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, a pupa 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 isofrom 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 ROHO (Thermo Scientific). Secondary antibodies were as follows: rabbit anti-mouse HRP (Abcam), goat anti-rabbit HRP (Abcam), Alexa Fluor 488 goat 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 was performed using a Mastercycler® ep replex (Eppendorf) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). qRT-PCR primers were as follows: Rac1 primer, 5′-ACGGAGCTTGGTGTAAACCT-3′ (forward) and 5′-AGACGGTGGGAGTCTCTC-3′ (reverse); RhoA primer, 5′-AGCTGTGTGTTAAGACATGCCTG-3′ (forward) and 5′-GTGTCCCAATAGGCSAATCCTC-3′ (reverse); Zpo2 primer, 5′-CAAGGTGCTGAAGATGCT-3′ (forward) and 5′-GGACAGTGTTATTCCGT-3′ (reverse); and E-cadherin primer, 5′-AGCTCTAAAGGAGATGCTCCG-3′ (forward) and 5′-CAGTGGTTACATTCTCGGT-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 para-}

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

fin 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 × 10^6 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. 1A). We also detected Zpo2 expression in embryonic lung and kidney (Fig. 1B).

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. 2C). 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. 2C). 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-
Immunostaining for endogenous ZPO2 in EpH4.9 cells indicated that ZPO2 is strictly targeted to the nucleus (Fig. 2D). 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 Zn²⁺/H11001 finger domain, followed by a proline-tyrosine (PT)-rich domain in the C-terminal region (Fig. 2E). 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. 2E). 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. 2F). 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. 2G).

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, ΔZn²⁺/H11001 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...
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. 5A). 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. 5A). 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. 5B). Interestingly, down-regulation of Zpo2 completely inhibited the cellular motility, and the colonies failed to demonstrate the invasive phenotype (Fig. 5B). 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. 6A). 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. 6B). Conversely, down-regulation of Zpo2 decreased FAK activity, as demonstrated by lower pFAK staining in the Western blot analysis (Fig. 6B). 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
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). 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). Additionally, Zpo2-overexpressing cells showed a 40-fold increase in Ki67 levels by
qRT-PCR, whereas targeted knockdown of Zpo2 via shRNA significantly reduced Ki67 levels (Fig. 8C). Moreover, immuno-
staining 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. 9A). 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. 9B). 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. 9C). 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 $5.0 \times 10^4$ 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. 9D). 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...
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
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-
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

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

The closely related gene \textit{Zpo1} is also important in mammary gland homeostasis and tumor development (5). We demonstrated that \textit{Zpo2} functions similarly to \textit{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 \textit{Zpo1} and \textit{Zpo2} is the establishment of their expression during mammary gland development. \textit{Zpo1} expression is detected as early as E11.5 (5), 1 day earlier than \textit{Zpo2}. Interestingly, although \textit{Zpo1} is expressed at similar levels in both basal and luminal epithelial compartments, \textit{Zpo2} demonstrated a biased expression in the basal cells. Moreover, \textit{Zpo1} is present in both the cytoplasm and nucleus (5), whereas \textit{Zpo2} appeared to be strictly a nuclear protein. Although it appears that \textit{Zpo1} and \textit{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 \textit{Zpo1} and \textit{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 \textit{Zpo2} functionality and suggest that \textit{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 \textit{Zpo2} serves as a candidate gene in initiating disease progression, such as in breast cancer.

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