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The Transcriptional Repressor *ZNF503/Zeppo2* Promotes Mammary Epithelial Cell Proliferation and Enhances Cell Invasion*

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Background: *ZNF503/Zeppo2* regulates the development of the hindbrain and limbs.
Results: *Zeppo2* is a transcriptional repressor that promotes mammary cell proliferation and migration.
Conclusion: *Zeppo2* plays a role in mammary gland homeostasis.
Significance: Deregulation of *Zeppo2* could result in breast cancer development.

The NET (nocA, Nlz, elB, TLP-1) subfamily of zinc finger proteins is an important mediator during developmental processes. The evolutionary conserved zinc finger protein *ZNF503/Zeppo2* (zinc finger elbow-related proline domain protein 2, *Zpo2*) plays critical roles during embryogenesis. We found that *Zpo2* is expressed in adult tissue and examined its function. We found that ZPO2 is a nuclearly targeted transcriptional repressor that is expressed in mammary epithelial cells. Elevated *Zpo2* levels increase mammary epithelial cell proliferation. *Zpo2* promotes cellular invasion through down-regulation of *E-cadherin* and regulates the invasive phenotype in a RAC1-dependent manner. We detect elevated *Zpo2* expression during breast cancer progression in a MMTV-PyMT transgenic mouse model. Tumor transplant experiments indicated that overexpression of *Zpo2* in MMTV-PyMT mammary tumor cell lines enhances lung metastasis. Our findings suggest that *Zpo2* plays a significant role in mammary gland homeostasis and that deregulation of *Zpo2* may promote breast cancer development.

Proper embryogenesis relies on the strict regulation of key transcriptional regulatory genes. Recently, the members of the NET subfamily of zinc finger proteins that are related to the Sp family of transcription factors have been identified as important regulators during development. In particular, *ZNF503*/*Zeppo2* (zinc finger elbow-related proline domain protein 2, *Zpo2*/*Nolz1/Zfp503*) is a regulatory factor that is conserved across many species. In *Drosophila*, elbow, a protein closely related to ZPO2, serves as a transcriptional repressor that is involved in tracheal development (1). In zebrafish, the ZPO2 homologous protein Nlz2 (Znf503) is required for proper hindbrain development (2). In mice, expression of *Zpo2* is detected during limb and striatum development, highlighting the importance of *Zpo2* during organogenesis (3, 4). Although these examples demonstrate that *Zpo2* plays key roles in multiple developmental processes, the role of *Zpo2* in adult tissue (homeostasis) remains elusive.

Zpo2 is a member of a two-gene subfamily along with *ZNF703/Zpo1* (5) and is 54% identical (mouse) in sequence. Zpo1 is overexpressed because of genomic amplification in 25% of breast cancer cases and results in a poor prognosis (5–9). In mammary epithelial cells, overexpression of *ZPO1* induces cellular invasion and promotes breast cancer metastasis through alteration of p120-CATENIN isoform expression (5). Although *Zpo1* plays a significant role in mammary gland homeostasis, little is known about the role of *Zpo2* in the mammary gland.

In this report, we demonstrate that ZPO2 is a transcriptional repressor that is expressed in a diverse array of tissues, including the mammary gland. We show that, in mammary epithelial cells, ZPO2 regulates cellular migration and proliferation. Moreover, *Zpo2* expression is up-regulated during breast cancer progression, and overexpression of *Zpo2* leads to higher tumor seeding in recipient lungs during mammary tumor transplant experiments. Our findings identify *Zpo2* as a new candidate gene in the maintenance of mammary gland homeostasis.

EXPERIMENTAL PROCEDURES

Cell Culture—EpH4.9 mammary epithelial cells were cultured in DME-H21 medium supplemented with 5% fetal bovine serum, insulin (5 μg/ml), and antibiotics. MMTV-PyMT mammary tumor cells were cultured in DME-H21 medium supplemented with 5% fetal bovine serum and antibiotics. For three-dimensional Matrigel cultures, EpH4.9 or PyMT cells were placed in low-adhesion plates to form aggregates. The larger aggregates were broken into smaller aggregates by pipetting several times, and the aggregates were separated to form single cells by gravity. The cell aggregates were resuspended in cold Matrigel (BD Biosciences) and plated in 50-μl 12-well plates. The Matrigel was allowed to solidify at 37 °C for 30 min, and then DMEM:F12 with 1× Insulin-Transferrin-Selenium (Invitrogen) and 50 ng/ml EGF (Invitrogen) was added to the cultures.

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Two-dimensional Scratch Assay—5.0×10^5 cells/well were plated in a 6-well plate and allowed to form a confluent monolayer. A p200 pipette tip was used to create a "scratch" by scraping the monolayer in a straight line. The cells were washed once to remove the dislodged cell debris. Fresh medium was added to the cells, and cell migration was recorded over 72 h.

Immunostaining—Cultured cells on coverslips were washed twice with cold PBS and fixed with 4% paraformaldehyde for 20 min. The cells were washed twice with cold PBS and blocked with PBS containing 5% goat serum and 0.25% Triton X-100 for at least 1 h at room temperature. Primary antibody was diluted in PBS plus 5% goat serum and incubated overnight at 4 °C. Cells were washed with PBS, incubated with secondary antibody for 1 h at room temperature, and then washed with PBS and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Primary antibodies were as follows: phospho-histone H3 (R&D Systems), V5 mouse monoclonal antibody (Invitrogen), Znf503 (Sigma), Actin-HRP (Santa Cruz Biotechnology), E-Cadherin (BD Biosciences), RAC1 (Upstate Biotechnology), and RHOA (Thermo Scientific). Secondary antibodies were as follows: rabbit anti-mouse HRP (Abcam), goat anti-rabbit HRP (Abcam), Alexa Fluor 546 goat anti-mouse (Molecular Probes), and Alexa Fluor 488 goat anti-mouse (Molecular Probes).

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR—RNA isolation was performed using TRIzol reagent (Invitrogen) according to the protocol of the manufacturer. cDNA was generated using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad). qRT-PCR[3] was performed using a Mastercycler® ep realplex (Eppendorf) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). qRT-PCR primers were as follows: *RacI* primer, 5′-ACGGAGCTGTTGGTAAAACCT-3′ (forward) and 5′-AGACGGTGGGGATGTACTCTC-3′ (reverse); *RhoA* primer, 5′-AGCTTGTGGTAAGACATGCTTG-3′ (forward) and 5′-GTGTCCCATAAAGCCAACTCTAC-3′ (reverse); *Zpo2* primer, 5′-CAAGGTGCTGAAGATGCTGACG-3′ (forward) and 5′-GAGAGTTTGGAA GAAGG CGAAGG-3′ (reverse); and *E-cadherin* primer, 5′-AGCTCTAAGGACAGTGGTCAT-3′ (forward) and 5′-CAGTGCTTTACATTCCTCGGT-3′ (reverse).

Co-IP Experiments and Transcription Assays—EpH4.9 cells were cotransfected with 5 μg of V5-tagged *Zpo2* and 5 μg of *Grg4* FLAG-tagged in 10-cm culture dishes using FuGENE6 transfection reagent. 48 h post-transfection, the cells were collected, and co-IP was performed using the universal magnetic co-IP kit (Active Motif) as directed by the protocol. The co-IP samples were examined via Western blot analysis. Transcription assays were performed as described previously (5).

siRNA and RNA in Situ Hybridization of Mouse Tissues—2×10^5 cells/well in 6-well plates were transfected with 1 μg of *Rac1* siRNA (Santa Cruz Biotechnology) or 1 μg of *RhoA* siRNA (Invitrogen). Replicate experiments were used for three-dimensional culture analysis or expression analysis via qRT-PCR and Western blot analysis. RNA *in situ* hybridization on paraffin sections of mouse embryos was performed as described in Ref. 10. *In situ* hybridization on whole-mount embryo CD1 mouse embryos was performed as described in Ref. 11.

Flow Cytometry—To sort primary mouse mammary epithelial cells into basal and luminal fractions, mammary glands from adult virgin female mice were digested with collagenase. Organoids were collected by brief centrifugation and digested with trypsin to dissociate into single cells. The cells were stained with antibodies against CD49f, CD24, and lineage markers (CD45, CD31, and Ter119) (eBioscience, catalog nos. 17-0495-82, 48-0242-82, 12-0451, 11-0311-85, and 11-5921-82, respectively). Analysis and cell sorting were performed on a LSRII instrument (BD Biosciences) and analyzed using FlowJo (TreeStar) and FACS Diva software (BD Biosciences).

Rac1 Activity Assay—The Rac1/Cdc42 activation assay kit (Millipore) was used to measure RAC1 activity in EpH4.9 cell lysates.

Experimental Metastasis—1×10^4 control or *Zpo2*-overexpressing MMTV-PyMT tumor cells were transplanted into 6 week-old syngeneic FVB female mice via tail vein injection. 6 weeks post-transplant, the animals were harvested, and the lungs were analyzed for tumor seeding.

RESULTS

Zpo2 Expression Is Detected in Mammary Placode and Adult Mammary Epithelial Cells—We first examined *Zpo2* expression in normal mouse embryos. Whole-mount *in situ* hybridization showed that *Zpo2* expression in mammary placodes was evident as early as embryonic day 12.5 (E12.5) and was clearly detectable by E13.5 (Fig. 1*A*). We also detected *Zpo2* expression in embryonic lung and kidney (Fig. 1*B*).

Zpo2 expression persisted in adult mammary epithelial cells. We fractionated basal and luminal mammary epithelial cells from 6-week-old female mice using FACS (Fig. 2, *A* and *B*) and examined *Zpo2* expression via qRT-PCR (Fig. 2*C*). *Zpo2* mRNA was expressed in both mammary basal and luminal epithelial compartments. However, *Zpo2* was more abundant in basal cells compared with luminal cells (Fig. 2*C*). To validate our findings in human breast tissue, we utilized the Human Protein Atlas database to examine *Zpo2* expression in adult mammary glands. On the basis of histological analysis provided by the Human Protein Atlas database, *Zpo2* expression was apparent in both mammary epithelial compartments with higher levels in the basal cell layer than the luminal cell compartment. We also examined *ZPO1* expression via the Human Protein Atlas database. Interestingly, unlike *Zpo2*, *Zpo1* expression was detected equally in both mammary cell compartments.

In addition to the mammary gland, *ZPO2* expression was also present in other tissues. The Human Protein Atlas database indicated a high or medium *Zpo2* expression level in 52 of 81 analyzed adult tissues, including lung, kidney, and intestine. Overall, these observations suggest that *Zpo2* may play a crucial role in tissue homeostasis because its expression initiates during development and continues through adulthood in most tissues, including the mammary gland.

Zpo2 Is a Nuclear Protein That Functions as a Transcriptional Repressor—To investigate a functional role for Zpo2 in mammary epithelial cells, we first determined its cellular local-

[3] The abbreviations used are: qRT-PCR, quantitative RT-PCR; NET (nocA, Nlz, elB, TLP-1); co-IP, coimmunoprecipitation; FAK, focal adhesion kinase; SFK, Src family of kinases; CDK, cyclin-dependent kinase; E, embryonic day.

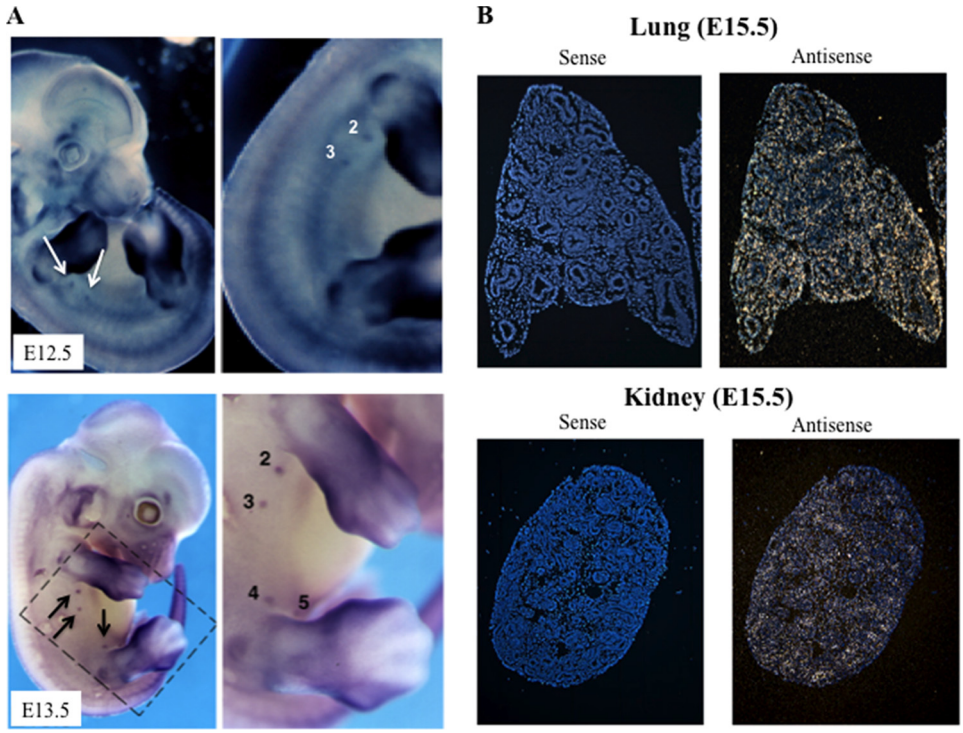

FIGURE 1. ***Zpo2* is expressed in embryonic tissues.** *A*, whole-mount RNA *in situ* hybridization of mouse embryos demonstrates *Zpo2* expression in E12.5 and E13.5 mammary placodes (*arrows*). *Zpo2* expression is present in mammary glands 2, 3, 4, and 5 of the E13.5 embryo. *B*, *in situ* hybridization indicating *Zpo2* expression in E15.5 lung and kidney.

ization. Immunostaining for endogenous *ZPO2* in EpH4.9 cells indicated that *ZPO2* is strictly targeted to the nucleus (Fig. 2*D*). Interestingly, analysis of the *Zpo2* sequence suggests that *Zpo2* lacks a *bona fide* nuclear localization signal. Therefore, we examined the protein domain(s) required for nuclear targeting of *ZPO2*. Similar to the mammalian *ZPO1* and the *ZPO2* ortholog in zebrafish Nlz2 (12), *ZPO2* contains an Sp-box domain on the N terminus and a single Zn^{2+} finger domain, followed by a proline-tyrosine (PT)-rich domain in the C-terminal region (Fig. 2*E*). We generated several truncated V5-tagged *Zpo2* constructs and examined the cellular localization of the expressed proteins. Although full-length V5-tagged *ZPO2* clearly localized to the nucleus, the *Zpo2* construct lacking the PT domain in the C terminus failed to localize to the nucleus and appeared cytoplasmic (Fig. 2*E*). These data suggest that the C-terminal domain plays a pivotal role in nuclear localization of *ZPO2*.

Transcriptional repression by the NET subfamily of zinc finger proteins may be mediated through its interaction with the corepressor *Groucho* (*Grg*) (1, 12). Analysis of the ZPO2 protein sequence suggests a predicted *Grg4* binding site on the N terminus flanking the Sp-Box protein domain. Therefore, a potential interaction between ZPO2 and GRG4 could promote transcriptional repression activity by ZPO2 in mammary cells. We found that *Grg4* is expressed in the developing mammary gland (5), and this prompted us to further investigate whether ZPO2 and GRG4 interact in mouse mammary epithelial cells. Co-IP of V5-tagged *Zpo2* and FLAG-tagged *Grg4* in EpH4.9 lysates followed by Western blot analysis using ZPO2 and GRG4 antibodies indicated that ZPO2 and GRG4 exist in a complex (Fig. 2*F*). Additionally, to demonstrate transcriptional activity of ZPO2, we generated a DNA construct containing full-length *Zpo2* coupled to a *Gal4* DNA-binding domain (*Zpo2-Gal4*). We examined the effect of *Zpo2-Gal4* on gene expression using a GAL4-luciferase reporter gene in mammary EpH4.9 cells. We observed that increasing levels of *Zpo2-Gal4* reduced the reporter activity, suggesting that ZPO2 functions as a transcriptional repressor in mammary cells (Fig. 2*G*).

To identify the protein domain(s) responsible for transcriptional repression, we generated several truncated versions of *Zpo2* coupled to a *Gal4* DNA-binding domain. Reporter analysis using the GAL4-luciferase system indicated that *Zpo2* possesses two transcriptional regulatory domains (Fig. 3), one at the N terminus and the second at the C terminus. Both the N-terminal Sp-box domain (Zpo2 construct 3, ΔZn^{2+} finger ΔPT rich) and the C-terminal PT rich domain (Zpo2 construct 11, PT-rich domain) were individually sufficient to diminish the luciferase reporter activity (Fig. 3).

Zpo2 Enhances Cellular Motility and Invasiveness—Our previous studies indicated that *Zpo1* enhances cellular motility in mammary epithelial cells (5). However, it is not clear whether *Zpo2* exerts similar effects on the mammary cells. To gain insight into the effect of *Zpo2* expression on cellular behavior in mammary epithelial cells, we generated EpH4.9 and MMTV-PyMT (PyMT) cell lines that stably expressed full-length *Zpo2* or *Zpo2*-shRNA (shZpo2). The expression level of *Zpo2* in EpH4.9 and PyMT stable lines was verified by qRT-PCR and Western blot analysis (Fig. 4, *A–D*).

To evaluate whether *Zpo2* enhances cell motility in mammary epithelial cells, we performed scratch assays. *Zpo2*-overexpressing cells were significantly more motile than the control cells (Fig. 4, *E* and *G*). The *Zpo2*-expressing cells were able to

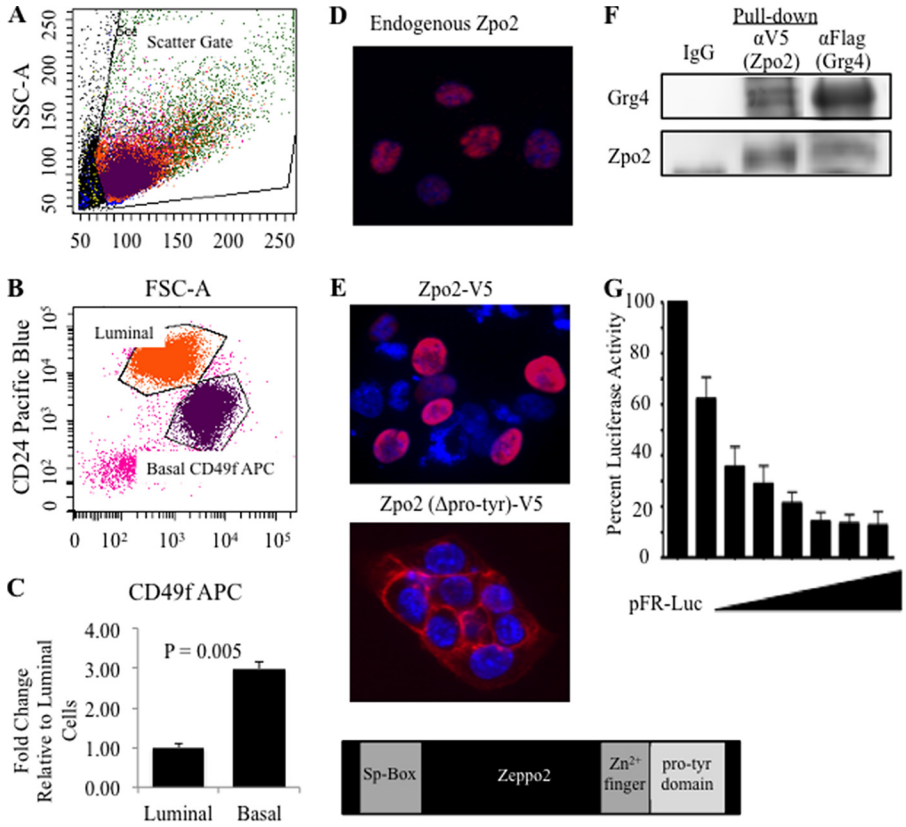

FIGURE 2. **Analysis of *Zpo2* expression in the adult mammary gland.** *A*, FACS plots showing the mammary epithelial sort strategy. The scatter plot indicates live primary mammary cells. *SSC*, side scatter; *FSC*, forward scatter. *B*, basal and luminal epithelial cells were fractionated on the basis of CD24 and CD49f surface markers. Allophycocyanin (APC). *C*, real-time qRT-PCR analysis of *Zpo2* expression in fractionated mammary epithelial cells showing that *Zpo2* expression is ~3-fold higher in basal cells compared with luminal epithelial cells. *D*, immunostaining using anti-ZPO2 antibody. Endogenous ZPO2 is localized to the nucleus. *Blue*, DAPI nuclear staining; *red*, ZPO2. *E*, immunostaining using an anti-V5 antibody indicates nuclear localization of ZPO2-V5. In the absence of the PT domain, ZPO2 fails to localize to the nucleus. *Blue*, DAPI nuclear staining; *red*, ZPO2. *F*, Western blot analysis of a co-IP experiment in EpH4.9 cells indicates ZPO2 and GRG4 interaction. *G*, GAL4-luciferase reporter assay. Increasing levels of ZPO2-GAL4 down-regulate the luciferase (*Luc*) reporter activity. *Error bars* represent mean ± S.E.

close the gap between the two invading fronts more efficiently than the control cells. Conversely, knockdown of *Zpo2* slowed the migration of cells, and the invading fronts did not close the gaps as efficiently as the control cells (Fig. 4, *F* and *H*).

Moreover, to further validate the effect of *Zpo2* on cell motility and migration, we performed three-dimensional culture assays. We placed aggregates of control and *Zpo2*-overexpressing cells in three-dimensional Matrigel cultures and examined their colony morphology. Although non-tumorigenic EpH4.9 control cells were non-invasive and formed tight spherical colonies, *Zpo2*-overexpressing cells were very invasive and dispersed throughout the Matrigel matrix 7 days post-seeding (Fig. 5*A*). Conversely, targeted knockdown of *Zpo2* inhibited the invasive phenotype, and shZpo2 cells formed colonies that were significantly more compact than the control cells 7 days post-seeding (Fig. 5*A*). PyMT mammary tumor cells are highly invasive, and, 7 days post-seeding, the control cells form many invading processes through the Matrigel matrix. However, overexpression of *Zpo2* in PyMT cells expedited the invasive phenotype. Within 3 days post-seeding, the *Zpo2*-overexpressing PyMT cells demonstrated the full invasive phenotype through the Matrigel matrix (Fig. 5*B*). Interestingly, down-regulation of *Zpo2* completely inhibited the cellular motility, and the colonies failed to demonstrate the invasive phenotype (Fig. 5*B*). These data indicate that *Zpo2* promotes cellular invasion and migration.

Induction of Cellular Invasion by Zpo2 Is Mediated through Down-regulation of E-cadherin and Depends on Src/Rac1 Activity—We next asked whether altered cell-cell adhesion contributed to the effects of *Zpo2* on cellular migration. Activation of focal adhesion kinase (FAK/PTK2) and down-regulation of *E-cadherin* play a pivotal role in initiating cellular migration and metastasis (13, 14). We first examined the activation state of FAK in *Zpo2*-expressing cells by performing immunostaining using phospho-FAK (pFAK) antibody. *Zpo2*-overexpressing cells demonstrated high levels of pFAK compared with the control (Fig. 6*A*). Additionally, Western blot analysis indicated that, in response to *Zpo2* expression, both EpH4.9 and PyMT cells increase FAK activity, as demonstrated by higher levels of pFAK staining (Fig. 6*B*). Conversely, down-regulation of *Zpo2* decreased FAK activity, as demonstrated by lower pFAK staining in the Western blot analysis (Fig. 6*B*). The immunostaining and Western blot analysis suggest a correlation between *Zpo2* expression levels and FAK activation in mammary cells.

Loss of cell-cell adhesion because of down-regulation of *E-cadherin* results in cell dispersal (15–18). We asked whether higher pFAK staining in cells expressing *Zpo2* correlates with

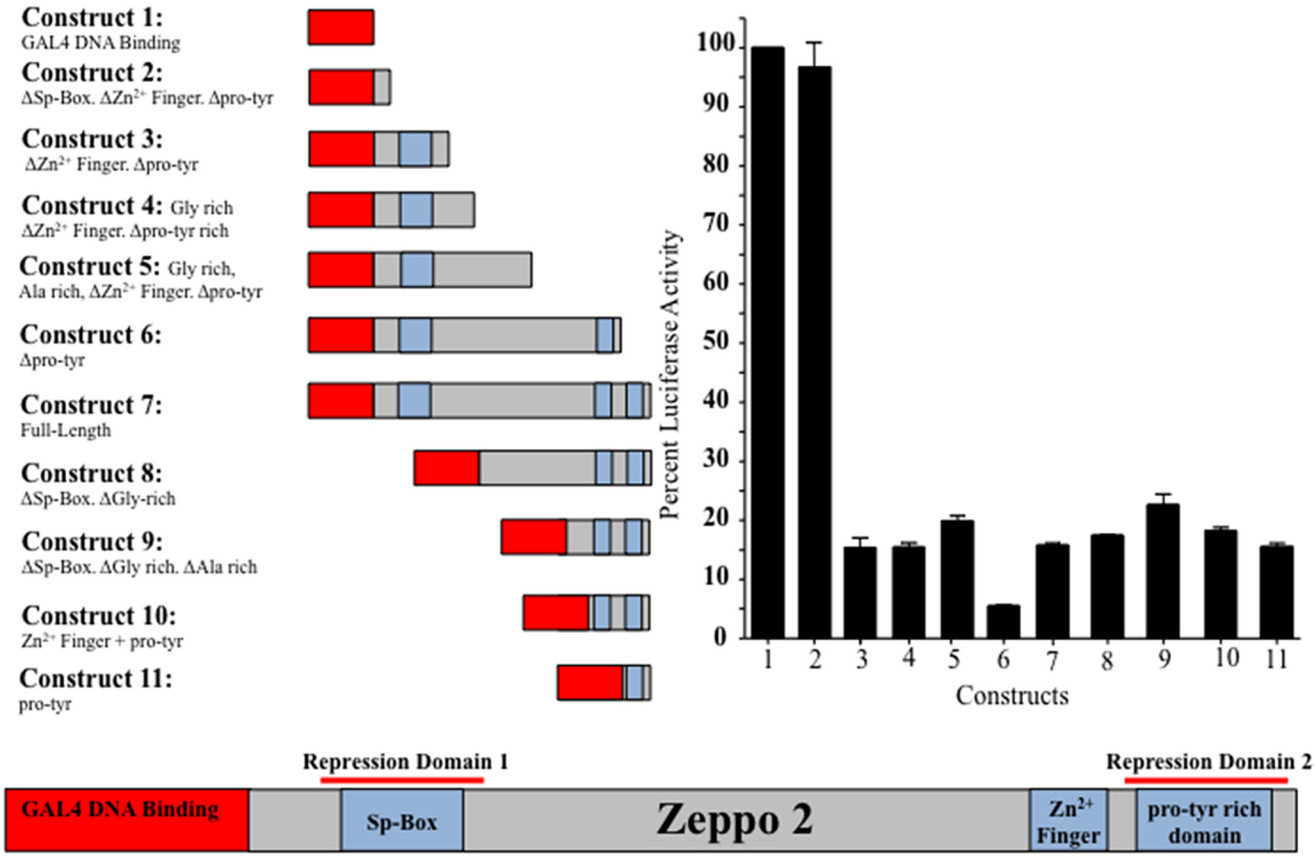

FIGURE 3. **Characterization of *Zpo2*-repressive domains.** Shown is a GAL4-luciferase reporter analysis of Zpo2 constructs. In total, 11 constructs were generated. The constructs increased in size from N-terminal to C-terminal or decreased in size from N-terminal to C-terminal. All constructs contained either the Sp-box domain or PT and repressed reporter activity. Interestingly, construct 3 containing only the Sp-box domain and construct 11 with only the PT domain were individually sufficient to inhibit reporter activity. *Error bars* represent mean ± S.E.

reduced *E-cadherin* levels. Immunostaining for E-cadherin in the EpH4.9 and PyMT stable cell lines indicated a decrease in overall E-cadherin staining in *Zpo2*-overexpressing cells compared with the control (Fig. 6*C*). Similarly, Western blot analysis indicated a reduction in *E-cadherin* expression in *Zpo2*-expressing cells compared with the control (Fig. 6*D*). However, down-regulation of *Zpo2* via shRNA enhanced E-cadherin levels in both EpH4.9 and PyMT cells, as detected by Western blot analysis (Fig. 6*D*). Overall, our data suggest that overexpression of *Zpo2* initiates cellular motility in part by activation of FAK and down-regulation of *E-cadherin* in mammary cells.

FAK activation influences the activity of Src family of kinases (SFK) as well as the RhoA GTPases RAC1 and RHOA (19–22). Moreover, a dynamic interaction and cross-regulation between E-cadherin, RAC1, and RHOA is well documented (3, 23–27) Because *Zpo2* activates FAK and down-regulates *E-cadherin*, we next determined whether the cellular invasiveness mediated by *Zpo2* is RAC1- and RHOA-dependent. Interestingly, overexpression of *Zpo2* in EpH4.9 cells up-regulated RAC1 and SFK protein levels and down-regulated the RHOA expression level (Fig. 7*A*). Moreover, in response to *Zpo2* overexpression, RAC1 demonstrated higher activity levels, as detected by presence of more GTP-bound RAC1 species compared with control EpH4.9 cells (Fig. 7*A*). Next we examined loss of *Rac1* or *RhoA* and the subsequent effect on cellular motility in *Zpo2*-overexpressing cells. We knocked down *Rac1* and *RhoA* separately in *Zpo2*-expressing EpH4.9 cells utilizing siRNA-mediated gene silencing (Fig. 7*B*). Although *Rac1* knockdown disrupted *Zpo2*-induced cellular invasion (Fig. 7*C*), RhoA knockdown dramatically increased the invasive phenotype exerted by *Zpo2* (Fig. 7*C*). These data suggest that the cellular invasion induced by *Zpo2* relies on the activity of RAC1 and inhibition of RHOA. Western blot analysis indicated that *Zpo2* up-regulates SFK expression levels. Because SFKs regulate Rho GTPase activity, we also inhibited SFK activity and examined cellular invasion in EpH4.9-Zpo2 cells. Inhibition of SFK by SU6656 also disrupted cellular invasion in *Zpo2*-expressing cells (Fig. 7*D*). Overall, our data indicate that *Zpo2* promotes cellular migration through activation of FAK and down-regulation of *E-cadherin*. Moreover, cellular motility induced by *Zpo2* is SFK/RAC1-dependent.

Zpo2 Promotes Mammary Cell Proliferation—To determine whether *Zpo2* influences the proliferative state of mammary epithelial cells, we first examined candidate cell cycle-associated gene expression levels in response to *Zpo2* expression. Induction of *Zpo2* in EpH4.9 cells increased cyclin D2 and cyclin E levels, as indicated by Western blot analysis (Fig. 8*A*). We also examined the levels of the cyclin-dependent kinases (Cdks) *Cdk1* and *Cdk2*. qRT-PCR analysis of T47D cells indicated that *Zpo2* expression elevates *Cdk1* and *Cdk2* levels compared with control cells (Fig. 8*B*). Additionally, *Zpo2*-overexpressing cells showed a 40-fold increase in *Ki67* levels by

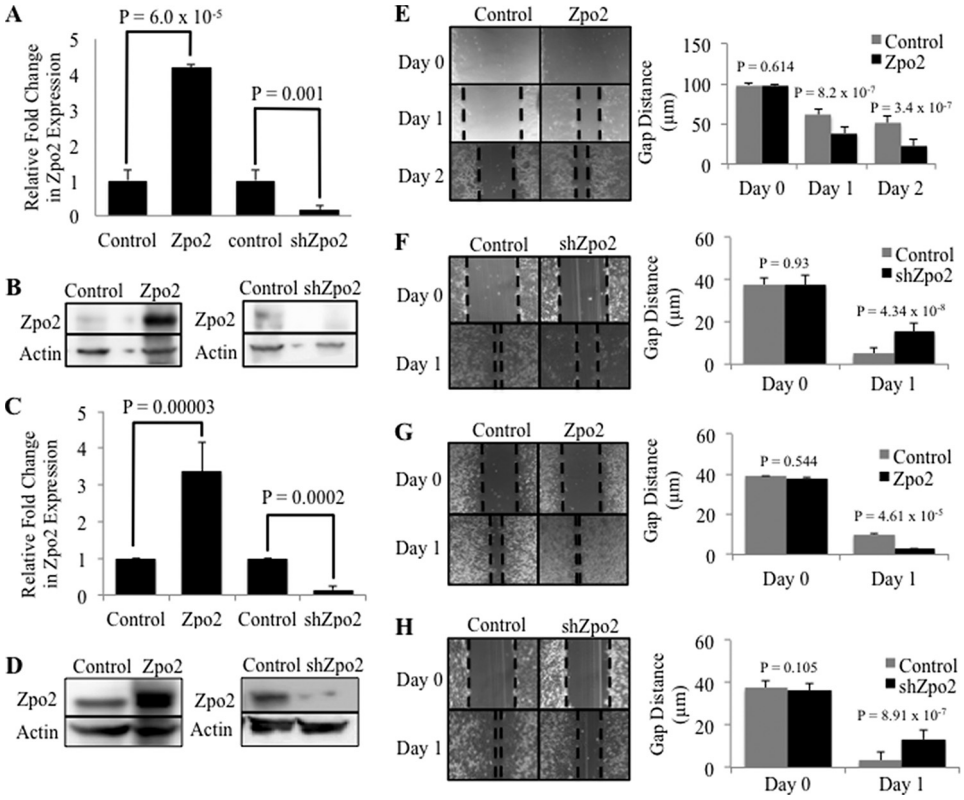

FIGURE 4. **Overexpression of *Zpo2* in EpH4.9 stable cells enhances cellular motility.** *A*, qRT-PCR analysis for *Zpo2* expression in EpH4.9 stable cell lines. *B*, Western blot analysis indicating ZPO2 expression in EpH4.9 control, EpH4.9-Zpo2, and EpH4.9-shZpo2 stable cell lines. *C*, analysis of *Zpo2* expression via qRT-PCR in PyMT mammary tumor stable cell lines. *D*, Western blot analysis for ZPO2 expression in PyMT stable cell lines. *E*, scratch assay. *Zpo2*-overexpressing EpH4.9 cells are more motile than control cells. Time lapse images indicate that *Zpo2*-overexpressing cells close the gap between the invading fronts significantly faster than control cells. *F*, down-regulation of *Zpo2* in EpH4.9 cells impedes cellular migration, as demonstrated in the two-dimensional scratch assay. *G*, PyMT stable cell lines. Overexpression of *Zpo2* significantly increases cellular migration in the two-dimensional scratch assay. *H*, scratch assay demonstrating that the knockdown of *Zpo2* expression in PyMT mammary tumor cells significantly slows cellular motility compared with control cells.

qRT-PCR, whereas targeted knockdown of *Zpo2* via shRNA significantly reduced *Ki67* levels (Fig. 8*C*). Moreover, immunostaining analysis for phospho-histone H3 (pH3) indicated that *Zpo2* indeed increased proliferation in EpH4.9 and PyMT cells (Fig. 8, *D* and *E*). Collectively, our data demonstrate that *Zpo2* promotes mammary cell proliferation.

Zpo2 Expression Is Elevated in Mammary Tumors, and Up-regulation of Zpo2 Enhances Tumor Seeding—Abnormal cellular proliferation results in tumorigenesis. Our data indicate that *Zpo2* increases mammary cell proliferation. Additionally, *in silico* analysis of ZPO2 expression in human cancer tissues using the Human Protein Atlas database indicated an association between *ZPO2* deregulation and tumor development in multiple tissues, including breast, colon, and lungs (Fig. 9*A*). Accordingly, we asked whether the *Zpo2* expression level changes during mammary tumor progression. We utilized the well established MMTV-PyMT transgenic mouse model to analyze *Zpo2* expression at several stages of mammary tumor development. We obtained tumors at 6 weeks (premalignant/hyperplasia), 12 weeks (early malignant), and 18 weeks (late carcinoma). Comparison of *Zpo2* expression in age-matched WT mammary gland and MMTV-PyMT tumors clearly indicated that *Zpo2* expression increases during mammary tumor progression and remains elevated throughout the late carcinoma stage (Fig. 9*B*). Western blot analysis for ZPO2 expression in age-matched WT mammary gland and late carcinoma stage MMTV-PyMT tumors also indicated much higher ZPO2 levels in the tumor cells (Fig. 9*C*). To further evaluate the importance of *Zpo2* in breast cancer development, we used *Zpo2*-overexpressing PyMT cell lines for tumor transplant experiments. In an experimental metastasis protocol, we transplanted 5.0×10^4 control or PyMT-Zpo2 cells via tail vein injection. The ability of cells to seed metastases in recipient lungs was analyzed 6 weeks post-transplant. qRT-PCR analysis using PyMT-specific primers indicated that *Zpo2*-overexpressing cells had 3-fold more seeding ability than the control cells (Fig. 9*D*). The *in silico* and *in vivo* analysis of *Zpo2* expression in mammary tumors suggests a relationship between elevated *Zpo2* expression levels and breast cancer progression and tumor development.

DISCUSSION

In this study, we determined that *Zpo2* is a new candidate gene that plays a role in mammary gland homeostasis. Our data indicate that *Zpo2* is expressed in both the developing and adult mammary gland. Expression of *Zpo2* in mammary placodes is detected as early as E12.5 and continues to persist throughout adulthood. In the normal adult mouse mammary gland, *Zpo2* is highly expressed in the basal cell compartment and, to a lesser extent, in terminally differentiated luminal epithelial cells. Our observation suggests that Zpo2 may play a role in mammary epithelial cell differentiation. Future studies should determine

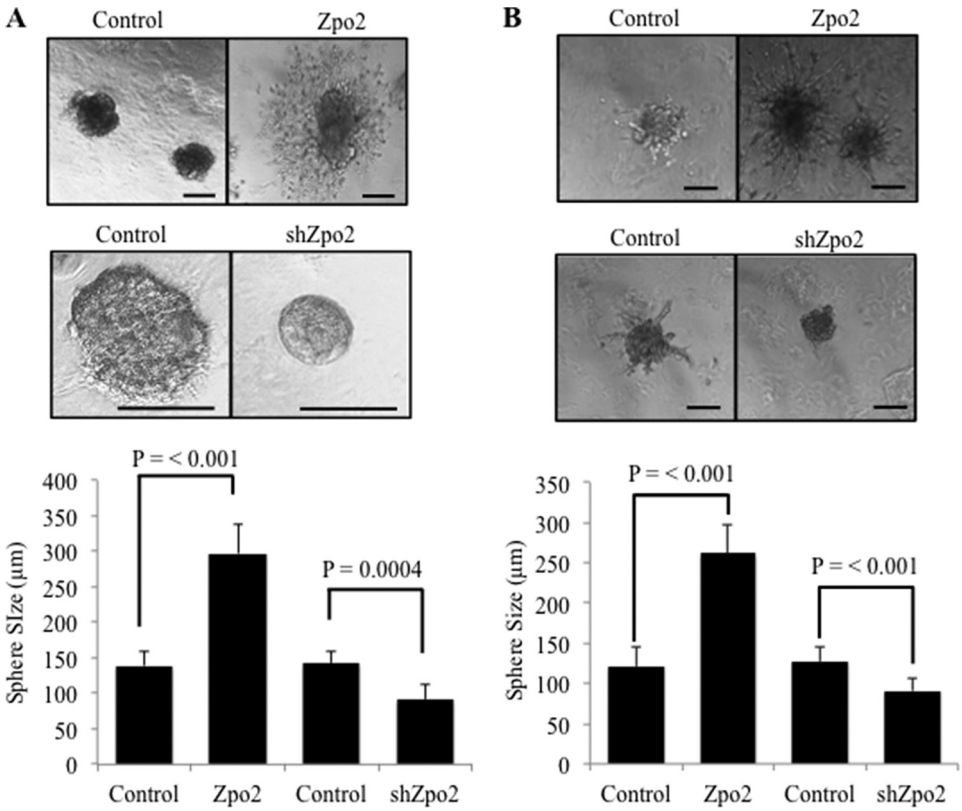

FIGURE 5. ***Zpo2* enhances cellular migration and invasion in three-dimensional Matrigel culture assays.** *A*, three-dimensional Matrigel culture assay 7 days post-seeding. Overexpression of *Zpo2* in EpH4.9 cells promotes cellular invasiveness through a Matrigel matrix, whereas *Zpo2* knockdown results in the formation of more compacted colonies. *Scale bars* = 100 μm. *B*, analysis of cellular invasion in three-dimensional Matrigel cultures. 3 days post-seeding, *Zpo2*-overexpressing PyMT cells demonstrate a significant invasive phenotype. Knockdown of *Zpo2* in PyMT disrupts the cellular invasion in PyMT tumor cells. *Scale bars* = 100 μm.

whether ZPO2 drives basal cell as opposed to luminal cell differentiation. It is possible that higher *Zpo2* expression levels maintain basal cell lineage and, because mammary epithelial cells differentiate to luminal cells, *Zpo2* levels are down-regulated. Additionally, we found that *Zpo2* is expressed in other branched tissues, such as lung and kidney during development and adulthood. It is possible that *Zpo2* may possess a common theme in other branched organs during development and adulthood. It will be interesting to investigate whether *Zpo2* also plays a role in the differentiation state of other branched organs and their cellular motility and proliferation.

We demonstrated that, in mammary epithelial cells, ZPO2 is a nuclear protein that functions as a transcriptional repressor. Although ZPO2 lacks a nuclear targeting sequence, it is interesting that its expression was only detected in the nucleus. We discovered that the PT-rich domain in the C terminus plays a pivotal role in targeting ZPO2 to the nucleus. However, the mechanism underlying ZPO2 translocation to the nucleus is not clear. It is very likely that ZPO2 interacts with other nuclearly targeted proteins and that this interaction facilitates ZPO2 nuclear localization. We also detected two transcriptional repressor domains in ZPO2. The region containing the Sp-box domain in the N terminus and the PT-rich domain in the C terminus were both capable of independently exerting transcriptional repression. Moreover, we also identified GRG4 as a ZPO2 binding partner in mammary epithelial cells. Sequence analysis of *Zpo2* indicates a predicted *Grg4* binding site flanking the Sp-box protein domain. Therefore, the transcriptional repression exerted by the truncated *Zpo2* clones containing only the Sp-box domain may be mediated through ZPO2/GRG4 interaction. However, we cannot rule out that other ZPO2-interacting partners may also promote ZPO2-mediated transcriptional inhibition. For example, in zebrafish, Nlz1 (*Zpo2* ortholog) is potentially able to bind HDAC1 and HDAC2 indirectly through Groucho to drive transcriptional repression. It is not clear how the C terminus region represses transcription. In the future, it would be valuable to identify additional ZPO2-interacting partners to provide insights into the mechanisms of subcellular targeting and transcriptional regulation exerted by ZPO2.

We observed that *Zpo2* overexpression enhances mammary epithelial cell motility and invasiveness. Overexpression of *Zpo2* in EpH4.9 or PyMT cells enhanced cellular motility and invasiveness in both two- and three-dimensional cultures. Conversely, knockdown of *Zpo2* levels in either EpH4.9 or PyMT cells reduced cellular motility and invasiveness compared with control cells. Interestingly, the invasive phenotype driven by *Zpo2* coincided with elevated FAK activity. FAK activation through phosphorylation elicits a variety of cellular activity, including the induction of cellular migration and enhanced tumor metastasis. Moreover, FAK activation reduces *E-cadherin* levels (13, 14). We also showed that, in response to *Zpo2* overexpression, *E-cadherin* expression levels diminished. Down-regulation of *E-cadherin* by *Zpo2* could either be the

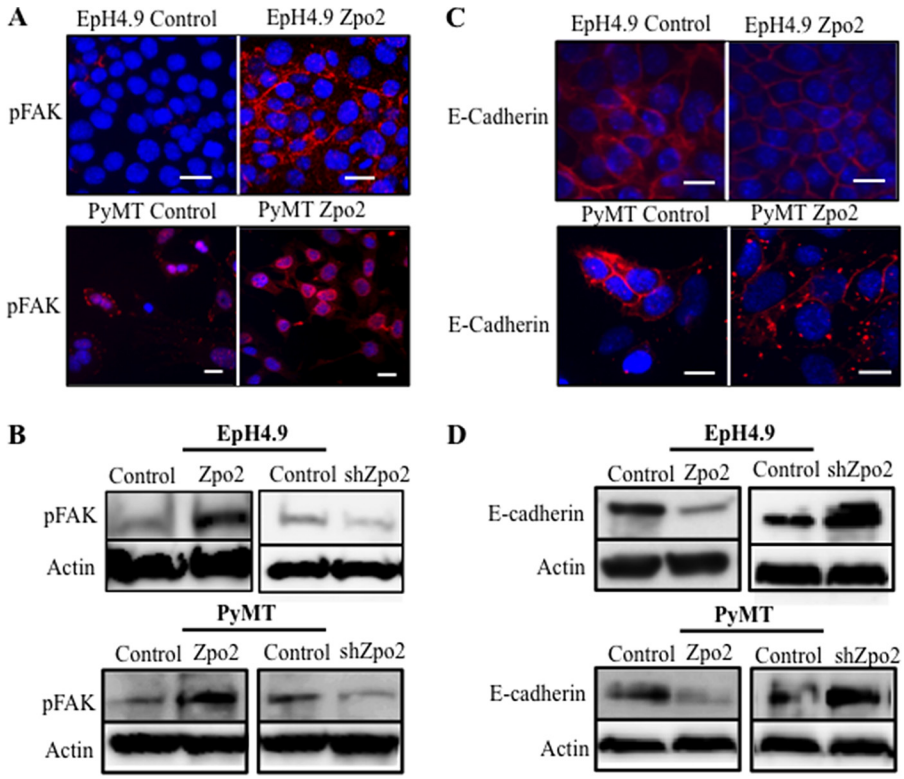

FIGURE 6. ***Zpo2* induces FAK activity and down-regulates *E-cadherin* expression levels.** *A*, immunofluorescence indicating pFAK staining in EpH4.9 and PyMT stable cell lines. Overexpression of *Zpo2* induces FAK activity. *Blue*, DAPI nuclear staining; *red*, pFAK. *Scale bars* = 20 μm. *B*, Western blot analysis indicating higher pFAK levels in EpH4.9 Zpo2 and PyMT Zpo2 cells than in control cells. Down-regulation of *Zpo2* via shRNA results in lowering of pFAK staining, as detected in the Western blot analysis. *C*, immunofluorescence analysis of E-cadherin in EpH4.9 and PyMT cells showing that overexpression of *Zpo2* down-regulates E-cadherin levels. *Blue*, DAPI nuclear staining; *red*, E-cadherin. *Scale bars* = 20 μm. *D*, Western blot analysis indicating E-cadherin levels in EpH4.9 and PyMT stable cell lines. Overexpression of *Zpo2* lowers E-cadherin levels compared with the control. Down-regulation of *Zpo2* results in elevation of E-cadherin expression compared with control cells.

outcome of direct transcriptional repression of *E-cadherin* by ZPO2 or, alternatively, the result of a secondary effect through FAK activation. Nevertheless, reduction of *E-cadherin* levels in response to *Zpo2* enhances cellular motility through loss of cell-cell adhesion. Moreover, FAK targets SFKs and influences RAC1 and RHOA activity (19–22). Here we showed that cellular invasion initiated by *Zpo2* depends on the activity of the Rho GTPases RAC1 and RHOA. We demonstrate that, in response to *Zpo2* expression, SFK and RAC1 protein levels increase. However, *Zpo2* overexpression led to decreased RHOA expression. Additionally, we showed that *Zpo2* not only increases RAC1 levels but also enhances RAC1 activity. We observed more GTP-bound RAC1 (active form) in *Zpo2*-expressing cells compared with the control. We further showed the importance of RAC1/RHOA activity as a component of ZPO2-mediated cellular migration in knockdown experiments in *Zpo2*-overexpressing cells. Inhibition of *Rac1* disrupted the ability of *Zpo2* to promote cellular invasion in three-dimensional Matrigel cultures. Conversely, inhibition of *RhoA* exacerbated cellular invasion, suggesting that activation of RAC1 and inhibition of RHOA mediate cellular invasion caused by ZPO2.

We also demonstrated that cellular invasion mediated by *Zpo2*-overexpressing cells also depended on the presence of SFK activity. Inhibition of SFKs completely abrogated cellular invasion in EpH4.9-Zpo2 cells. These observations suggest that cellular motility induced by ZPO2 correlates with activation of FAK, up-regulation of SFK and RAC1 protein levels, and increased RAC1 activity. These changes, collectively, could lead to cytoskeletal changes that promote cellular migration. However, it is not clear how ZPO2 enhances FAK activity. Our preliminary data suggest that Zpo2 up-regulates β1-integrin expression (data not shown). Up-regulation of β1-integrin, in turn, could result in activation of FAK, resulting in enhanced cellular migration. Another possible mechanism may involve the up-regulation of TGFβ by ZPO2, resulting in activation of FAK in the affected cells. In the future, it would be worthwhile to examine the molecular mechanisms underlying the interplay between ZPO2, FAK, SFKs, and Rho GTPases.

In silico analysis suggested that deregulation of *ZPO2* could play a role in tumorigenesis. However, the mode of ZPO2 activity and the underlying mechanism exerted by ZPO2, resulting in tumor development, has not been reported to date. Loss of cell cycle control and overproliferation of cells are the hallmarks of tumor progression. We demonstrated that induction of *Zpo2* up-regulates various cell cycle-associated genes. In response to *Zpo2* expression, Cyclin D2, Cyclin E, *Cdk1*, and *Cdk2* levels were elevated. Moreover, *Zpo2*-expressing cells demonstrated higher proliferative activity, as seen by enhanced *Ki67* levels and increased pH3 staining. Collectively, our data suggest that deregulation of *Zpo2* could play a role in breast cancer. We used the MMTV-PyMT mouse tumor model, which exhibits all stages of mammary tumor progression that resembles human breast cancer. At 6 weeks of age, MMTV-

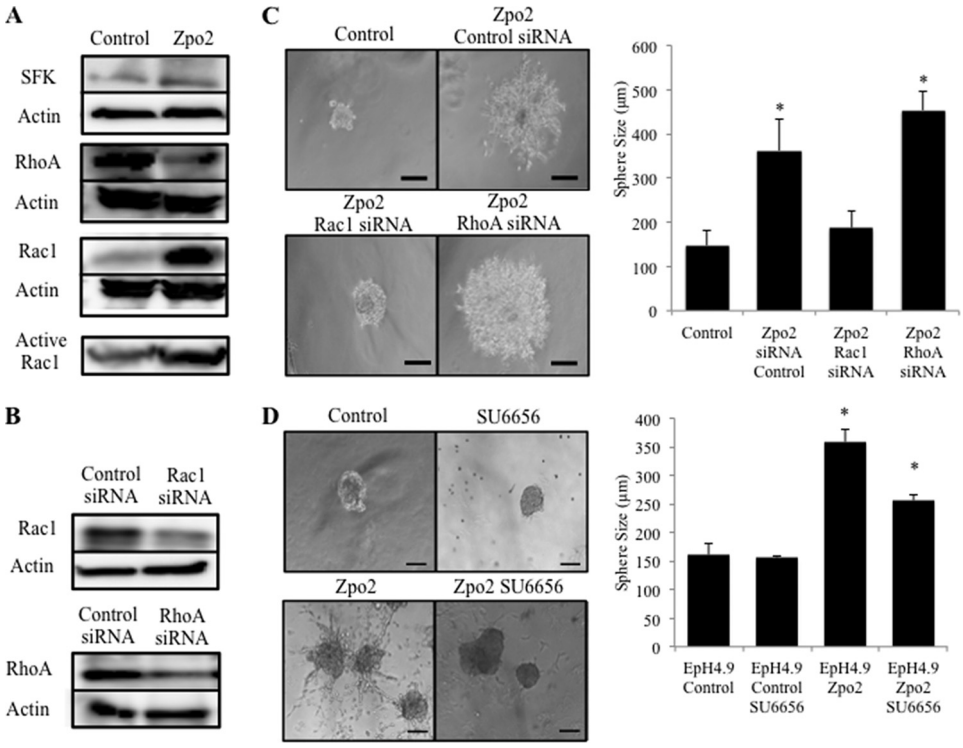

FIGURE 7. **Cellular invasion initiated by *Zpo2* is RAC1- and SFK-dependent.** *A*, Western blot analysis of EpH4.9 control and Zpo2 stable cell lines. Overexpression of *Zpo2* up-regulates SFKs and lowers RHOA protein levels. *Zpo2* overexpression elevates the RAC1 expression level and overall RAC1 activity compared with the control. *B*, Western blot analysis demonstrating down-regulation of RAC1 and RHOA by siRNA in EpH4.9 cells. *C*, three-dimensional Matrigel culture assay using EpH4.9 stable cell lines, 6 days post-seeding. *Rac1* knockdown disrupts *Zpo2*-mediated cellular invasion. Inhibition of *RhoA* promotes a more aggressive phenotype. *, $p < 0.05$. *Scale bars* = 100 μm. *D*, inhibition of SFK by SU6656 inhibits cell dispersal and migration mediated by *Zpo2* overexpression in EpH4.9 stable cell lines. *, $p < 0.05$. *Scale bars* = 100 μm.

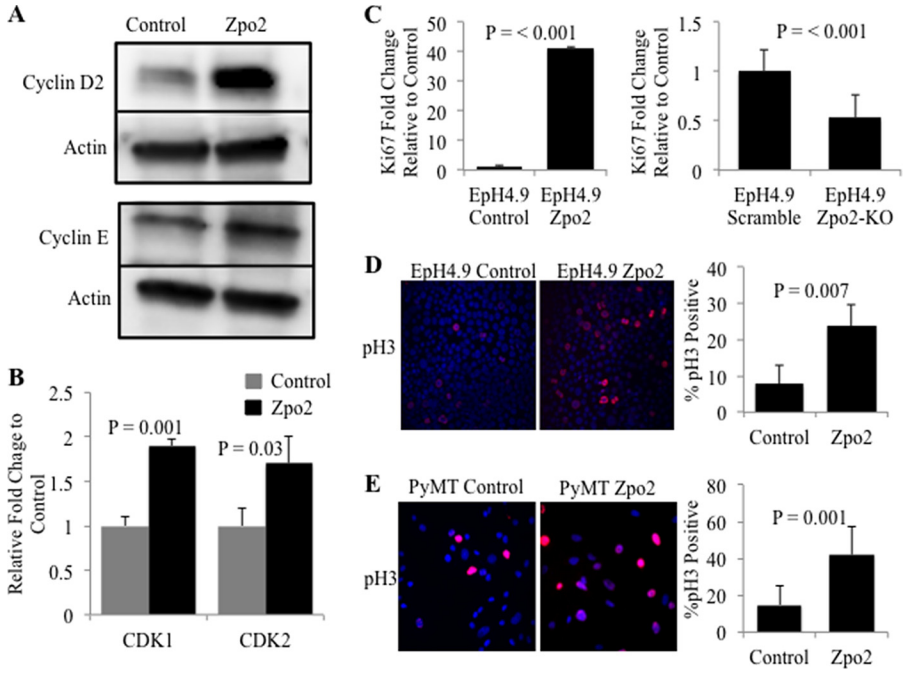

FIGURE 8. **Zpo2 promotes mammary cell proliferation.** *A*, Western blot analysis in EpH4.9 cells. Overexpression of *Zpo2* elevates cyclin D2 and cyclin E levels. *B*, qRT-PCR analysis of *CDK1* and *CDK2* in T47D cells. *ZPO2* up-regulates *CDK1* and *CDK2* levels compared with the control sample. *C*, qRT-PCR analysis of *Ki67* expression in EpH4.9 stable cell lines showing that *Zpo2* up-regulates *Ki67* levels and that targeted knockdown of *Zpo2* reduces *Ki67* levels. *D*, immunostaining for pH3 in EpH4.9 cells. *Blue*, DAPI; *red*, pH3. Quantitation of pH3-positive cells indicates that *Zpo2*-overexpressing cells are more proliferative and demonstrate a higher number of pH3-positive cells. *E*, immunostaining for pH3 in PyMT mammary tumor cells. *Blue*, DAPI; *red*, pH3. Overexpression of *Zpo2* increases cellular proliferation compared with the control.

PyMT mice demonstrate mammary gland hyperplasia or non-malignant tumor development. At this stage, we observed elevated Zpo2 levels compared with the age-matched WT control. *Zpo2* expression levels increased significantly at the early malignant stage (12 weeks) and remained elevated through the late carcinoma stage (18 weeks). Therefore, there is a direct

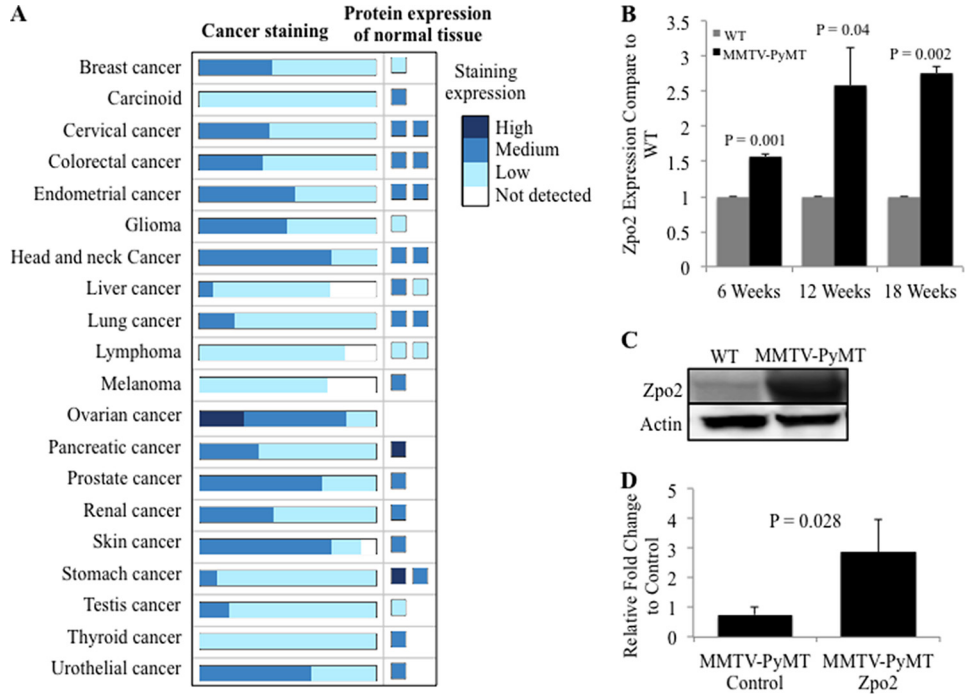

FIGURE 9. ***ZPO2* is expressed during breast cancer progression and enhances metastasis.** *A*, *in silico* analysis of *ZPO2* expression in human cancers using the Human Protein Atlas database. *ZPO2* overexpression is detected in multiple tumors, including breast cancer, colorectal cancer, and lung cancer. *B*, qRT-PCR analysis of *Zpo2* expression in age-matched WT mammary gland and MMTV-PyMT tumors. *Zpo2* is elevated in MMTV-PyMT tumors compared with the WT control. *C*, Western blot analysis of ZPO2 expression in an age-matched WT mammary gland MMTV-PyMT tumor (18 weeks). ZPO2 is highly elevated in MMTV-PyMT tumors compared with the control. *D*, effect of *Zpo2* on experimental metastasis assayed by qRT-PCR analysis via PyMT-specific primers. 5.0×10^4 control PyMT cells or *Zpo2*-overexpressing PyMT cells were transplanted via tail vein injection. Overexpression of *Zpo2* enhances PyMT tumor cell seeding in the recipient lungs compared with the control.

correlation between *Zpo2* expression level and MMTV-PyMT tumor progression. On the basis of our findings that elevated *Zpo2* could promote increased cell cycle activity and proliferation of mammary tumor cells, we asked whether higher *Zpo2* levels would enhance cellular metastasis and seeding. In an experimental metastasis assay, we observed that overexpression of *Zpo2* resulted in a much higher tumor seeding ability compared with the control. Therefore, *Zpo2* promotes cellular proliferation and enhances cellular migration, and these behaviors are clearly significant during tumor progression.

The closely related gene *Zpo1* is also important in mammary gland homeostasis and tumor development (5). We demonstrated that *Zpo2* functions similarly to *Zpo1*. Both genes function as transcriptional repressors and promote cellular proliferation and invasion in mammary cells. However, despite their similarities, there are differences between these two related genes. At the protein sequence level, ZPO1 and ZPO2 are 54% similar and possess similar protein domains. However, ZPO2 is slightly larger than ZPO1 (ZPO2, 647 amino acids; ZPO1, 594 amino acids). The difference in size and amino acid composition suggests that they may bind to different interacting partners to drive different cellular activities. Another noticeable difference between *Zpo1* and *Zpo2* is the establishment of their expression during mammary gland development. *Zpo1* expression is detected as early as E11.5 (5), 1 day earlier than *Zpo2*. Interestingly, although *Zpo1* is expressed at similar levels in both basal and luminal epithelial compartments, *Zpo2* demonstrated a biased expression in the basal cells. Moreover, ZPO1 is present in both the cytoplasm and nucleus (5), whereas ZPO2 appeared to be strictly a nuclear protein. Although it appears that ZPO1 and ZPO2 possess similar activity in mammary cells, it is not clear whether they function in a redundant manner or have separate roles. Therefore, deciphering the role of *Zpo1* and *Zpo2* and their importance in mammary cells is worth future investigations.

Taken together, our study emphasizes the importance of developmentally regulated genes in adult mammary tissue homeostasis. Our findings provide a better understanding of ZPO2 functionality and suggest that ZPO2 is a versatile regulator that plays a role during development and adult mammary epithelial cells and that its deregulation could lead to disruption of adult tissue homeostasis. Future studies should elucidate whether *Zpo2* serves as a candidate gene in initiating disease progression, such as in breast cancer.

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