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ZNF121 interacts with ZBRK1 and BRCA1 to regulate their target genes in mammary epithelial cells

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As one of the largest families of regulatory proteins in human cells, the Cys2-His2 (C2H2) zinc finger proteins have been reported to play important roles in development, differentiation, and human diseases [1]. However, due to their large numbers in the human genome, most of the zinc finger proteins are not well studied. The ZNF121 zinc finger protein is one such factor. A large-scale combination of tandem affinity purification with the mass spectral multidimensional protein identification technology (MudPIT) suggests that Zinc finger protein 121 (ZNF121) interacts with the MYC oncoprotein [2]. MYC has been demonstrated to be one of the most potent oncoproteins that participates in a broad range of cellular processes, such as cell proliferation, differentiation, stemness, apoptosis, cell migration, and metabolism [3,4] to regulate multiple aspects of tumorigenesis [5].

In our recent study, we used the yeast two-hybrid assay and other approaches to confirm that ZNF121 indeed interacts with MYC [6]. In addition, ZNF121 and MYC regulate each other’s protein expression or stability, and ZNF121 indeed regulates cell proliferation, apoptosis, and likely breast cancer development [6]. The ZNF121 mRNA level is also higher in breast

Abbreviations
ANG1, angiopoietin-1; BRCA1, breast and ovarian cancer susceptibility protein 1; Dox, doxycycline; GADD45α, growth arrest and DNA-damage-inducible 45 alpha; HMGA2, high-mobility group AT-hook 2; MMP9, matrix metallopeptidase 9; ZBRK1, zinc finger and BRCA1-interacting protein with a KRAB domain 1; ZNF121, zinc finger protein 121.
cancer samples compared to normal tissues. These findings suggest that ZNF121 likely plays an oncogenic role in breast cancer, yet very little has been reported about any aspects of ZNF121 in the literature.

In this study, we continued to characterize ZNF121-interacting proteins in the context of breast cancer development. A yeast two-hybrid assay was performed to identify the zinc finger and BRCA1-interacting protein with a KRAB domain 1 (ZBRK1) as a new interacting partner of ZNF121. Previous studies have demonstrated the interaction between ZBRK1 and the breast and ovarian cancer susceptibility protein 1 (BRCA1) breast cancer molecule in the transcriptional regulation of many BRCA1 target genes, including growth arrest and DNA-damage-inducible 45 alpha (GADD45a), a protein involved in cell cycle control and DNA damage response [7,8], angiopoietin-1 (ANG1) [9,10], and high-mobility group AT-hook 2 (HMGA2) [11–13]. The regulation of these genes depends on the binding of ZBRK1 to the canonical GGGxxxCAGxxxTTT motif on their promoters. We therefore further tested and verified the interaction between ZNF121 and ZBRK1 in mammalian cells. Interestingly, BRCA1 was also shown to bind with ZNF121. Similar to ZBRK1 and BRCA1, ZNF121 also modulated the expression of ANG1 and HMGA2. ZNF121 also affected the expression of ZBRK1 and BRCA1.

Materials and methods

Yeast two-hybrid assay

Yeast two-hybrid assay was conducted with the Matchmaker Gold Yeast Two-Hybrid System (Clontech, Mountain View, CA, USA). The full-length CDS sequence of ZNF121 was cloned into pGBlKT7 vector and transformed into the Y2HGold yeast strain. Mating was done with a universal human cDNA library in Y187 yeast strain. After mating, the yeast cells were seeded on SD/–Leu/–Trp/X-a-Gal/AbA (DDO/X/A) plates, and then, the positive colonies were transferred to SD/–Ade/–His/–Leu/–Trp/X-a-Gal/ AbA (QDO/X/A) plates. Positive colonies growing on QDO/X/A plates were expanded for plasmid extraction, transformed into DH5α-competent Escherichia coli cells, and then sequenced.

Cell lines and drugs

Cell lines used in this study were all from the American Type Culture Collection (ATCC, Manassas, VA, USA). HEK293T cells were maintained in Dulbecco’s Modified Eagle’s medium (Gibco, Shanghai, China), supplemented with 10% FBS. T-47D cells were cultured in RPMI1640 medium (Gibco) containing 10% FBS. MCF10A cells were cultured in F12/DMEM (Gibco) supplemented with 5% horse serum, 20 ng·mL⁻¹ EGF, 0.5 mg·mL⁻¹ hydrocortisone, 100 ng·mL⁻¹ cholera toxin, and 10 μg·mL⁻¹ insulin. Drugs used in this project included puromycin (P8833, Sigma, Shanghai, China), G418 (Genview, A-138-G), polybrene (H9268, Sigma), and doxycycline (D9891, Sigma).

ELISA

ELISA was performed using the Human Angiopoietin 1 (ANGPT1) ELISA kit (DLEVELOP, Wuxi, China) according to the manufacturer’s instructions. Briefly, cells were seeded and transfected with siRNAs in 48-well plates. Twenty-four hours after transfection, the cells were supplied with fresh medium and grown for another 24 h. Then, the cell medium was used for determination of the amount of ANG1. The concentration of ANG1 in each group was normalized to the cell number, which was determined using the Cell Counting Kit-8 (CCK-8) (Dojindo, Beijing, China) as previously reported [14].

Construction of stable cell lines overexpressing ZNF121

The coding sequence of ZNF121 was cloned into pLVX-AcGFP1-C1 vector (Clontech) to generate pLVX-AcGFP1-ZNF121 plasmid. Lentivirus was produced in HEK293T cells by transfecting the cells with pLVX-AcGFP1-ZNF121 and the packaging plasmids psPAX2 and pMDG2. Twenty-four hours after transfection, the cells were replenished with fresh medium, and another 24 h later, the cell medium containing the virus was filtered and used to co-infect MCF10A cells with lentivirus containing rtTA in the presence of 8 μg·mL⁻¹ polybrene. Twenty-four hours after infection, the cells were cultured in normal medium containing 1 μg·mL⁻¹ puromycin and 200 μg·mL⁻¹ G418. Pooled stable cells were designated as conditional MCF10A cells and used for further study. The expression of ZNF121 was induced with 1 μg·mL⁻¹ doxycycline (Dox).

Co-immunoprecipitation (co-IP) and western blotting (WB)

Cells were seeded and transfected with Entranyser-D reagent (Engreen Biosystems, Beijing, China) in 60-mm plates. Twenty-four hours after transfection, the cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.4, 1% NP-40, 10% glycerin, 1 mM EDTA, cocktail protease inhibitor (Roche, Basel, Switzerland) with PMSF added immediately before use). After centrifugation, 10% of supernatant was reserved as the input and the remainder
was incubated overnight either with anti-FLAG M2 affinity gel (Sigma), anti-c-Myc magnetic beads (Thermo Fisher, Rockford, IL, USA), or agarose-conjugated anti-GFP (D153-8, MBL International, Woburn, MA, USA). Then, the gel or agarose was washed with chilled lysis buffer 5 times, resuspended in 60 μL 1 x SDS sample buffer and boiled for 5 min, and chilled on ice and centrifuged, and the supernatant was used for SDS/PAGE. For endogenous IP, 293T cells were harvested in lysis buffer and incubated overnight with 3 μg BRCA1 antibody (14823S, Cell Signaling Technology, Danvers, MA, USA) or normal rabbit IgG at 4 °C, followed by incubation with Protein A/G Plus-Agarose (sc-2003, Santa Cruz, Shanghai, China) for 2 h. The agarose was then washed and eluted as above. After electrophoresis, the proteins were transferred to poly(vinylidene difluoride) membrane and blocked with 5% skim milk in TBST (TBS+0.1% Tween-20). After blocking, the membrane was incubated with primary antibodies overnight and HRP-conjugated second antibody for 2–3 h, washed with TBST for 3 x 6 min, and developed with WesternBright ECL HRP substrate (K-12045-D50, Advansta, Menlo Park, CA, USA). Primary antibodies used include the following: ZNF121 (ab58156, Abcam, Cambridge, UK), Myc-tag (c3956, Sigma), FLAG-tag (F7425, Sigma), GFP (sc-8334, Santa Cruz), and BRCA1 (sc-6954, Santa Cruz).

**Immunofluorescence staining**

293T cells were seeded on 12-mm glass coverslips in a 24-well plate at about 30% confluence. Twenty-four hours later, cells were cotransfected with FLAG-ZNF121 and Myc-ZBRK1 plasmids, and the medium was replaced 12 h after transfection. Twenty-four hours after transfection, cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature (RT), permeabilized with 0.5% Triton X-100 for 15 min at RT, and blocked with 2% BSA for 30 min. Cells were then incubated simultaneously with FLAG-tag antibody and Myc-tag antibody at 4 °C overnight. After washing with PBS 3 times, cells were incubated with FITC-labeled goat anti-mouse IgG and TRITC-labeled goat anti-rabbit IgG at RT for 2 h. Nuclei were stained with DAPI, coverslips were mounted, and photographs were taken with a Zeiss LSM710 laser microscope.

**Plasmids and siRNA transfection, quantitative real-time PCR (qRT-PCR) and immunofluorescence**

Details for these assays were the same as previously reported [6]. The CDS sequence of ZBRK1 was obtained by PCR from the total cDNA of 293T cells and cloned into other vectors, including pGADT7, pCI-Myc, pEGFP-C1, and pCMV-tag2b, pEGFP-C1-BRCA1 plasmid and the relevant truncation constructs were cloned from a pCMV-tag2B-BRCA1 plasmid that was used in a previous study [15]. siRNA for ZBRK1 (siZBRK1, HSS184234) was purchased from Invitrogen (Carlsbad, CA, USA), and the sequence is as follows: 5′-GAACUCUGUUGAGUGUACUGGA AU-3′. Sequences of primers for qRT-PCR were as follows: GAPDH, 5′-GGTTGCTCTCCTCTGACATTCAAC-3′ and 5′-GTTGGCTGAGCCAAATCATTGTGTG-3′; ZNF121, 5′- TTTGCCCTTATTCGTGTTG-3′ and 5′-AATGTTGTTGAG GTGCTGAC-3′; ZBRK1, 5′-CCCAGTTGAACTGTGTT CTTCC-3′ and 5′-CCACTCTCCTCCTAGG-3′; ANG1, 5′-AGGCTCAACTGGGACAGCGGAA-3′ and 5′-CAGCAAGCTATCAACCACG-3′; MPP9, 5′-GCACCTGCAAGATCTAGG-3′ and 5′-ACGACG TCTCCAGTACCAGA-3′; HMGA2, 5′-AAAGCAGCTCA AAAGAAAGCA-3′ and 5′-TGTTGTTGCCCCATTCCCA GT-3′.

**ZNF121 shRNAs**

The shRNA constructs for ZNF121 were MISSION shRNA clones from Sigma. The sequences were as follows: shZNF121-1 (TRCN0000117780), AACGTGGATAGGACAAA; and shZNF121-2 (TRCN0000117779), CTTTG TGTGATCAGTCACAT. Control shRNA plasmid encodes a scramble sequence that targets no genome sequence. Production of Lentivirus and selection of stable cell populations followed the same procedure as ZNF121-overexpression in cells.

**Statistical analysis**

Statistical analysis was conducted in Microsoft Excel. Student’s t-test was used to compare the differences between variables. P values < 0.05 were considered significant.

**Results**

**Identification of ZBRK1 as an interacting protein of ZNF121**

To explore how ZNF121 functions, we carried out a yeast two-hybrid assay to screen for its potential interacting partners. Initial screening resulted in 39 positive colonies corresponding to 27 proteins, including ZBRK1, RNF2, ZNF17, ZNF200, ZNF198, and ZNF420. Besides ZBRK1, described below, RNF2 was confirmed to be a false-positive colony (data not shown), and other proteins remain to be confirmed. The full list of potential ZNF121-interacting proteins is shown in Table 1.

To confirm the positive interaction between ZNF121 and ZBRK1, a cotransformation assay was performed in Y2HGold yeast cells. Cells cotransformed with ZNF121 and ZBRK1 plasmids resulted in a signal comparable to the signal from the positive control
cells. No signal was observed in the negative control cells, indicating an interaction between these two proteins in yeast cells (Fig. 1A). We then tested the interaction between ZNF121 and ZBRK1 in mammalian cells. FLAG-ZNF121 plasmid was cotransfected into 293T cells with either Myc-ZBRK1 plasmid or the control vector. Western blotting following co-IP detected Myc-ZBRK1 in the immunoprecipitates of FLAG-ZNF121 (Fig. 1B). Consistently, FLAG-ZNF121 was also detected in the immunoprecipitates of Myc-ZBRK1 using the same approach, suggesting that FLAG-ZNF121 and Myc-ZBRK1 interact with each other in 293T cells (Fig. 1C). These data provide additional evidence supporting ZBRK1 as a new interacting protein of ZNF121.

To map the regions of ZBRK1 that mediate its interaction with ZNF121, we cloned different regions of ZBRK1 into the pEGFP-C1 vector and directly tested the interaction of these truncated ZBRK1 proteins with the endogenous ZNF121 in 293T cells. Western blotting following co-IP showed that GFP-ZBRK1-Zn, GFZNF121 interacts with BRCA1

Considering that BRCA1 is one of the main known interacting proteins of ZBRK1 and that BRCA1 plays important roles in cellular processes and carcinogenesis, we tested the interaction between ZNF121 and BRCA1 in 293T cells. Western blotting clearly detected endogenous BRCA1 protein in immunoprecipitates pulled down by the FLAG antibody in cells transfected with FLAG-ZNF121 but not in cells transfected with vector control (Fig. 2A). Similarly, Myc-tagged ZNF121 was detected in the immunoprecipitates in cells transfected with GFP-BRCA1 but not in cells transfected with GFP (Fig. 2B). In addition, endogenous ZNF121 was also detected in the immunoprecipitates of BRCA1 antibody (Fig. 2C). These observations suggest a physical interaction between ZNF121 and BRCA1 in 293T cells.

We mapped the regions of BRCA1 that mediate its interaction with ZNF121. Different regions of BRCA1 were cloned into the pEGFP-C1 vector and co-expressed with FLAG-ZNF121 in 293T cells. Western blotting following co-IP revealed that the BR-2 region (amino acids 341–748), which mediates BRCA1's interaction with ZBRK1 [7], mediated the binding of BRCA1 to ZNF121 (Fig. 2D).

ZNF121 regulates the expression of ANG1 in both MCF10A and T-47D cells

Zinc finger and BRCA1-interacting protein with a KRAB domain 1 and BRCA1 regulate the expression of a group of genes involved in human cancer by forming a complex with CtIP in MCF10A cells [11–13]. After confirming the interactions of ZNF121 with ZBRK1 and BRCA1, we tested whether ZNF121 also regulates the expression of the common target genes of ZBRK1 and BRCA1, such as ANG1. We used a Tet-On inducible system to express ZNF121 coding region was fused with GFP, and the expression was successfully induced by Dox at both the RNA and protein levels (Fig. 3A). The mRNA level of ANG1 was

Table 1. List of potential ZNF121-interacting proteins suggested by the yeast two-hybrid screening.

<table>
<thead>
<tr>
<th>Name</th>
<th>Designations</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZNF23</td>
<td>Zinc finger protein 23 (KOX 16)</td>
</tr>
<tr>
<td>ZNF350</td>
<td>Zinc finger and BRCA1-interacting protein with a KRAB domain 1</td>
</tr>
<tr>
<td>ZNF420</td>
<td>ATM and p53-associated KZN protein</td>
</tr>
<tr>
<td>RNF2</td>
<td>Ring finger protein 2</td>
</tr>
<tr>
<td>ZBTB38</td>
<td>Zinc finger and BTB domain containing 38</td>
</tr>
<tr>
<td>ZNF198</td>
<td>Homo sapiens Zinc finger, MYM-type 2 (ZMYM2)</td>
</tr>
<tr>
<td>ZNF200</td>
<td>Zinc finger protein 200</td>
</tr>
<tr>
<td>ZNF438</td>
<td>Zinc finger protein 438</td>
</tr>
<tr>
<td>ARFGEF2</td>
<td>ADP-ribosylation factor guanine nucleotide-exchange factor 2</td>
</tr>
<tr>
<td>PAN2</td>
<td>Homo sapiens PAN2 poly(A) specific ribonuclease subunit homolog</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>RFX5</td>
<td>Homo sapiens regulatory factor X,5</td>
</tr>
<tr>
<td>EAPP</td>
<td>Homo sapiens EZF-associated phosphoprotein</td>
</tr>
<tr>
<td>SNX5</td>
<td>Homo sapiens sorting nexin 5</td>
</tr>
<tr>
<td>ZNF622</td>
<td>Zinc finger-like protein</td>
</tr>
<tr>
<td>ZNF214</td>
<td>Zinc finger protein 214</td>
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<tr>
<td>ZNF17</td>
<td>Zinc finger protein 17</td>
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<td>ZNF251</td>
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<tr>
<td>ZNF302</td>
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<td>ZNF63</td>
<td>Zinc finger protein 83</td>
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<tr>
<td>ZNF661</td>
<td>Zinc finger protein 561</td>
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<tr>
<td>ZKSCAN5</td>
<td>ZKSCAN5 Zinc finger with KRAB and SCAN domains 5</td>
</tr>
<tr>
<td>PIFO</td>
<td>Primary cilia formation</td>
</tr>
<tr>
<td>SPATA22</td>
<td>Homo sapiens spermatogenesis associated 22</td>
</tr>
<tr>
<td>SPATA4</td>
<td>Homo sapiens spermatogenesis associated 4</td>
</tr>
<tr>
<td>GSG1</td>
<td>Homo sapiens germ cell associated 1</td>
</tr>
<tr>
<td>TSC21</td>
<td>Testis-specific conserved protein of 21 kDa</td>
</tr>
<tr>
<td>ARFGEF2</td>
<td>ADP-ribosylation factor guanine nucleotide-exchange factor 2</td>
</tr>
<tr>
<td>PAN2</td>
<td>Homo sapiens PAN2 poly(A) specific ribonuclease subunit homolog</td>
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<td>SPATA4</td>
<td>Homo sapiens spermatogenesis associated 4</td>
</tr>
<tr>
<td>GSG1</td>
<td>Homo sapiens germ cell associated 1</td>
</tr>
<tr>
<td>TSC21</td>
<td>Testis-specific conserved protein of 21 kDa</td>
</tr>
</tbody>
</table>

P-ZBRK1-C1, and full-length ZBRK1 (GFP-ZBRK1-FL) were able to bind to ZNF121, suggesting that the zinc finger region of ZBRK1 is the key segment mediating its interaction with ZNF121 (Fig. 1D).

We also analyzed the cellular localization of these two proteins by immunofluorescence staining, and found that both Myc-ZBRK1 and FLAG-ZNF121 were localized in the nucleus, which suggests that their interaction occurs in the nucleus (Fig. 1E).

ZNF121 interacts with BRCA1

Considering that BRCA1 is one of the main known interacting proteins of ZBRK1 and that BRCA1 plays important roles in cellular processes and carcinogenesis, we tested the interaction between ZNF121 and BRCA1 in 293T cells. Western blotting clearly detected endogenous BRCA1 protein in immunoprecipitates pulled down by the FLAG antibody in cells transfected with FLAG-ZNF121 but not in cells transfected with vector control (Fig. 2A). Similarly, Myc-tagged ZNF121 was detected in the immunoprecipitates in cells transfected with GFP-BRCA1 but not in cells transfected with GFP (Fig. 2B). In addition, endogenous ZNF121 was also detected in the immunoprecipitates of BRCA1 antibody (Fig. 2C). These observations suggest a physical interaction between ZNF121 and BRCA1 in 293T cells.

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significantly reduced by ZNF121 overexpression (Fig. 3A). Consistently, knockdown of ZNF121 by RNAi significantly increased ANG1 mRNA level in MCF10A cells (Fig. 3B). Similarly, ZNF121 knockdown also increased the expression of ANG1 in T-47D breast cancer cells while the expression of matrix metalloproteinase 9 (MMP9), another ZBRK1 target gene whose expression is independent of BRCA1, was decreased [16] (Fig. 3C). Because the ANG1 protein is a secreted factor, we examined the level of ANG1 protein in the cell culture medium using an ELISA. ZNF121 knockdown in T-47D cells also significantly increased ANG1 protein level in the medium (Fig. 3D). These results suggest that ZNF121 plays a role in the regulation of ANG1 expression in breast epithelial cells including breast cancer cells.

ZNF121, ZBRK1, and BRCA1 coordinate to regulate the expression of ANG1

Zinc finger and BRCA1-interacting protein with a KRAB domain 1 has been previously shown to

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**Fig. 1.** ZBRK1 interacts with ZNF121. (A) Demonstration of ZNF121’s interaction with ZBRK1 in yeast. Y2HGold yeast cells were respectively transformed with: a, pGBK7-p53+pGADT7 (positive control); b, pGBK7-ZNF121+pGADT7-ZBRK1; and c, pGBK7-ZNF121+pGADT7 (negative control). Transformed yeasts were spread onto QDO/X/A plates and cultured at 30 °C for 4 days and then photographed. (B) FLAG-ZNF121 interacts with Myc-ZBRK1 in 293T cells. 293T cells were cotransfected with pCI-Myc-ZBRK1 (Myc-ZBRK1) and pCI-FLAG-ZNF121 (FLAG-ZNF121). Twenty-four hours later, the cell lysates were prepared for immunoprecipitation with FLAG antibody and WB was performed with Myc-tag antibody. (C) 293T cells were transfected and analyzed as in B, but immunoprecipitation was done with Myc-tag antibody. (D) Indicated ZBRK1 constructs (left) were respectively cotransfected into 293T cells together with Myc-ZNF121 plasmid. Twenty-four hours later, cell lysates were prepared for co-IP with GFP antibody and WB with the indicated antibodies. (E) ZNF121 and ZBRK1 colocalize in 293T cells. Cells were transfected as in C, and then, immunofluorescence was performed and photographed with a Zeiss LSM710 laser microscope. Scale bars, 5 μm.
regulate its downstream genes by directly binding to gene promoters. Taken together with the findings of ZNF121-ZBRK1 interaction and ZNF121 regulation of ANG1 expression, we tested whether the regulation of ANG1 by ZNF121 depends on ZBRK1. We found that in T47-D cells, knockdown of either ZNF121 or ZBRK1 increased ANG1 mRNA expression, and an additive effect was observed when both ZNF121 and ZBRK1 were knocked down simultaneously (Fig. 4A), suggesting that ZNF121 and ZBRK1 regulate the expression of ANG1 cooperatively. Unexpectedly, knockdown of ZNF121 significantly increased the
mRNA level of ZBRK1 (Fig. 4A). Surprisingly, knockdown of either ZNF121 or ZBRK1 decreased the expression level of BRCA1, and their dual knockdown showed an additive effect, even though the decrease in mRNA level of BRCA1 was not as significant as in the protein level (Fig. 4A). Similar changes were detected in MCF10A cells (data not shown). We also used another siRNA to knock down ZNF121, and similar effects were detected on the expression of BRCA1 protein and ZBRK1 mRNA in both T-47D cells and MCF10A cells (data not shown). The effects of ZNF121 knockdown on the expression of ANG1, ZBRK1, and BRCA1 were also confirmed in T-47D cells expressing different ZNF121 shRNAs (Fig. 4B). In addition, we also found ZNF121 stable knockdown increased the expression of two other BRCA1 and ZBRK1 targets, HMGA2 and GADD45α (Fig. 4B).

We studied the cooperation between ZNF121 and ZBRK1 in the regulation of ANG1 expression in MCF10A cells with Tet-On inducible expression of
ZNF121 interacts with ZBRK1

As expected, induction of ZNF121 expression indeed increased the protein level of BRCA1 and decreased the mRNA level of ANG1 in these cells (Fig. 4C). The inhibitory effect of ZNF121 overexpression on the mRNA expression of both ANG1 was not affected by the knockdown of ZBRK1 (Fig. 4C). In

Fig. 4. Cooperative regulation of BRCA1 and ANG1 by ZNF121 and ZBRK1. (A) T-47D cells were transfected with the indicated siRNAs, and 48 h later, the cells were harvested both for WB with antibodies for ZNF121 and BRCA1 and for semiquantitative PCR and qRT-PCR with the indicated primers. The level of actin protein or GAPDH mRNA served as the internal control. (B) Stable knockdown of ZNF121 increases the common target genes of ZBRK1 and BRCA1 in T-47D breast cancer cells. T-47D cells expressing control shRNA (shControl) or ZNF121 shRNAs (shZNF121-1, -2) were prepared for RNA extraction and then qRT-PCR with the indicated primers (right). The efficiency of ZNF121 shRNAs was confirmed by WB with ZNF121 antibody, and the protein level of BRCA1 was also examined (left). (C) Conditional MCF10A cells were respectively transfected with siCtrl or siRNA for ZBRK1 (siZBRK1) either in the presence or absence of Dox. Forty-eight hours after transfection, the cells were harvested both for WB and semiquantitative PCR and qRT-PCR with the indicated antibodies or primers. For A-C, data represent Mean ± SD (n = 3). Two-tailed Student's t-test, *P < 0.05; **P < 0.01.
addition, we also found ZNF121 overexpression inhibited the mRNA level of HMG-A2, an observation which was not affected by ZBRK1 knockdown (Fig. 4C).

Discussion

Regulatory interactions between tumor suppressors and oncogenes create and maintain different cellular activities. For example, as one of the most potent oncogenes, MYC was found to bind to and regulate BRCA1 to control gene expression [17–19].

In this study, we performed a yeast two-hybrid screen to identify potential ZNF121-interacting proteins and focused on one such protein, ZBRK1, given its established interaction with the BRCA1 breast cancer gene and its role in human cancer development [16,20]. In addition, our previous study suggests that ZNF121 is an oncogene [6]. We confirmed the interaction between ZNF121 and ZBRK1 through the yeast two-hybrid assay, combination of co-IP and western blotting, and mapping of the ZBRK1 protein region that mediates its interaction with ZNF121 (Fig. 1).

We demonstrated that ZNF121 interacts with BRCA1 and regulates the expression of BRCA1 and two common target genes of BRCA1 and ZBRK1 (Figs. 2–4). Taken together with previous studies that have demonstrated the interaction between ZBRK1 and BRCA1 in gene regulation, these results suggest that ZNF121 also cooperates with ZBRK1 and BRCA1 in the regulation of their common target genes. Indeed, transient knockdown of ZNF121 significantly promoted the expression of ANG1 in both the T-47D breast cancer cell line and the MCF10A non-neoplastic human breast epithelial cell line (Fig. 3). In MCF10A cells, stable overexpression of ZNF121 inhibited the expression of ANG1 and HMG-A2 (Figs 3 and 4). In addition, in T-47D cells when ZNF121 was stably knocked down using shRNA, not only mRNA levels of both ANG1 and HMG-A2 but also the expression of GADD45α were increased in comparison to the control cells (Fig. 4B). ZNF121 is thus likely another member of the protein complex composed of ZBRK1/BRCA1/CtIP.

Although it remains to be tested whether ZNF121 interacts with CtIP and whether ZNF121 is indeed a member of the ZBRK1/BRCA1/CtIP complex, the relationships among these proteins appear to be more complicated than expected. For example, knockdown of either ZNF121 or ZBRK1 led to reduced BRCA1 protein expression, and knockdown of both ZNF121 and ZBRK1 demonstrated an additive effect. However, knockdown of ZBRK1 appeared to erase the effect of ZNF121 on the expression of ANG1 and HMG-A2, two common target genes of ZBRK1 and BRCA1 (Fig. 4C). It is possible that there are different complexes involving these proteins.

Interestingly, we found that ZNF121 knockdown increased the level of ZBRK1 mRNA, but we were unable to detect a change at the protein level due to the lack of a suitable antibody. It is thus unknown whether a direct regulatory effect or a feedback effect is responsible for the potential effect of ZNF121 on ZBRK1 expression. As for the regulation of BRCA1 by ZNF121, we propose a mechanism that is dependent on protein interaction, because the change in BRCA1 mRNA caused by ZNF121 alteration was not always consistent with the change in BRCA1 protein expression. This possibility is in agreement with our unpublished data in which the reduction in BRCA1 protein by the knockdown of ZNF121 in T-47D cells could be partly blocked by proteasome inhibitor treatment.

From the perspective of function, we propose that ZNF121 likely plays a role in tumor angiogenesis because of the increase in ANG1 protein in the medium of T-47D cells with the knockdown of ZNF121. Considering that ZNF121 is overexpressed in breast cancer and knockdown of ZNF121 inhibits cell proliferation [6], it seems somewhat surprising that knockdown of ZNF121 also increased the expression of ANG1, an angiogenesis modulator. Nevertheless, enhanced expression of ANG1 has been shown to inhibit tumorigenesis [21,22], which supports the hypothesis that ZNF121 affects tumorigenesis by regulating both MYC and ANG1.

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Author contributions

AL and JD conceived and designed the study. AL performed most of the experiments, analyzed the data, and drafted the manuscript. KZ and YZ contributed to construction and purification of plasmids. ZZ contributed to yeast two-hybrid assay. LF contributed to the preparation of most reagents. JD supervised the project and finalized the manuscript.
Conflict of interest
The authors declare no conflict of interest.

References