INTRODUCTION

Tumor initiation and progression often involve the dysregulation of developmentally important genes in cancer cells, endowing them with stem cell-like features such as enhanced self-renewal, invasiveness, and an aberrant differentiation state [1]. Triple-negative breast cancer (TNBC) cells that lack the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [2], often exhibit the molecular and functional traits of cancer stem cells [3–9]. Despite advances in breast cancer therapy, the survival of TNBC patients remains poor, due to aggressive cancer progression, therapy resistance, metastasis, and recurrence, likely due to increased cancer stemness [10]. Consequently, in order to develop effective targeted therapy, there is a pressing need to identify all molecular regulators that define cancer stem cell traits in TNBC.

The MYC proto-oncogene is a transcription factor essential in stem/progenitor cell maintenance and differentiation [11]. MYC is frequently overexpressed in many cancers, and drives a cancer stem cell phenotype with enhanced cell growth, metastasis, and metabolic reprogramming [11–13]. In breast carcinomas, MYC is one of the most frequently dysregulated oncogenes, particularly in BRCA1-mutated, basal-like TNBCs, where MYC amplification is reported in up to 50% of cases [14–16]. In these cancers, the BRCA1 gene acts as a tumor suppressor to repress the expansion of basal stem cells and basal-like breast cancers [17–19].

We previously reported a MYC-centered regulatory network in mouse embryonic stem (ES) cells that accounts for the similarity between the ES cells and cancer cells, and is associated with a worse prognosis in cancer, including cancers of the breast [20]. We found zinc finger protein 148 (ZNF148, also called Zfp148 and ZBP-89), a ubiquitously expressed Krüppel-like zinc finger transcription factor 148 – also called Zfp148 and ZBP-89), a ubiquitously expressed Krüppel-like zinc finger protein, as a target of MYC-regulatory network in mouse ES cells [20]. Partial deletion of the ZNF148 locus causes defective primordial germ cell development in mice [21], though a complete deletion in the C57BL/6 mouse strain leads to postnatal lethality [22]. In malignant tumors, ZNF148 has been reported to be a suppressive [23–25] or oncogenic [26–28] factor, depending on the tumor cell of origins and tissue types. Despite ZNF148 being an established node of the MYC-network in ES cells, and the significance of MYC
in driving cancer stem cells traits, the role of ZNF148 in breast cancer remains elusive. The Inhibitor of DNA binding 3 (ID3) is a member of the ID protein family, consisting of four members (ID1 to ID4). ID1 and ID3 share the closest sequence homology, and they are functionally redundant [29, 30]. Developmentally, it is well established that expression of the ID members is strongest in stem/progenitor cells, while their levels decrease upon differentiation. However, their expression frequently becomes reactivated in cancer, providing cancer cells with stem cell-like traits [31, 32]. In TNBC, ID1 and ID3 are requisite for self-renewal, metastasis, tumor re-initiation, and colonization, making them attractive targets for cancer stem cell therapy [30, 33–35].

Herein, we provide evidence for a direct transcriptional circuitry that functionally incorporates MYC, ZNF148, and ID1/3, regulating cancer stem cell traits in breast cancer. MYC actively represses ZNF148 expression, and in turn, the depletion of ZNF148 leads to de-repression of ID1/3, which drives the cancer stem cell phenotype. Thus, ZNF148 functions as a tumor suppressor, promoting the differentiated state of breast cancer cells, and suppressing cell proliferation, metastasis, and biosynthetic programs associated with cancer stem cells.

MATERIALS AND METHODS
Cell culture and In vitro clonogenic assay
MCF7, HCC1806, BT549 and MDA-MB-231 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% FCS, 2 mM Glutamine and 2% penicillin-streptomycin (P/S). HEK293T cells were cultured in DMEM High Glucose with 10% FCS and 2% P/S. MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 0.5 mg/ml hydrocortisone. All cell lines are sourced from ATCC and tested for mycoplasma contamination. Cells were maintained at 20–80% confluence at 37 °C with 5% CO2 air atmosphere. Clonogenic assays were performed as previously described [36]. In brief, 1 × 104 MDA-MB-231 cells were seeded into 100 mm dishes and cultured for 12 days before cells were fixed with 100% methanol for 10 min and stained with 0.5% (w/v) crystal violet in 25% methanol.

Expression constructs and oligonucleotides
The human ZNF148 cDNA was amplified from MGC clone 4223572 (GE Healthcare Dharmaco, Inc.) and cloned into the pEF-Biotag vector [37] for biotinylation-mediated chromatin immunoprecipitation followed by sequencing (BioChIP-seq). The cDNA was cloned into the pLeGO-iG2 vector [38] (a gift from Boris Fehse, Addgene plasmid # 27341), for transient transfection and stable lentivirus transduction. Oligonucleotides used for polymerase chain reactions (PCR) are listed in Supplemental Table 1.

Western blotting
The ZNF148-N14 rabbit polyclonal antibody was raised as previously reported [37]. Antibodies specific for MYC (9402, Cell Signaling) and GAPDH (sc-25778, Santa Cruz Biotechnology Inc.) were purchased commercially. Western blotting (WB) was performed as described [37] using 10–25 μg of nuclear protein in each lane of 4–12% Bis-Tris gels (Invitrogen). All chemicals were purchased from Merk/Sigma-Aldrich, unless noted otherwise.

Animal studies
All mammary orthotopic xenograft experiments were conducted in six to eight-week-old female NOD.Cg-Prkd corkout Il2rg<sup>−/−</sup>Cgaj2Rz/Arc (NSG<sup>™</sup>) mice (Animal Resources Center, WA, Australia). To generate mammary tumors in mice, 0.5 × 10<sup>6</sup> MDA-MB-231-Luc cells stably transduced with empty vector control (EV) or ZNF148 cDNA (ZNF148<sup>OE</sup>), were resuspended in 2 mg/ml Matrigel<sup>™</sup>-HC (BD BioSciences) and injected into inguinal mammary fat pads of mice. To visualize the xenograft tumors in vivo, D-Luciferin (14681, Cayman Chemical) was injected intra-peritoneally (i.p.) into mice at 150 mg/kg of body weight, 12 min before the live imaging. To minimize the animal use, we performed power analysis using repeated-measures ANOVA with an F test for between-within subjects with Greenhouse-Geisser correction. Five mice per group provided sufficient power (>0.8) to detect an effect size of one. For imaging, randomized mice were placed under isoflurane induced anesthesia and orientated ventral side down to facilitate oxygen and isoflurane flow through the nose cones. Images were taken using IVIS Lumina II multispectral imaging system and analyzed with Living Image 4.2 Software (Caliper LS, Hopkinton, MA, USA). Xenograft experiments were not performed in a blinded manner. All experiments involving mice were approved by the Animal Ethics Committee at Harry Perkins Institute of Medical Research (Perth, WA, Australia).

Biotinylation-mediated chromatin immunoprecipitation (BioChIP)
BioChIP was performed broadly as previously described [22]. MCF7 and MDA-MB-231 cells were grown to 60–80% confluence and cross-linked with 1% formaldehyde (methanol-free) for 10 min at room temperature and quenched with 125 mM glycine. The cells were washed and sequentially lysed in PIPEs and SDS lysis buffer. Genomic DNA was sonicated at 4 °C with a Covaris m220 sonicator to 200 to 1000 base pair (bp) for quantitative ChIP-PCR and 200–400 bp for BioChIP-sequ. Post immunoprecipitation, biotin-protein-DNA complexes were recovered with Dynabeads<sup>™</sup> M-280 Streptavidin (11205D, Thermo Fisher Scientific), before washing and de-crosslinking and DNA purification for subsequent analyses.

Cellular proliferation, viability, cell cycle, and scratch wound closure assays
MTT cell proliferation and viability assays (Roche) were performed as per the manufacturer’s instructions. In brief, breast cancer cells were seeded into 96-well plates at a density of 2 × 10<sup>4</sup> cells/well, cultured for 96 h before being processed for MTT assay. Cell cycle analyses were performed on an AccurFlow flow cytometer (BD Biosciences) using the BrdU kit (BD Biosciences) in accordance with the manufacturer’s protocol. For cell proliferation assays using IncuCyte<sup>®</sup> live-cell imaging (Essen BioScience), 6 × 10<sup>4</sup> MDA-MB-231 and 4 × 10<sup>4</sup> BT549 cells were seeded into 96-well plates and cultured for 100 h. For scratch wound closure assays, the cells were cultured in IncuCyte<sup>®</sup> ImageLock 96-well plates until a monolayer formed. The cells were serum starved 24 h prior to creating the scratch on the cell monolayer using WoundMaker (Essen BioScience). All images and confuence measurements were performed on an IncuCyte<sup>®</sup> live-cell imager and processed with the IncuCyte Zoom software (Essen BioScience).

Quantitative RT-PCR and ChIP-PCR
RNA samples were prepared using RNeasy Mini Kit (QIAGEN) or SV Total RNA Isolation system (Promega). QuantiTect Reverse Transcription Kit (QIAGEN) was used to synthesize cDNA. Eluates from ChIP were purified using UltraPure<sup>™</sup> Phenol:Chloroform:isoamyl Alcohol (25:24:1, v/v) (Invitrogen) and ethanol precipitated using a standard protocol. Quantitative PCR was performed on a Rotor-Gene 6000 thermocycler (Qiagen) using SensiMixPlus SYBR (Bioline). The expression levels of genes quantified by RT-PCR (RT-qPCR) are presented relative to that of GAPDH; while the enrichments of regulatory regions assayed by quantitative ChIP-PCR (ChIP-qPCR) were calculated relative to an unrelated region in exon 6 of the ACTB locus, as previously described [20, 22, 39].

Transfection, RNA interference, CRISPR/Cas9, and virus transduction
Transfection of plasmid vectors was performed using FuGENE<sup>®</sup> HD (Promega) and Lipofectamine<sup>™</sup> 2000 transfection reagent (Thermo Fisher Scientific) as per the manufacturer’s protocol. Stable transduction of lentiviral short hairpin RNA (shRNA) knockdown in breast cancer cells was performed as described previously [22]. Short hairpin RNAs (shRNAs) seed sequences against MYC and ZNF148 are shown in Supplemental Table 2. Retroviral transduction of pMXs-GFP and pMXs-cMYC (human) vectors was performed essentially as previously described [37] using a pCL-10A1 packaging vector (Novus Biologicals). The CRISPR/Cas9 gRNA plasmid targeting exon 4 of ZNF148 (Target ID: HS0000451815) was purchased from Sigma-Aldrich.

ALDEFLUOR assay
The Aldehyde dehydrogenase (ALDH) activity in MDA-MB-231 cells was measured using ALDEFLUOR<sup>™</sup> kit (01700, STEMCELL technologies), following the manufacturer’s protocol. In brief, 5 × 10<sup>3</sup> cells were subjected
to the ALDEFLUOR assay with 2.5 μL of activated ALDEFLUORTM reagent in the presence or absence of 5 μL of diethylaminobenzaldehyde (DEAB). Flow cytometry analysis was performed using AccuriTM instruments (BD Biosciences).

Sequencing analysis

RNA-sequencing (RNA-seq) was performed on Illumina HiSeq2000 system, and the sequenced reads were aligned to the human (GRCh38) genome using Salmon (v.0.7.2) [40]. Differentially expressed genes (DEGs) were determined using DEseq2 package. BioChIP-seq was performed on NextSeq 500 and the sequenced read were mapped to human (GRCh38) genome using Bowtie2 (v 0.5.9-r16) [41]. Peaks were called using MACS2 with a q-value cut off of 0.01 (v2.1.1) [42]. DNA binding motif analysis was performed using HOMER [43]. Binding and Expression Target Analysis (BETA) [44] was used to integrate and analyze BioChIP-seq and DEG data. GREAT (v4.0.4) [45], ShinyGO (v0.61) [46] and BETA were used for gene ontology functional annotation analysis.

Statistical analyses

Student’s t-test (two-tailed) and Two-way ANOVA (ordinary) statistical analyses were performed using GraphPad Prism. Replicate experiments, sample size (n), mean values, SEM, SD, or CI error bars are indicated in the figure legends.

RESULTS

ZNF148 is repressed by MYC in breast cancer

To investigate if MYC transcriptionally regulates ZNF148 in breast cancer, we first examined the ChIP-seq data in the Encyclopedia of DNA Elements (ENCODE) database [47, 48]. Confirming our previous observation in mouse ES cells [20], MYC occupancy was observed at the proximal promoter of ZNF148 in both MCF7 (ER positive, luminal subtype) and MCF10A (normal-like) breast cancer cells (Fig.1A), and this region harbored several canonical and non-canonical E-box sequences (Supplemental Fig. 1A). To validate and extend this observation in TNBC cells, we performed ChIP-qPCR for MYC using MCF7 and a metastatic TNBC cell line, MDA-MB-231. This confirmed a significant enrichment of MYC at −750, −2400, and −4250 bp upstream regions of the ZNF148 locus (Fig. 1B, C).

Next, we performed a loss-of-function study using short hairpin RNAs (shRNAs) against MYC to understand the impact of MYC depletion on ZNF148 gene regulation. Two different shRNAs significantly reduced both MYC mRNA and protein levels in MCF7 and MDA-MB-231 cells, compared to the control samples transduced with an shRNA targeting firefly luciferase (shCtr) (Fig. 1D, E). While the majority of MYC transcriptional regulation has been linked to gene activation [11, 12], silencing of MYC, surprisingly resulted in increased ZNF148 mRNA and protein expression (Fig.1D, E). Analysis of publicly available ChIP-seq data revealed the simultaneous presence of H3K27me3 repressive and H3K4me3 active histone marks on the ZNF148 promoter, consistent with a bivalent epigenetic state, suggesting the ZNF148 gene is poised for repression and activation (Supplemental Fig. 1B). To ascertain if this negative relationship also occurred in clinical specimens, we queried the patient-derived gene expression data in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) datasets. Indeed, an inverse correlation of ZNF148 and MYC mRNA levels and is depicted with a linear trend line in “red”: Student’s t-test, *P < 0.05. **P < 0.01. ***P < 0.001. ****P < 0.0001. Error bars indicate mean ± SD.

Fig. 1 MYC represses ZNF148 expression by direct chromatin occupancy. A Schematic representation of ZNF148 locus on chromosome 3q21 reverse strand. Peak calls for MYC in MCF7 (purple bars), and MCF10A (yellow bars) cells from ENCODE [47] are indicated. B Quantitative ChIP-PCR (ChIP-qPCR) validation of MYC occupancy at −750, −2400, and −4250 bp upstream of ZNF148 promoter region in MCF7 cells (n = 3). C As in “B” for MDA-MB-231 cells. D Analysis of MYC and ZNF148 mRNA and protein in MCF7 cells transduced with the short-hairpin (sh) luciferase control (Ctr) and shMYC #1 and #2 lentiviruses, by RT-qPCR (top panel, n = 3) and Western blot (bottom panel). E As in “C” for MDA-MB-231 cells. F Scatter plot of MYC and ZNF148 mRNA levels (log2) from 1904 breast cancer patient samples in METABRIC cohort [56, 78]. Spearman correlations of −0.24 (p = 5.29 ×10−27) and Pearson correlations of −0.25 (p = 1.90 ×10−27) indicate an inverse correlation of ZNF148 and MYC mRNA levels and is depicted with a linear trend line in “red”: Student’s t-test, *P < 0.05. **P < 0.01. ***P < 0.001. ****P < 0.0001. Error bars indicate mean ± SD.

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correlation between MYC and ZNF148 mRNA levels in breast cancer patients was observed (Fig. 1F). Collectively, these results support a direct transcriptional role for MYC in suppressing the expression of ZNF148 in breast cancer through chromatin occupancy.

To determine the functional significance of ZNF148 in breast cancer, we used lentivirus to stably express ectopic ZNF148 cDNA into ER+ breast cancer (MCF7), TNBC (MDA-MB-231, BT549, and HCC1806), and normal-like breast (MCF10A) cell lines. RT-qPCR
and Western blot (WB) analysis confirmed the ectopic expression of ZNF148 in these cells (Fig. 2A–E). IncuCyte cell proliferation assay showed no change in the proliferation rate in MCF10A, but a marginal increase in ER+ MCF7 cells that stably express ectopic ZNF148 (Fig. 2A, B). Interestingly, we observed a reduction in cell growth in all three TNBC cell lines that overexpress ZNF148 (Fig. 2C–F). BrdU cell cycle analysis of MDA-MB-231 cells expressing ectopic ZNF148 showed a significant reduction in the synthesized (S) phase as well as increased gap (GO/G1) phases compared to the empty vector (EV) control cells, confirming the anti-proliferative effect of ZNF148 (Fig. 2G). Subsequently, the impact of ZNF148 on cell migration in TNBC cell lines was investigated by the IncuCyte scratch assay. To account for ZNF148’s anti-proliferative effect, both EV controls and the ZNF148OE cells were serum-starved for 24 h for cell cycle synchronization prior to the migration assay. Under this synchronized state, MDA-MB-231-ZNF148OE, BT549-ZNF148OE, and HCC1806-ZNF148OE showed significantly slower wound closure compared with the control (Fig. 2H, I and Supplemental Fig. 1).

The above in vitro cellular assays indicate that ZNF148 exhibits classical tumor-suppressive traits with anti-proliferative and anti-migratory effects on TNBC cells. To validate this in vivo, we established five biological replicates, which were then employed the BioChIP-seq approach [49] to map the transcriptional repressor complex was the transcriptional and epigenetic mechanism to restrict cellular proliferation.

**ZNF148 regulates cell differentiation and migration**

To determine the clinical relevance of the observed cell line data, we examined the relapse-free survival (RFS) of breast cancer patients based on the expression level of ZNF148. In all breast cancer subtypes, patients with a higher level of ZNF148 had a better survival chance, consistent with the observed tumor suppressive and anti-migratory effect of ZNF148 (Fig. 3F). Of particular note, improved RFS associated with high ZNF148 expression was observed only in patients with lymph node-positive, but not in the lymph node-negative cases of breast cancer (Fig. 3G, H). These results suggest that ZNF148’s tumor suppressive role is more significant in metastatic disease, which is typically associated with the aggressive TNBC subtype.
potential clonal biases and allow the detection of immediate gene expression changes, RNA-seq was performed on total RNA isolated from early passage MDA-MB-231-shCtr and MDA-MB-231-shZNF148 cells. These analyses revealed 229 significantly upregulated genes (Padj < 0.05, Fold change > 1.5) and 298 downregulated genes (Padj < 0.05, Fold change < −1.5) upon the depletion of ZNF148 (Fig. 5A). We identified additional direct and indirect target genes of ZNF148 by combined analysis of BioChIP-seq and RNA-seq data. ZNF148 occupancy was identified in 78% of the downregulated genes, indicating that these genes are positively regulated by ZNF148. Similarly, 59% of the genes upregulated in MDA-MB-231-shZNF148 were the direct binding targets of ZNF148, identifying these as negatively regulated by ZNF148 (Fig. 5B). The plot of cumulative fraction of genes (%) versus rank of genes based on regulatory potential score (from high to low), predicted that ZNF148 functions as both an activator and repressor (Fig. 5C). GO term biological process analysis of the genes that are directly repressed by ZNF148 showed a...
Fig. 4  Genomic targets of ZNF148. A Doughnut chart showing the percentage distribution of ZNF148 chromatin occupancy peak locations determined by BioChIP-seq. B Consensus DNA binding motif analysis of ZNF148 occupied sequences (HOMER) [43]. The top 5 representative DNA binding sequence motifs were ranked by p-value and known transcription factors. C Representative BioChIP-seq signals at the bone morphogenetic protein 4 (BMP4) locus in MDA-MB-231 cells expressing BirA alone or BirA and FB-ZNF148. D As in “C” for SMAD family member 6 (SMAD6) locus. E As in “C” for discoidin domain receptor tyrosine kinase 1 (DDR1) locus. F GO biological process analysis of ZNF148 top 1500 peaks. G GO cellular component analysis of ZNF148 top 1500 peaks.
enrichment for various macromolecule modification, biosynthesis, and metabolic processes, which are normally required for cell growth (Supplemental Fig. 6A). In contrast, genes directly activated by ZNF148 showed an enrichment for cellular component organization, organelle organization, inhibition of metabolic process, and anatomical structure morphogenesis, which are reminiscent of cellular differentiation processes (Supplemental Fig. 6A).

To gain further insight into the collective biological consequences of ZNF148 loss, including the direct and indirect changes, additional GO term analysis (biological processes) was performed on the 298 downregulated and 229 upregulated genes (Fig. 5D, E and Supplemental Tables 3, 4). While the downregulated genes were enriched in cytoskeleton and extracellular matrix organization-related terms, the upregulated genes were enriched in terms related to inhibiting development and differentiation (Fig. 5D, E). In line with the enhanced migration phenotype observed with the depletion of ZNF148 in vitro, we found enrichment of cell and tissue migration GO terms (Fig. 5E). These data collectively support the notion that ZNF148 is required for breast cancer stemness and inhibiting migration and differentiation processes in breast cancer cells.

**Loss of ZNF148 enhances stemness of breast cancer cells**

A high level of MYC drives stemness and an aggressive phenotype in breast cancer [11, 12, 14, 15, 56]. Based on our finding, we posit that MYC negatively regulates ZNF148 (Fig. 1) to repress biological processes involving cellular differentiation and development (Fig. 5), fostering cancer stem-cell-like features in breast cancer cells. To test this hypothesis, we measured aldehyde dehydrogenase (ALDH) enzymatic activity, which is elevated in cancer stem cell populations [4, 5, 9]. Upon silencing ZNF148 using shRNA, a significant increase in the percentage of ALDH-positive (ALDH+) cells was observed in MDA-MB-231-shZNF148, compared with the basal level in MDA-MB-231-shCtrl cells (Fig. 6A). Consistent with this, the percentage of ALDH+ cells in MDA-MB-231-ZNF148KO cells likewise increased significantly compared to the control cells (Fig. 6B). In addition, clonogenic assays, which measure stem-like cell growth in vitro [57, 58], revealed increased colony formation in MDA-MB-231-ZNF148KO cells compared to the control, further validating our hypothesis that ZNF148 is a negative regulator of stemness in breast cancer (Fig. 6C).

**ZNF148 transcriptionally represses ID1 and ID3**

As ZNF148 suppresses stemness in MDA-MB-231 cells, we reasoned that certain target gene(s) of ZNF148 promoting stemness in cancer cells, are repressed by ZNF148. To identify downstream target gene(s) that enhance stem-like features in breast cancer, we ranked ZNF148 ChIP-enrichment scores, and the expression fold changes of genes within the enriched GO terms (Fig. 5E) and identified Inhibitor of DNA binding 3 HLH protein (ID3) as a potential candidate. ID1 and ID3 proteins are functionally redundant and have been shown to promote breast cancer cell self-renewal, tumor cell dissemination, and metastatic colonization of the lung and tumor re-initiation [33–35]. Importantly, the ZNF148 occupancy was significantly enriched at the promoter and gene body of ID3 (Fig. 7A). A closer examination of the other family members of ID genes, ID1, ID2, and ID4 loci, also showed ZNF148 occupancy at the promoter and gene body, similar to ID3, albeit statistically less significant in MACS2 peak calling scores (Supplemental Fig. 7). Upon silencing of ZNF148 mRNA, we observed a dramatic increase of ID3 and ID1 transcription (Fig. 7B). Conversely, the overexpression of ZNF148 significantly decreased ID3 and ID1 (Fig. 7C). These data collectively provide compelling evidence that ZNF148 actively represses ID1/3 genes in breast cancer cells.

To demonstrate the MYC-ZNF148-ID1/3 regulatory axis, we overexpressed MYC and GFP (control) cDNA in untransformed MCF10A breast epithelial cells using retroviruses and monitored the changes in MYC, ZNF148, and ID1/3 transcripts. Consistent with the repressive role of MYC on ZNF148, the MYC overexpression reduced the ZNF148 transcript level, subsequently
conveying an increase in ID3 expression (Supplemental Fig. 8A). Next, we used lentivirus-mediated shRNA transduction to downregulate ZNF148 in MCF10A-GFP and MCF10A-MYCOE cells (Supplemental Fig. 8B). The ZNF148 depletion increased the MYC transcript level in MCF10A-GFP cells, consistent with the inverse expression correlation found in breast cancer patients (Supplemental Figs. 8B and 1F). Furthermore, the ID3 expression increased with the ZNF148 depletion, or with the overexpression of MYC, while simultaneous depletion of ZNF148, and MYC overexpression led to an additive increase in ID3 transcripts. For ID1, the gene expression changes did not follow that of ID3 upon MYC overexpression and ZNF148 depletion, indicating an additional layer of regulatory mechanisms in MCF10A cells. Collectively, these data demonstrate that the genetic relationship between the MYC-ZNF148-ID3 axis is conserved across mammary epithelial cells irrespective of their transformed status.

Finally, we queried the METABRIC breast cancer patient data to examine the correlation between MYC, ZNF148, and ID1/3 expression levels. Indeed, an inverse correlation between ZNF148 and both ID1/3 was observed (Fig. 7D). Furthermore, a direct proportional expression between MYC and ID1/3 was observed (Fig. 7E), further strengthening the existence of a MYC, ZNF148, and ID1/3 regulatory axis in controlling the stemness in breast cancer (Fig. 7F, G).

**DISCUSSION**

Despite the importance of ID genes driving the cancer stem cell phenotype in a broad array of epithelial cancers, their regulatory mechanism remains incompletely understood. In this study, we uncover a novel role of ZNF148 in suppressing TNBC cell growth and metastasis and provide evidence for a direct regulatory circuitry between MYC, ZNF148, and ID1/3 that impacts stem cell traits in breast cancer. We show ID1 and ID3 are transcriptionally repressed targets of ZNF148, and that silencing of ZNF148 derepresses their expression, resulting in increased stemness of TNBC cells.

Patients with a higher level of ZNF148 show improved survival, but interestingly, the benefit of having a high ZNF148 gene dosage is accentuated only in metastatic, lymph-node positive disease (Fig. 3). It is possible that the effect of ZNF148 dosage in breast cancer is more pronounced in the context of MYC, stemness, and metastatic state of the cells. Our in vitro cell line data suggests that the anti-proliferative effect of ZNF148 is significant in TNBC but not in ER+ cells (Fig. 2). MDA-MB-231, in...
downregulating tumor suppressor genes and cell cycle regulators in cancer \[11, 61, 62\], while concomitantly MYC-driven cancer stem cell features \[59, 60\].

In particular, is a highly metastatic TNBC cell line with pronounced 

Oncogenic MYC alters global transcriptional programs to drive stem cell-like cancer [63–65]. Mechanistically, polycomb repressive complex 2 (PRC2) has been shown to interact with MYC proteins to induce transcriptional silencing of the target genes [66, 67]. SUZ12 and EZH2 are core subunits of PRC2; indeed, both were enriched at the promoter region of ZNF148 (Supplementary Fig. 18). SUZ12 and EZH2 occupancy, along with E-box motifs and MYC occupancy, and H3K27me3 marks at the regulatory region of the ZNF148 locus provide a possible mechanism for MYC-induced repression of ZNF148 in breast cancer.

MYC also represses lineage-specific transcription factors in mammary tissues, such as GATA3 and ESR1 to drive cancer stem cell-like states in breast cancer [68]. We previously reported physical interaction between ZNF148 and GATA3 proteins and possible functional cooperation between them in hematopoietic cells [37]. It is possible that ZNF148 may cooperate with GATA3 to drive mammary tissue development, but without a mammary-specific mouse knockout study, whether ZNF148 also plays a lineage-specific transcriptional role remains unclear. Our current study supports a role for ZNF148 as a tumor suppressor gene in metastatic TNBC, that is actively repressed by MYC.

The level of MYC expression is exceptionally high in basal-like TNBCs that harbor BRCA1 mutation [14–16]. The BRCA1 gene suppressed basal stem cell expansion during mammary tumor development [17–19] and downregulated MYC expression [69], quenching the MYC-driven oncogenic pathways. Interestingly, the same study identified ZNF148 as a gene significantly upregulated by BRCA1 [69]. Although it was not clear whether BRCA1 directly contributed to transcriptional activation of ZNF148, the inverted gene expression pattern between ZNF148 and MYC is consistent with our findings. It is possible that the tumor suppression by BRCA1 may be in part mediated by ZNF148 activation as a result of MYC downregulation.

ZNF148 binds to GC-rich DNA motifs and shares the most similarity with ZNF281 (Fig. 4B). We recently reported similar GC-rich DNA consensus motifs, unique and overlapping chromatin occupancy sites for ZNF148 and ZNF281, and their functional redundancy in myelogenous leukemia cells [22]. Recently, ZNF281 was shown to activate epithelial to mesenchymal transition (EMT) transcription factors, ZEB1 and SNAI1 via activation of the TGF-β pathway, promoting EMT and metastasis in breast cancer [70]. Despite the similar DNA binding motifs between ZNF148 and ZNF281, we did not observe the chromatin occupancy of ZNF148 on ZEB1 or SNAI1 loci. Moreover, contrary to the ZNF281 metastatic role, ectopic expression of ZNF148 inhibited cell migration (Fig. 2H, I and Supplemental Fig. 2). ZNF281 is 80% homologous to ZNF148 in the DNA-binding domains, but only 3 to 40% similar in the N-terminal and C-terminal domains. These

**Fig. 7 ZNF148 represses ID1/3.** A BioChIP-seq signals at the inhibitor of DNA binding 3 (ID3) locus in MDA-MB-231 cells expressing BirA alone or BirA and FB-ZNF148. B RT-qPCR analysis for ZNF148 (left panel, n = 6), ID3 (middle panel, n = 12) and ID1 (right panel, n = 6) mRNA transcripts, relative to GAPDH and shControl (shCtr), in shZNF148 MDA-MB-231 cells. C RT-qPCR analysis for ZNF148 (left panel, n = 6), ID3 (middle panel, n = 4) and ID1 (right panel, n = 9) mRNA transcripts, relative to GAPDH and empty vector (EV) control, in ZNF148 overexpressing MDA-MB-231 cells. D Scatter plots of ID3 vs. ZNF148 (Pearson r = −0.3776, p < 0.0001), and ID1 vs. ZNF148 (Pearson r = −0.1761, p < 0.0001) mRNA levels (log2) from 1904 breast cancer patient samples in METABRIC cohort [56, 78]. E Scatter plot of ID3 vs MYC and ID1 vs MYC as in “D”, with Pearson r = 0.3359 (p < 0.0001) and Pearson r = 0.1590 (p < 0.0001) respectively, indicating a direct correlation of ID3/1 and MYC mRNA levels. F Schematic diagram of ZNF148 actively repressing ID1/3 gene expression. G Schematic diagram of regulatory circuitry between MYC, ZNF148, and ID1/3 in MYC-driven stem cell-like cancer. Student’s t-test, ***, p < 0.001. ****, p < 0.0001. Error bars indicate ± SD.
structural features may provide a foundation for shared chromatin occupancy but a divergent role at the occupied sites. There may be competition for occupancy at the same regulatory loci between ZNF148 and ZNF281, but mediate different functions by recruiting different cofactors, resulting in different net phenotypic outcomes.

The TGF-β pathway has a dual role acting as either tumor suppressor or oncogenic during carcinogenesis [71]. As a tumor suppressor, TGF-β reduces the breast cancer stem cell population, and cell proliferation via MYC, ID1, and ID3 genes [72, 73]. Given the MYC-ZNF148-ID1/3 regulatory axis found in this study, ZNF148 may be involved in the TGF-β downstream pathways, mediating the repression of ID1/3 genes, thus reducing the cancer stem cell properties in breast cancer. In the breast tumor microenvironment, TGF-β is oncogenic by priming cancer cells for metastasis to the lungs [74], facilitating the dissemination and colonization of cancer cells that have undergone EMT via ID1 mediated mesenchymal to epithelial transition (MET) [75]. Considering the recent finding that ZNF281 promotes metastasis via the TGF-β pathway, and an opposing role of ZNF148 involving MYC and ID genes, both ZNF148 and ZNF281 may be integral transcriptional suppressors that MYC actively represses, particularly in breast cancer. Currently, various therapeutic approaches are being investigated to target both MYC and the ID proteins [12, 31, 76]. Our findings suggest that upregulating ZNF148 in breast cancer could also be beneficial. Sodium butyrate, an HDAC inhibitor was shown to increase ZNF148 expression in colorectal cancer cells [77], however, it did not change the ZNF148 levels in breast cancer cells (data not shown). Considering the ubiquitous nature of ZNF148 expression, the MYC, ZNF148, ID1/3 regulatory axis may be present in a broad array of cancers driven by MYC and ID proteins. Future work to identify therapeutic agents that upregulate ZNF148 could be of clinical importance, not only in breast cancer, but in various cancers with aggressive cancer stem cell-like traits.

DATA AVAILABILITY
The RNA-seq and BisoChip-seq data generated in this study have been deposited in the Gene Expression Omnibus (GEO) public database under the accession numbers GSE132953 and GSE147020, respectively.

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**AUTHOR CONTRIBUTIONS**

Conceptualization: AJW; methodology, AJW, LE, DCV, JYW, and JK; investigation, AJW, MK, B-KL, MH, KR, and MS; writing—first draft, AJW; writing—review & editing, MK, JK, DCV, LE, JW, PB, and PJL; supervision, AJW and PJL.

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ADDITIONAL INFORMATION

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