015**, *32*, 857–866. [\[CrossRef\]](#)
70. DeFranciscis, V.; Amabile, G.; Kortylewski, M. Clinical applications of oligonucleotides for cancer therapy. *Mol. Ther.* **2025**, *33*, 2705–2718. [\[CrossRef\]](#) [\[PubMed\]](#)
71. Chirnomas, D.; Hornberger, K.R.; Crews, C.M. Protein degraders enter the clinic—A new approach to cancer therapy. *Nat. Rev. Clin. Oncol.* **2023**, *20*, 265–278. [\[CrossRef\]](#) [\[PubMed\]](#)
72. Nalawansa, D.A.; Crews, C.M. PROTACs: An Emerging Therapeutic Modality in Precision Medicine. *Cell Chem. Biol.* **2020**, *27*, 998–1014. [\[CrossRef\]](#) [\[PubMed\]](#)
73. Samarasinghe, K.T.G.; Crews, C.M. Targeted protein degradation: A promise for undruggable proteins. *Cell Chem. Biol.* **2021**, *28*, 934–951. [\[CrossRef\]](#) [\[PubMed\]](#)
74. Hnisz, D.; Abraham, B.J.; Lee, T.I.; Lau, A.; Saint-Andre, V.; Sigova, A.A.; Hoke, H.A.; Young, R.A. Super-enhancers in the control of cell identity and disease. *Cell* **2013**, *155*, 934–947. [\[CrossRef\]](#)
75. Saint-Andre, V.; Federation, A.J.; Lin, C.Y.; Abraham, B.J.; Reddy, J.; Lee, T.I.; Bradner, J.E.; Young, R.A. Models of human core transcriptional regulatory circuitries. *Genome Res.* **2016**, *26*, 385–396. [\[CrossRef\]](#)
76. Wei, L.; Chen, J.; Song, C.; Zhang, Y.; Zhang, Y.; Xu, M.; Feng, C.; Gao, Y.; Qian, F.; Wang, Q.; et al. Cancer CRC: A Comprehensive Cancer Core Transcriptional Regulatory Circuit Resource and Analysis Platform. *Front. Oncol.* **2021**, *11*, 761700. [\[CrossRef\]](#)
77. Zhou, B.; Fang, F.; Zhang, Y.; Li, Z.; Hu, Y.; Li, Y.; Jiao, W.; Wu, Y.; Wan, X.; Yang, Y.; et al. Core transcriptional regulatory circuitry molecule ZNF217 promotes AML cell proliferation by up-regulating MYB. *Int. J. Biol. Sci.* **2025**, *21*, 1966–1983. [\[CrossRef\]](#)

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