

ZNF503/Zpo2 drives aggressive breast cancer progression by down-regulation of GATA3 expression

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The transcription factor GATA3 is the master regulator that drives mammary luminal epithelial cell differentiation and maintains mammary gland homeostasis. Loss of GATA3 is associated with aggressive breast cancer development. We have identified ZNF503/ZEPPO2 zinc-finger elbow-related proline domain protein 2 (ZPO2) as a transcriptional repressor of GATA3 expression and transcriptional activity that induces mammary epithelial cell proliferation and breast cancer development. We show that ZPO2 is recruited to GATA3 promoter in association with ZBTB32 (Repressor of GATA, ROG) and that ZBTB32 is essential for down-regulation of GATA3 via ZPO2. Through this modulation of GATA3 activity, ZPO2 promotes aggressive breast cancer development. Our data provide insight into a mechanism of GATA3 regulation, and identify ZPO2 as a possible candidate gene for future diagnostic and therapeutic strategies.

breast cancer | GATA3 | ZNF503/ZPO2 | ZBTB32 | tumor metastasis

The transcription factor GATA3 is a key mediator of luminal cell fate determination and homeostasis in adult mammary gland (1–3). *GATA3* is one of top three altered genes during breast cancer progression (4). *GATA3* expression is detected in estrogen receptor (ER)-positive mammary tumors (5–12). ER⁺ tumor cells morphologically resemble more differentiated luminal cells, and affected patients generally have a promising survival outcome. Mammary tumor cells that exhibit diminished levels of *GATA3* expression have less differentiated cellular morphology and give rise to more aggressive breast cancers characterized by larger tumor size, higher histological grade, and enhanced metastasis (7, 13–16).

DNA mutations are one cause of *GATA3* deregulation. Frame-shift mutations or sequence alteration near the highly conserved second zinc finger, which is required for DNA binding in the *GATA3* gene, may play a role in diminishing *GATA3* functionality, leading to breast cancer progression (4, 17–19). Other regulatory pathways or transcriptional inhibitors may influence *GATA3* expression and transcriptional activity as well.

The NET subfamily of zinc finger proteins that are related to the Sp family of transcription factors plays a prominent role during development (20). We have identified *ZNF703/Zeppl1* (zinc finger elbow-related proline domain protein 2; *Zpo2/Nolz2/Zfp703*) and *ZNF503/Zeppl2* (zinc finger elbow-related proline domain protein-2; *Zpo2/Nolz1/Zfp503*) expression in mammary epithelial cells (21). *Zpo1* and *Zpo2* are closely related and have 54% protein sequence identity. Our work and that of others have shown that deregulation of *Zpo1* can lead to breast cancer (21–24); however, unlike *Zpo1*, our understanding of *Zpo2* involvement in mammary tumor development is minimal. Previous studies have indicated that *Zpo2* is critical for proper development in several species (25–27). We have shown that *Zpo2* is a transcriptional repressor expressed in both embryonic and adult mammary epithelial cells, with significantly higher expression levels in the basal cell compartment than in luminal epithelial cells (28). *Zpo2* promotes mammary epithelial cell proliferation and enhances cellular migration and invasion. Elevated *ZPO2/ZNF503* levels correlate with breast cancer progression and

increased metastasis (28). The underlying mechanism of ZPO2-induced breast cancer remains elusive, however. Moreover, whether *Zpo1* and *Zpo2* function redundantly or exert different cellular effects during mammary tumorigenesis is unclear.

Here we report an interplay among *Zpo2*, *ZBTB32*, and *GATA3* in mammary epithelial cells. We show that *ZBTB32* facilitates *Zpo2* targeting to the *GATA3* promoter, leading to down-regulation of *GATA3* expression and activity. Modulation of *GATA3* by *Zpo2* in turn results in the development of aggressive breast cancers.

Results

In Silico Analysis of Breast Cancer Databases Suggests Interplay Between ZPO2 and GATA3. Deregulation of *GATA3* is one of the most prominent associations with breast cancer. Analysis of 823 patient samples from the TCGA database via cBioportal (cbioportal.org/) indicated that *GATA3* expression is altered in 14.2% of cases (Fig. S1A). These alterations are due to amplification, mutation, mRNA down-regulation, and changes in protein levels. Of these, roughly 7% are due solely to down-regulation of *GATA3* independent of any mutations (Fig. S1B). These data suggest that unknown regulatory mechanisms may down-regulate wild-type (WT) *GATA3* expression, resulting in the development of breast cancer. Analysis of TCGA database via Regulome

Significance

Transcription factor *GATA3* has emerged as one of top three altered genes in mammary tumors. In breast cancer, *GATA3* expression has been associated with an estrogen receptor (ER)-positive (ER⁺/luminal) phenotype. ER⁺ tumor cells resemble more differentiated cells, and affected patients are more responsive to therapy and have overall better survival outcomes. Loss of *GATA3* correlates with ER⁻, less differentiated, and more aggressive tumors with poorer prognosis. We have identified zinc-finger elbow-related proline domain protein 2 (ZPO2) as a transcriptional repressor that down-regulates *GATA3* and promotes aggressive breast cancer. Thus, ZPO2 could be an important oncogenic target for prediction of aggressive breast cancer.

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Explorer (explorer.cancerregulome.org) for protein network association indicated a pairwise association between *Zpo2* and several key mammary epithelial cell regulators including *GATA3* (Fig. 1A). Intriguingly, *ZPO2/ZNF503* levels were higher in breast cancer samples with WT *GATA3* than in those with mutated *GATA3* (Fig. 1B).

Analysis of the GSE1378 database (29, 30) indicated a correlation between higher *ZPO2* levels and poorer survival in breast cancer patients (Fig. 1C). In addition, we analyzed distant metastasis-free survival (DMFS) using patient samples from the DMFS database expressing high and low *ZPO2*. Again, higher *ZPO2* levels correlated with poor patient survival (Fig. 1D). We also observed poor prognosis in patients with higher *ZPO2* when analyzed from GSE19615 (31) (Fig. S2). Analysis of a TCGA dataset composed of 825 patient samples for *GATA3*, *ZPO1/ZNF703*, and *ZPO2* expression levels revealed an inverse correlation between *ZPO2* expression and *GATA3* or *ZPO1* level. In the tumor samples, where *ZPO2* levels were the highest, *GATA3* and *ZPO1* expressions were the lowest (Fig. 1E).

Collectively, our *in silico* analyses indicate that *ZPO2* and *GATA3* reside within the same protein network, and that there is an inverse relationship of their expression in breast cancer samples. Thus, *ZPO2* is a candidate gene that may play a potential role in mammary tumor induction via down-regulation of *GATA3*.

Zpo2 Down-Regulates GATA3 Expression in Mammary Epithelial Cells.

We next examined the effect of *Zpo2* overexpression or *Zpo2* knockdown on *GATA3* levels in murine mammary cell lines. We used EpH4.9 and MMTV-PyMT (PyMT) mammary stable cell lines that either overexpressed full-length V5-tagged *Zpo1* or *Zpo2* constructs or expressed *Zpo2*-shRNA (sh*Zpo2*) (21, 28). Both cell lines had detectable levels of endogenous *GATA3*. We found that *Zpo2* down-regulated *GATA3* levels, whereas shRNA-mediated *Zpo2* knockdown enhanced *GATA3* expression (Fig. 2A–E). Modulation of *GATA3* levels was specific to *Zpo2* activity; *Zpo1*

overexpression did not alter *GATA3* RNA and protein levels (Fig. 2A and C). Furthermore, transient transfection of full-length V5-tagged *Zpo2* construct in MCF7, NMuMG, and EpH4.9 cells, which have detectable levels of endogenous *GATA3*, also revealed reduced *GATA3* protein levels (Fig. S3).

GATA3 functions as a tumor suppressor in part by blocking epithelial-mesenchymal transition (EMT)-associated gene expression and interfering with cellular invasiveness (13, 15, 32–34). In contrast, *Zpo2* promotes mammary cell proliferation and invasiveness (28). We found that *Zpo2* overexpression resulted in significant up-regulation of *Twist*, *Vimentin*, *Snail*, *ZEB1*, and *ZEB2* and lowered *E-cadherin* mRNA levels (Fig. 2F). In addition, *Zpo2* overexpression increased matrix metalloproteinase-9 (MMP9) expression levels and activity (Fig. 2G). Thus, our data indicate that *Zpo2* blocks *GATA3* and interferes with its transcriptional activity as a tumor suppressor.

Zpo2 Forms a Complex with ZBTB32 to Target and Repress GATA3 Expression.

Zpo2 is a transcriptional repressor targeted to the nucleus (28). Because *Zpo2* is in the same protein network as *GATA3* and inhibits *GATA3* expression and transcriptional activity, we investigated the underlying mechanism of *Zpo2*-mediated *GATA3* modulation. We performed yeast two-hybrid experiments to uncover potential *Zpo2* interacting partners that might link *Zpo2* to *GATA3* pathway. We used full-length *Zpo2* as bait and screened against an expression library generated from MMTV-PyMT mouse tumors. We found 14 candidate molecules that indicated strong interactions with *Zpo2*. These candidate molecules were classified into three groups: transcriptional regulators consisting of DP103, HDGF, *Zpo1*, and *ZBTB32*; sumoylation pathway-specific macromolecules; and a third group comprising a mix of proteins, such as Aha1, Dor1, PK2, SPK-2, and GSN (Table S1).

Among the potential candidate genes, *ZBTB32* provides a plausible link between *Zpo2* and *GATA3*. In lymphocytes,

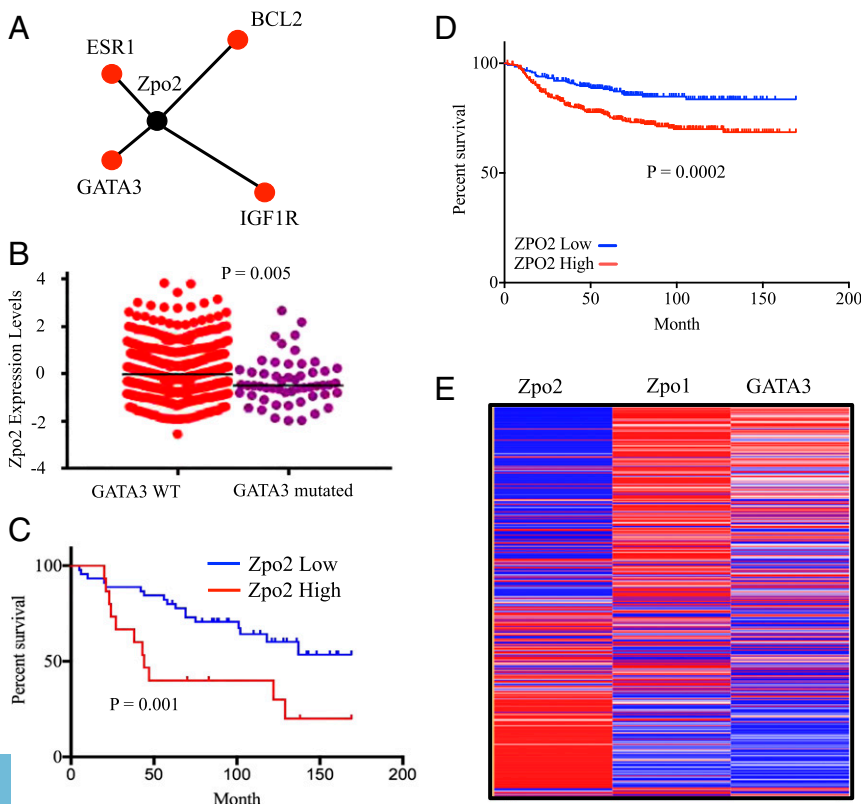


Fig. 1. *In silico* analysis of *ZPO2* expression using a breast cancer database. (A) Protein network analysis indicating pairwise association between *ZPO2* and *GATA3*. The analysis was performed with Regulome Explorer using the TCGA database. (B) TCGA database analysis of *ZPO2* expression in breast cancer samples with either WT or mutated *GATA3*. WT *GATA3*: mean \pm SD, 0.093 ± 1.056 ; SEM, ± 0.049 . Mutated *GATA3*: mean \pm SD, -0.328 ± 0.982 ; SEM, ± 0.134 . (C) Patient survival analysis based on high or low *ZPO2* expression. The GSE1378 breast cancer dataset was downloaded from the National Center for Biotechnology Information's Gene Expression Omnibus database. We divided the patients into two groups with different survival curves using *ZPO2* gene expression (quartile cutoff was set at 25% high and 75% low *ZPO2* expression). Higher *ZPO2* expression was associated with poor prognosis. (D) Kaplan–Meier analysis of distant metastasis free survival using the DMFS database. HR, 1.99; 95% CI, 1.38–2.86; $P = 0.0002$. (E) Heat map of the TCGA database analysis for *ZPO2*, *ZPO1*, and *GATA3* expression in breast cancer patients.

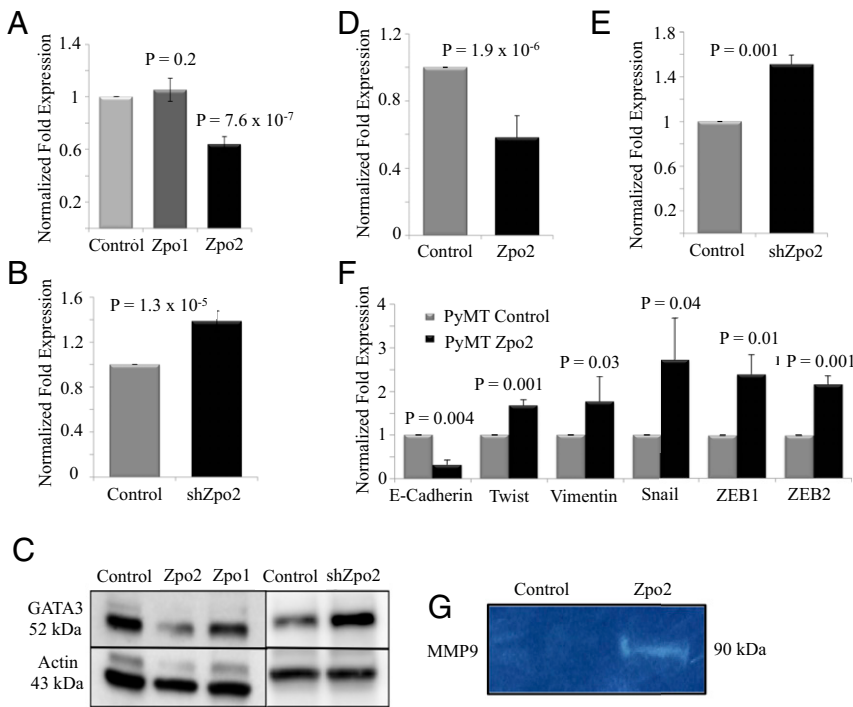


Fig. 2. Effect of elevated Zpo2 levels on Zpo1-, GATA3-, and EMT-associated genes. (A) qRT-PCR analysis indicating GATA3 levels in Eph4.9 cells overexpressing Zpo1 and Zpo2. (B) qRT-PCR analysis indicating that shRNA-mediated down-regulation of Zpo2 increases Gata3 levels. (C) Western blot analysis in Eph4.9 mammary epithelial cell extracts. Zpo2 overexpression lowers GATA3 levels. Zpo1 overexpression does not alter GATA3 levels. Down-regulation of Zpo2 elevates GATA3 levels. (D) qRT-PCR analysis of Gata3 expression in PyMT cells overexpressing Zpo2. Zpo2 down-regulates Gata3 levels. (E) qRT-PCR analysis in PyMT cells. Down-regulation of Zpo2 results in increased Gata3 levels. (F) qRT-PCR analysis of EMT-associated genes in PyMT cells. Overexpression of Zpo2 alters EMT-associated gene expression (G) Zymogram analysis indicating increased MMP9 levels in Eph4.9 mammary epithelial cells overexpressing Zpo2.

ZBTB32 specifically targets the *GATA3* promoter and inhibits GATA3 transcriptional activity (35). Therefore, we cotransfected full-length V5-tagged *Zpo2* and full-length Myc-tagged *Zbtb32* constructs into Eph4.9 cells to determine whether Zpo2 and ZBTB32 form a complex that targets and represses GATA3 expression and transcriptional activity. Coimmunoprecipitation (co-IP) with anti-Myc (*ZBTB32*) or anti-V5 tag (*ZPO2*) antibodies, followed by Western blot analysis using anti-V5 tag or anti-Myc antibodies to pull down ZPO2 or ZBTB32 proteins and examine ZPO2/ZBTB32 interaction, demonstrated that ZPO2 and ZBTB32 form a complex in mammary cells (Fig. 3A).

We also performed co-IP experiments using endogenous ZPO2 and ZBTB32 proteins followed by Western blot analysis to examine the ZPO2–ZBTB32 interaction (Fig. S4). Co-IP experiments using endogenous proteins also indicated a ZPO2–ZBTB32 interaction in Eph4.9 mammary cells. To validate that the Zpo2/ZBTB32 complex is targeted to the *Gata3* promoter, we performed chromatin immunoprecipitation (ChIP) analysis using endogenous ZPO2 and ZBTB32 proteins in Eph4.9 cells. Quantitative RT-PCR (qRT-PCR) using primers specific to *Gata3* promoter verified that both ZPO2 and ZBTB32 occupy the *Gata3* promoter (Fig. 3B). In addition, we cotransfected Eph4.9 cells with full-length *Zpo2* and *Zbtb32* constructs. ChIP analysis of transfected cells also demonstrated that ZPO2 and ZBTB32 occupy the *Gata3* promoter region (Fig. S5).

We next examined whether ZBTB32 is necessary for the inhibitory effect of Zpo2 activity. Knockdown of *Zbtb32* rescued *Gata3* levels in Eph4.9 cells overexpressing *Zpo2* (Fig. 3C). To further investigate the importance of ZBTB32 for Zpo2 functionality, we performed 3D Matrigel culture assays using control or Zpo2-expressing Eph4.9 cells. As shown previously (28), overexpression of *Zpo2* in Eph4.9 cells resulted in the formation of invasive colonies in 3D Matrigel cultures. Interestingly, knockdown of *Zbtb32* interfered with the invasive phenotype exerted by Zpo2, and colonies failed to infiltrate throughout the Matrigel matrix (Fig. 3D). To demonstrate the specificity of ZBTB32, we conducted a similar experiment in cells overexpressing *Zpo1*. Whereas Zpo1 induces cellular migration and promotes an invasive phenotype (21), similar to colonies expressing *Zpo2*, the knockdown

of *Zbtb32* in *Zpo1*-overexpressing cells did not change their invasive phenotype (Fig. S6). Taken together, our data indicate that Zpo2 relies on ZBTB32 to down-regulate GATA3 and promote cellular invasion.

Zpo2 Enhances in Vivo Tumor Growth and Metastasis. We next analyzed the effect of *Zpo2* on tumor growth and metastasis in vivo in orthotopic transplants formed after injecting 2.5×10^5 PyMT control cells or overexpressing full-length V5-tagged *Zpo2* cells into the mammary fat pads of 6-wk-old female recipient mice. We also transplanted 2.5×10^5 control or shZpo2 PyMT tumor cells in separate sets of mice. At 6 wk posttransplantation, we verified Zpo2 expression in the transplanted tumors using Zpo2- and V5-tag-specific antibodies via immunostaining (Fig. S7), and determined tumor size and weight (Fig. 4A and B). Overexpression of *Zpo2* resulted in larger tumor size compared with control, whereas knockdown of *Zpo2* resulted in smaller tumor size. Using PyMT-specific primers to perform qRT-PCR analysis on recipient lung tissue samples, we observed that PyMT cells expressing *Zpo2* exhibited enhanced tumor metastasis to the lungs (Fig. 4C), whereas knockdown of *Zpo2* in PyMT tumor cells rendered them less metastatic (Fig. 4C). Histological analysis of recipient lungs demonstrated more metastatic colonies in mice transplanted with PyMT tumor cells overexpressing *Zpo2* (Fig. 4D). In addition, when we transplanted 2.5×10^5 control or ZPO2-overexpressing human T47D cells orthotopically in 6-wk-old nude mice, at 4 wk after transplantation, T47D cells that overexpressed ZPO2 also generated larger tumors compared with controls (Fig. S8).

In further support of our in vivo tumor data, we used patient-derived xenograft (PDX) tumor models to examine ZPO2 levels in breast cancer. We compared ZPO2 levels in a nonmetastatic PDX line (SU2C-17) with those in PDX lines exhibiting tumor metastasis (i.e., HCI-001, HCI-004, and HCI-010) (36, 37). qRT-PCR analysis revealed higher ZPO2 expression in the more aggressive tumors (Fig. 4E). Interestingly, PDX tumor lines with higher ZPO2 expression also demonstrated lower GATA3 levels (Fig. 4F). Taken together, our in vivo data emphasize the importance of ZPO2 in modulating GATA3 and breast cancer development.

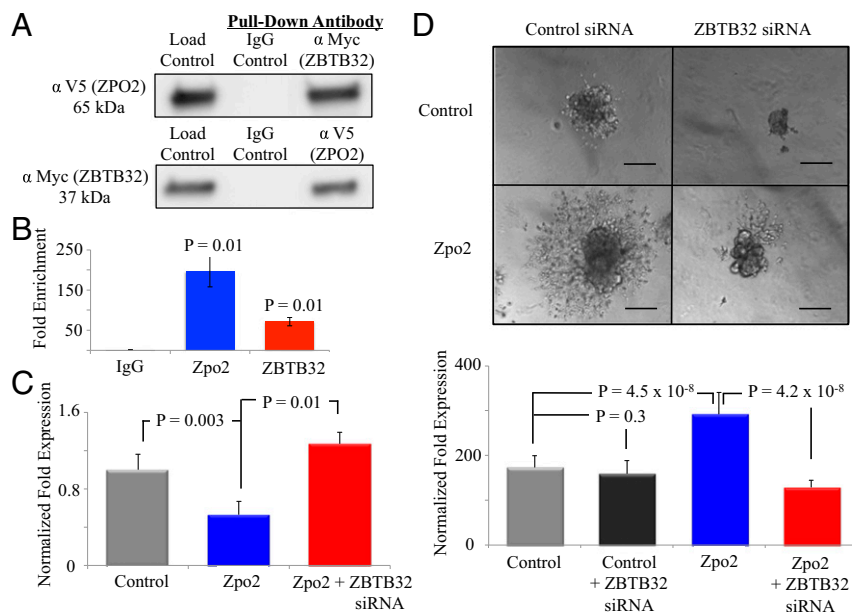


Fig. 3. Analysis of Zpo2 and ZBTB32 interaction. (A) Co-IP experiment indicating Zpo2 and ZBTB32 interaction. Eph4.9 cells were cotransfected with V5-tagged Zpo2 and Myc-tagged Zbtb32 constructs. Pull-down was performed with control IgG, anti-Myc (ZBTB32), or anti-V5 tag (ZPO2) antibodies. Western blot analysis for the presence of Zpo2 or ZBTB32 was performed with anti-V5 tag or anti-Myc antibodies, respectively. (B) ChIP analysis indicating the presence of Zpo2 and ZBTB32 on the *Gata3* promoter. qRT-PCR analysis was performed using primers specific to the *Gata3* promoter. $n = 4$. (C) qRT-PCR analysis for *Gata3* expression in Eph4.9 control or Eph4.9 Zpo2-overexpressing cells in the presence or absence of ZBTB32. Inhibition of Zbtb32 restored *Gata3* levels. (D) 3D Matrigel culture assay of control or Zpo2-overexpressing Eph4.9 cells. Inhibition of Zbtb32 interferes with cellular invasion mediated by Zpo2. (Scale bar: 150 μ m.)

Discussion

GATA3 has emerged as a prominent transcription factor required for the maintenance of mammary gland homeostasis. Loss of GATA3 is associated with aggressive breast cancer development. Whereas genomic mutations in *GATA3* lead to down-regulation or loss of GATA3 activity in approximately 14% of breast cancers, in our study we found that roughly 7% of tumors demonstrate *GATA3* mRNA down-regulation without any sequence mutation or alterations. Our analysis points to interplay between the transcriptional repressor Zpo2 and GATA3. Our in silico data suggest that Zpo2 resides in the same protein network as GATA3. We found that Zpo2 down-regulates GATA3 expression. Whereas Zpo2 and its related protein Zpo1 have high protein sequence similarities and Zpo1 induces aggressive breast cancer (21), modulation of GATA3 was specific to Zpo2 activity, given that overexpression of Zpo2, but not Zpo1, led to down-regulation of GATA3 in mammary cells. Thus, it appears that Zpo1 and Zpo2 are capable of contributing to breast cancer in nonredundant pathways.

We also characterized the relationship between *ZPO2* and *GATA3* in breast tumor samples segregated based on the presence of either WT or mutated *GATA3*. Interestingly, *ZPO2* transcript levels were higher in tumors with WT *GATA3* DNA, and higher *ZPO2* expression correlated with lower *GATA3* transcript levels in the same analyzed samples. Samples with high *ZPO2* expression also had low *ZPO1* transcript levels and vice versa. Moreover, patient tumors with higher *ZPO2* transcript levels had a poorer prognosis, again highlighting the importance of *ZPO2* in aggressive breast cancer.

GATA3 inhibits aggressive breast cancer through strict control and blockage of EMT-associated genes. Suppression of GATA3 levels could partially lift the inhibitory transcriptional control, thereby allowing for up-regulation of EMT-associated genes in the mammary cells. Given that down-regulation of *Gata3* via *Zpo2* coincided with up-regulation of EMT-associated genes and MMP9, these observations suggest that uncontrolled *Zpo2* expression results in the formation of aggressive tumors with more mesenchymal properties. Moreover, up-regulation of MMP9 activity also suggests that *Zpo2* could influence the microenvironmental remodeling that allows the invasive tumor cells to escape the main body of the tumor and begin to metastasize. Taken together, these data suggest that *Zpo2*

contributes to aggressive breast cancer development by exerting a variety of protumor activities through suppression of GATA3 in mammary cells.

Zpo2 translocates to the nucleus and functions as a transcriptional repressor, suggesting that Zpo2 can interact and form complexes with other proteins. However, because Zpo2 contains one zinc finger protein domain, it may lack DNA-binding ability. Using yeast two-hybrid analysis, we identified ZBTB32 as the most plausible candidate for targeting Zpo2 to the *Gata3* promoter sequence. In immune cells, during CD4⁺ T helper 1 (Th1) and T helper 2 (Th2) differentiation, *GATA3* expression is critical for Th2 cell fate commitment, whereas GATA3 activity must be blocked for proper Th1 differentiation (38). ZBTB32 plays a crucial role during Th1 and Th2 specification by negatively regulating GATA3 activity (35). ZBTB32 directly binds to the *GATA3* promoter sequence and inhibits the activation of GATA3 target genes. Our co-IP and ChIP analyses indicated that in mammary cells, Zpo2 and ZBTB32 form a complex targeted to the *Gata3* promoter. Intriguingly, the inhibitory effect of Zpo2 on GATA3 is ZBTB32-dependent. Knockdown of *Zbtb32* restored *GATA3* levels in cells overexpressing *Zpo2*. *Zpo2* overexpression led to the development of invasive colonies dispersed throughout the 3D Matrigel matrix, which was inhibited by knockdown of *Zbtb32*. *Zbtb32* knockdown restored the WT colony phenotype, even in the presence of *Zpo2* overexpression. Interestingly, loss of ZBTB32 did not alter the ability of Zpo1-expressing cells to form invasive colonies, lending further support to the idea that Zpo1 and Zpo2 function through non-redundant mechanisms. Our data suggest that ZBTB32 facilitates targeting of Zpo2 to *GATA3* promoter to down-regulate GATA3 target genes in breast cancer. Because GATA3 autoregulates itself (39, 40), repression of GATA3 transcriptional activity could result in an overall decrease in *GATA3* levels in mammary cells.

In vivo tumor transplant experiments further support the aggressive nature of *ZPO2* in breast cancer development. We found that overexpression of *Zpo2* increased tumor growth and aggressiveness in MMTV-PyMT and T47D tumor models. We also found elevated *ZPO2* expression in more aggressive breast cancer PDX tumor lines, coinciding with lower *GATA3* levels. Thus, the tumor-suppressive capability of the GATA3 pathway may be further enhanced once the restrictive influence of Zpo2

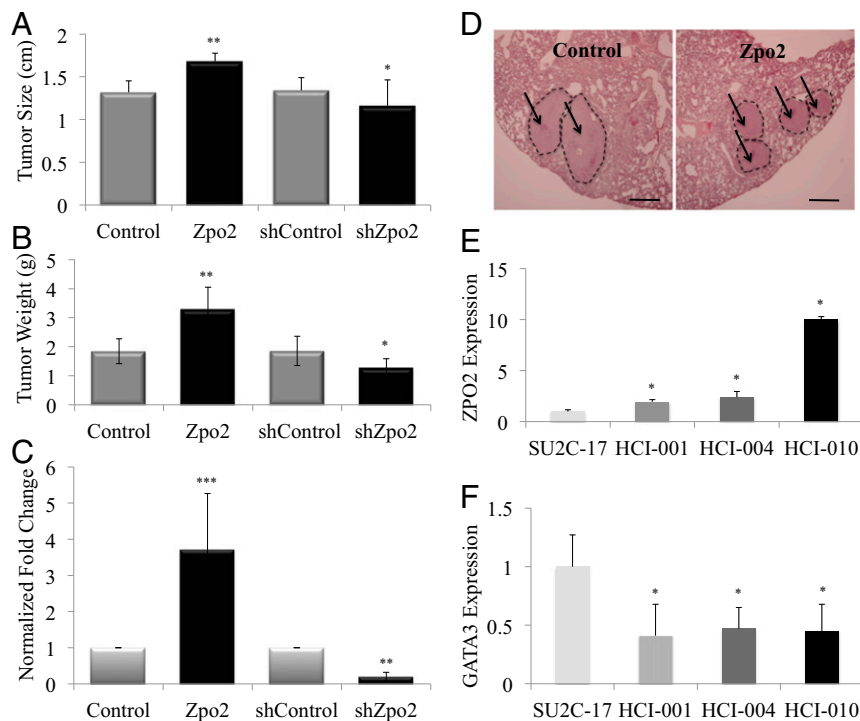


Fig. 4. Zpo2 promotes aggressive mammary tumor development. (A) Tumor measurement of orthotopically transplanted PyMT tumor cells. Zpo2 overexpression enhances tumor growth. Knockdown of Zpo2 reduces tumor size compared with controls. Zpo2-overexpressing tumors, $**P \leq 0.01$. Zpo2 knockdown tumors, $*P < 0.05$. $n = 10$. (B) Zpo2 overexpression leads to increased tumor weight, and knockdown of Zpo2 decreases tumor weight compared with controls. Zpo2-overexpressing tumors, $**P < 0.01$; Zpo2 knockdown tumors, $*P < 0.05$. $n = 10$. (C) qRT-PCR analysis using PyMT-specific primers for the presence of metastatic PyMT cells in the lung. Zpo2 enhances tumor metastasis. Knockdown of Zpo2 reduces the metastatic ability of PyMT cells to the lung. Zpo2-overexpressing tumors, $***P < 0.0001$. Zpo2 knockdown tumors, $**P < 0.001$. $n = 10$. (D) H&E staining of lung tissue. Overexpression of Zpo2 in PyMT cells enhances metastatic colony formation in the recipient lungs. The arrows point at metastatic colonies in the recipient lung. (Scale bar: 200 μm .) (E) qRT-PCR analysis of ZPO2 expression in PDX tumor lines. More aggressive tumor lines indicate higher ZPO2 expression. $*P < 0.001$. (F) qRT-PCR analysis of GATA3 levels in PDX tumor lines. GATA3 expression is reduced in more metastatic tumor lines. $*P < 0.001$.

on GATA3 is eliminated. This observation provides a promising avenue for designing new therapeutic strategies by focusing on the Zpo2/GATA3 signaling axis.

It is also noteworthy that Zpo2 could potentially interact with proteins involved in other regulatory pathways in the cells. Zpo2 interactions show a strong preference for components of the sumoylation pathway. Future studies may provide further insight into the importance of Zpo2 during translational modification, as well as its effect in eventual protein targeting and functioning. Moreover, Zpo2 and Zpo1 can form a complex, the significance of which merits future investigation. Our preliminary data indicate that Zpo1 and Zpo2 negatively control each other. Whether Zpo1/Zpo2 complex formation plays a role in negative regulation of these proteins remains unclear.

Early detection of aggressive tumors is a key step in the preparation and design of successful therapeutic strategies against cancer. We have identified ZPO2 as a negative regulator of GATA3, thus providing an alternative mechanism that results in a reduction in or perhaps even loss of GATA3 during breast cancer development. Overexpression of ZPO2 has been detected in breast cancer patients (28), and up-regulation of ZPO2 could supersede GATA3 down-regulation at much earlier time points during breast cancer development. In addition to modulating GATA3, we have shown that ZPO2 could alter multiple pathways, such as pFAK, Rac1/RhoA, and cell cycle-associated genes, in breast cancer (28). Therefore, our data suggest that ZPO2 could be considered an important predictive molecular marker for early detection of aggressive breast cancer.

Materials and Methods

Cell Culture and PDX Cell Lines. EpH4.9 cells were cultured in DME-H21 medium supplemented with 5% (vol/vol) FBS, insulin (5 $\mu\text{g}/\text{mL}$), and antibiotics. T47D tumor cells were cultured in RPMI medium 1640 supplemented with 10% (vol/vol) FBS, insulin, and antibiotics. MMTV-PyMT tumor cells were cultured in DME-H21 medium supplemented with 5% (vol/vol) FBS and antibiotics. The 3D Matrigel culture assay was performed as described previously (28).

Generation and propagation of PDX lines were done as described previously (36, 37). The nonmetastatic PDX line (SU2C-17) was generated from primary samples from a perineal lymph node provided by Dr. Hope Rugo. The tumor cells were negative for ER, PR, and HER2/neu. The tumor samples were propagated, and a PDX line was generated from these cells.

RNA Extraction, cDNA Synthesis, and qRT-PCR. RNA extraction was performed using TRIzol reagent (Invitrogen) following the manufacturer's protocol. cDNA synthesis was performed using iScript Reverse Transcription Supermix for qRT-PCR (Bio-Rad). qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with a Mastercycler ep realplex PCR instrument (Eppendorf).

qRT-PCR Primers and ZBTB32 siRNA. A list of these primers is provided in [SI Materials and Methods](#).

ChIP Assay. For ChIP analysis using endogenous ZPO2 and ZBTB32 proteins, EpH4.9 cells were grown to near-confluency in a 150-mm culture dish. ChIP analysis was performed using the ChIP-IT High-Sensitivity Kit (Active Motif) following the manufacturer's protocol. ChIP sample quality control was maintained with the ChIP-It Control qPCR Kit (Active Motif) and ChIP-It qPCR Analysis Kit (Active Motif). ZPO2 and ZBTB32 ChIP analyses were performed using anti-Zpo2 (Znf503) antibody (Sigma-Aldrich) and anti-ZBTB32 (ROG-M300) antibody (Santa Cruz Biotechnology). qRT-PCR

analysis for the *Gata3* promoter sequence was performed using two different primer sets: 5'-GGGTTTGGGTTGCAGTTTCCTGTG-3' (forward), 5'-GCGACGCAACTAAGGAGGTTCTA-3' (reverse) and 5'-CGCCAGATCTGTCAGTTCA-3' (forward), 5'-AGGAGAAACAGCGAGGGAAT-3' (reverse).

Co-IP. Eph4.9 cells were cultured in 10-cm culture dishes and cotransfected with 7 µg of V5-tagged full-length Zpo2 and 7 µg of Myc-tagged ZBTB32 using FuGENE6 transfection reagent (Promega). At 48 h posttransfection, cells were collected and co-IP was performed using a Universal Magnetic Co-IP Kit (Active Motif), followed by Western blot analysis.

Tumor Transplantation. All animal experiments were performed under approval of University of California San Francisco's Institutional Animal Care and Use Committee. Here 2.5×10^5 MMTV-PyMT cells from each stable cell lines were placed in 10 µL of Matrigel (Corning) and separately transplanted via intramammary injection using a Hamilton syringe in 6-wk-old syngeneic female FVB mice. Once tumors were palpable, tumors were measured regularly to avoid tumor growth above the allowable 2.0 cm. At 6 wk posttransplantation, animals were killed, and tumors and lungs were collected for analysis.

Immunostaining, Histology Analysis, and Antibodies. Tissues were fixed in 4% (vol/vol) paraformaldehyde overnight and then embedded in paraffin. Sections were cut (5–7 µm thickness) and treated following the standard protocol for H&E staining. Immunostaining of sections was performed using a sodium citrate antigen retrieval protocol. Sections were blocked with 5% (vol/vol) goat serum and 0.25% Triton X-100 for at least 1 h at room temperature. Primary antibodies were diluted in PBS containing 5% (vol/vol) goat serum and then incubated overnight at 4 °C. Sections were washed with PBS and incubated with secondary antibody for 1 h at room temperature. Following secondary antibody incubation, sections were washed with

PBS and mounted using Vectashield mounting medium containing DAPI (Vector Laboratories). A Nikon C1si confocal microscope was used to capture images. Image analysis was performed using ImageJ. The following anti- bodies were used for immunostaining and Western blot analysis: actin-HRP (Santa Cruz Biotechnology), Alexa Fluor 546 anti-mouse (Molecular Probes), GATA3 (R&D Systems), V5 mouse monoclonal antibody (Invitrogen), Zpo2 (Znf503; Sigma-Aldrich), and ZBTB32 (ROG; Santa Cruz Biotechnology).

MMP9. Eph4.9 control or Zpo2-overexpressing cells were lysed and loaded on 10% (wt/vol) Ready Gel Zymogram gel (BioRad) using Zymogram sample buffer (Bio-Rad). The gel was processed using Zymogram Renaturation Buffer (BioRad) for 30 min at ambient temperature, followed by overnight incubation in Zymogram Development Buffer (Bio-Rad). Gels were visualized with Coomassie Brilliant Blue R-250 (Bio-Rad) staining for 1 h at room temperature, followed by Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad).

Statistical Analysis. Statistical analysis was conducted using Graph Pad Prism 4 software. Statistical significance between two groups was calculated using Student's *t* test. A *P* value < 0.05 was considered to indicate statistical significance.

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