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Regulation of PCNA and Cyclin D1 Expression and Epithelial Morphogenesis by the ZO-1-Regulated Transcription Factor ZONAB/DbpA†

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The tight junction protein ZO-1 inhibits G_1/S-phase transition by cytoplasmic sequestration of a complex formed by CDK4 and the transcription factor ZONAB. Canine ZONAB is the homologue of human DbpA, an E2F target gene that is overexpressed in different carcinomas. Since the ZONAB target genes that are involved in G_1/S-phase transition are unknown, we employed the mammary epithelial cell line MCF-10A and cDNA arrays to screen for such genes. We identified genes encoding cell cycle and replication proteins whose expression was altered due to increased ZONAB expression. For proliferative cell nuclear antigen and cyclin D1 genes, we show that increased mRNA levels resulted in increased protein levels and we identified ZONAB-responsive elements in their promoters by using different approaches, including chromatin immunoprecipitation assays. RNA interference and overexpression of ZONAB affected the proliferation of both MCF-10A and MDCK cells as well as the differentiation of MDCK cells into polarized cysts in three-dimensional cultures. These results indicate that ZONAB regulates the transcription of genes that are important for G_1/S-phase progression and links tight junctions to the transcriptional control of key cell cycle regulators and epithelial cell differentiation.

Tight junctions (TJs) restrict paracellular diffusion in epithelial and endothelial cells and allow these cells to form selective barriers between compartments of different compositions (12, 65, 67). TJs are composed of transmembrane components that mediate adhesion and form the paracellular diffusion barrier. These proteins interact with a cytoplasmic plaque that consists of adaptor proteins, such as ZO-1, that recruit signaling molecules and interact with the actin cytoskeleton (14, 57). These components recruit different types of signaling proteins that regulate junctional functions as well as cell behavior (46).

TJ-associated proteins have also been linked to the differentiation of epithelial cells. By using MDCK cells, evolutionarily conserved junctional components that regulate cell polarity have been associated with the regulation of epithelial differentiation into cysts in three-dimensional (3-D) cultures (55, 58). Cysts are spheres with an internal lumen formed by a single layer of polarized cells that expose the apical membrane to the internal lumen (74). The capability of epithelial cells to form such cysts is important for the morphogenesis of many epithelial tissues. However, it is not known whether junctional signaling pathways that regulate proliferation and gene expression influence the morphogenesis of epithelial cysts.

ZONAB is a Y-box transcription factor that binds to the SH3 domain of ZO-1 and regulates the expression of the growth factor coreceptor erbB-2 and cell proliferation in a cell density-dependent manner (9–11). The manipulation of ZONAB activity also alters the final cell densities of mature MDCK cell monolayers (9). ZO-1 inhibits ZONAB by cytoplasmic sequestration. Since ZONAB binds CDK4, cytoplasmic sequestration of ZONAB results in reduced nuclear CDK4; hence, the regulation of CDK4 localization is one way by which ZO-1 and ZONAB regulate G_1/S-phase transition (9). Because ZONAB is a transcription factor, it is tempting to speculate that ZONAB may also affect cell proliferation by regulating the expression of cell cycle genes. However, such genes are not known.

Y-box transcription factors are multifunctional regulators of gene expression and have been proposed to play a role in promoting proliferation (26, 35). The human Y-box transcription factors DbpA and DbpB/YB-1 were identified since they bind to DNA probes that are derived from the human c-erbB-2 promoter (56). DbpA, the human homologue of canine ZONAB, is upregulated in cancer tissues (29, 50). In vitro, ZONAB binds to a different subset of inverted CCAAT box sequences than does DbpB/YB-1 (11), suggesting that ZONAB/DbpA binds to Y-box sequences that have different flanking regions than the ones recognized by YB-1/DbpB. However, the physiological relevance of these differences with in vitro DNA interactions is unknown and DbpA/ZONAB-regulated genes that are directly involved in cell proliferation have not been reported.

To identify genes that are regulated by ZONAB and TJs, we developed human mammary epithelial cell lines by using MCF-10A cells that overexpress ZONAB or express the SH3 domain of ZO-1, which inhibits ZONAB function. RNA that was isolated from these cell lines was used to screen for differential expression of proliferation genes by using cDNA arrays. Pro-

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liferating cell nuclear antigen (PCNA) and cyclin D1 genes were among those that were up-regulated by ZONAB, and different assays suggest that ZONAB directly regulates the expression of these genes. To analyze the role of the ZONAB-signaling pathway on epithelial differentiation, we tested our different cell lines for the morphogenetic potential to form cysts in collagen gels and found that ZONAB and ZO-1 indeed regulate epithelial differentiation in this model system. Our data thus link the ZO-1/ZONAB-signaling mechanism to the regulation of important cell cycle regulators, cell proliferation and epithelial differentiation.

MATERIALS AND METHODS

Cell lines and proliferation assays. The human nontransformed mammary epithelial cell line MCF-10A was a gift of Adrian Harris (University of Oxford, Institute of Molecular Medicine, United Kingdom). The cell line was subcloned, resulting in the cell line MCF-10A-95, which forms functional TJs (see below). A second MCF-10A cell line from Joan Brugge's laboratory (Department of Cell Biology, Harvard Medical School) was used for comparison in some experiments and was kindly provided by Alan Hall (Laboratory for Molecular Cell Biology, University College London). This second strain was used only when specifically indicated and was named MCF-10A-JB for the purpose of these studies. MCF-10A cells were cultured in Dulbecco's modified Eagle's medium and F-12 supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 10 μg/ml insulin, hydrocortisone 500 ng/ml, and 100 ng/ml cholera enterotoxin (20, 61). The human retinal pigment epithelial cell line ARPE-19 was a gift of John Greenwood (Institute of Ophthalmology, University College London, United Kingdom) and cultured as described previously (21). For transfection, HA-SH3 and ZONAB expression vectors were used (9, 11). For all functional assays with clonal cell lines, at least three independent clones for each construct were analyzed. For the construction of lentiviral vectors, the ZONAB cDNA was ligated into a pENTRIA vector (Gateway cloning kit; Invitrogen). pENTRIA was recombined with the destination vector giving rise to LNT-ZONAB, which was used to make lentivirus. For the down-regulation of ZO-1, the conserved sequences AAGATAGTTTGGCAGCAAGAG (Z1) and AATGTCCCTGAT CTTTCTGAC (Z2) were first targeted with the mU6 plasmid, which contains a mouse U6 promoter, as described previously (9). For down-regulation of ZONAB, the conserved sequences that had been targeted with the mU6 plasmid described previously by Balda et al. (9) were used. The plasmids were first tested in MDCK cells, and both gave efficient down-regulation, whereas control RNA interference (RNAi) vectors did not affect protein expression and cell density. The RNA duplex cassette Z1 was then excised and cloned into the lentiviral pHR′SIN plasmid (with cPPT and the woodchuck posttranscriptional regulatory element and deleted of the 3′ long terminal repeat). The new plasmid, pHR′SIN-shZO-1, was used to make lentivirus. The basic pHR′SIN plasmid backbone and the recombinant lentivirus production protocol have been previously described (7, 64). Viral infections were performed at a multiplicity of infection of 50. For tetracycline-regulated RNAi in MDCK cells, a modified mU6 plasmid was used containing a tetracycline operator at the 3′ end of the promoter (2). The sequences to target ZO-1 were as described above, and those used for ZONAB were as previously described (1, 9). Cell proliferation and cell density of MCF-10A cells were analyzed as previously described for MDCK cells (9). For synchronization, cells cultured at a low density for 2 days were incubated in medium with 0.2% serum for 48 h.

cDNA arrays. The BD Atlas human cancer 1.2 array (BD Biosciences Clontech) was used according to the manufacturer's instructions. RNA was either isolated from MCF-10A cells that were overexpressing ZONAB or transfected with empty vector. Cells were plated at low confluence and then grown for 4 days, and three independent clones per type of cell line for each analysis were used. Three independent sets of arrays were incorporated into the analysis by using the AtlasImage 2.01 (BD Biosciences Clontech) software for the quantification and comparison of single and combined arrays.

Protein expression and pull-down assays. Total cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose for immunoblotting (11). The following antibodies were used: for ZONAB, anti-C-terminus antibody (11); for ZO-1, antipeptide antibody (13); for anti-C-terminus cyclin D1, rabbit polyclonal antibody (Medical & Biological Laboratory); for PCNA, monoclonal antibody PC10 (Cancer Research UK); and for α-tubulin, monoclonal antibody 1A2 (36). The hemagglutinin-tagged SH3 domain was detected by immunofluorescence using a monoclonal antihemagglutinin (anti-HA) antibody (11, 18). The pull-down assays and fusion proteins used were previously described (8, 11). The cDNAs encoding the SH3 domains of ZO-2 (amino acid residues 586 to 665) and ZO-3 (amino acid residues 464 to 548) were amplified by PCR from an MDCK expression library and cloned into pGEX-4T-3.

Promoter analysis. The electrophoretic mobility shift assays were performed as described previously (11). The reporter gene plasmid of the human PCNA promoter (−560 to +60) in pGL2 was a gift of Michael B. Mathews (New Jersey Medical School) (43). The inverted CCAAT box at nucleotides −90 to −86 was replaced by AAAAA by PCR. The cyclin D1 promoter plasmids were based on the previously published plasmid (4, 69). The inverted CCAAT box at position −527 to −532 was also substituted by AAAAA. For reporter gene assays, ARPE-19 cells were seeded in 48-well dishes at 45,000 cells per well, cultured for 48 h, and then transfected by the calcium phosphate coprecipitation method overnight. The indicated firefly luciferase plasmids and expression vectors were cotransfected with a control plasmid driving renilla luciferase expression (24). After 24 h, luciferase determinations were performed using the dual-luciferase reporter system (Promega, Madison, WI). The relative firefly luciferase activities were calculated by normalizing according to the renilla luciferase activities. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments. For chromatin immunoprecipitation assays, MCF-10A cells were grown to 50 to 60% confluence before cross-linking with 1% formaldehyde (70). Immunoprecipitations were performed by using anti-ZONAB antibody covalently coupled to Sepharose beads (11). A total of 5% bovine serum albumin (BSA) was used to reduce nonspecific protein binding to the Sepharose. Coprecipitated promoter sequences were then analyzed after extraction of the DNA by PCR using the following primers: erbB-2, 5′-GGG CAG AGT CAC CAG CCT CTG-3′ and 5′-CCC ATG GCT CCG GCT GGA CC-3′; PCNA, 5′-AAG CTT TAT ACA ATG AAC GAT TGA GTG-3′ and 5′-CGA CGA CCG GCT GAG ACC TAG-3′; cyclinD1, 5′-GTC GCA TCT TGC TGT GAG CAC C-3′ and 5′-CTC TGC CTG GGA CAA GAC CAC C-3′; and β-actin, 5′-AAA CTC TCC CTC CTC CTC TTC C-3′ and 5′-TCG AGC CAT AAA AGG CAA CTT-3′.

3-D cultures. MDCK cell cysts were grown in 3-D collagen gels with modifications of a previously described method (47, 54, 71). Briefly, MDCK cells were trypsinized, diluted 1:3 with serum-containing medium to inactivate trypsin, spun, and then resuspended to a single cell suspension and counted. Cells (20,000 in 5 μl) were mixed with 100 μl collagen-Matrigel master mix that was prepared by neutralizing 60 μl of ice-cold solution containing 1 mg/ml calf skin type I collagen (Sigma; C8919) with 10 μl 10× Dulbecco's modified Eagle's medium, 2 μl HEPES 1 M (pH 7.4), and 2 to 3 μl of 2 M NaOH that was then mixed with 10 μl of 100% fetal bovine serum and 16 μl of Matrigel (growth factor reduced; BD Biosciences). The cell-collagen-Matrigel mix was plated in a well of a 48-well dish containing a coverslip that had been covered with 100 μl of the collagen-Matrigel master mix for 1 h at 37°C. The plated mixture was then allowed to form a solidified gel at 37°C prior to the addition of culture medium. The medium was replaced every 2 days, and the cysts were allowed to develop over 5 to 6 days. For fluorescence labeling, cells were fixed with 3% paraformaldehyde for 20 min at room temperature and were then washed twice with phosphate-buffered saline (PBS). Then, the cells were blocked and permeabilized for 30 min at room temperature with 2% BSA, 1% Triton X-100, and 25 mM Tris in PBS, followed by incubation overnight at 4°C with a goat anti-beta-catenin antibody. After three washes with 2% BSA, 1% Triton, 25 mM Tris in PBS, the samples were incubated with a Cy3-conjugated anti-goat antibody, fluorescein isothiocyanate (FITC)-phalloidin and Hoechst 33528. After three washes with PBS, cells were mounted with Mowiol. For quantification of the different structures, low-magnification pictures from the actin stainings were taken, which allowed for differentiation between the different types of structures as described in Results. At least 10 pictures of each cell line and condition were quantified from two experiments.

RESULTS

Proliferation of MCF-10A cells is regulated by ZONAB. As a first step to identifying genes that are regulated by ZONAB, we developed MCF-10A cell lines that either overexpress ZONAB or express the HA-tagged SH3 domain of ZO-1, which functions as a ZONAB inhibitor (11). Several clones with high levels of expression of the transfected proteins were selected by immunoblotting for ZONAB and by immunofluo-

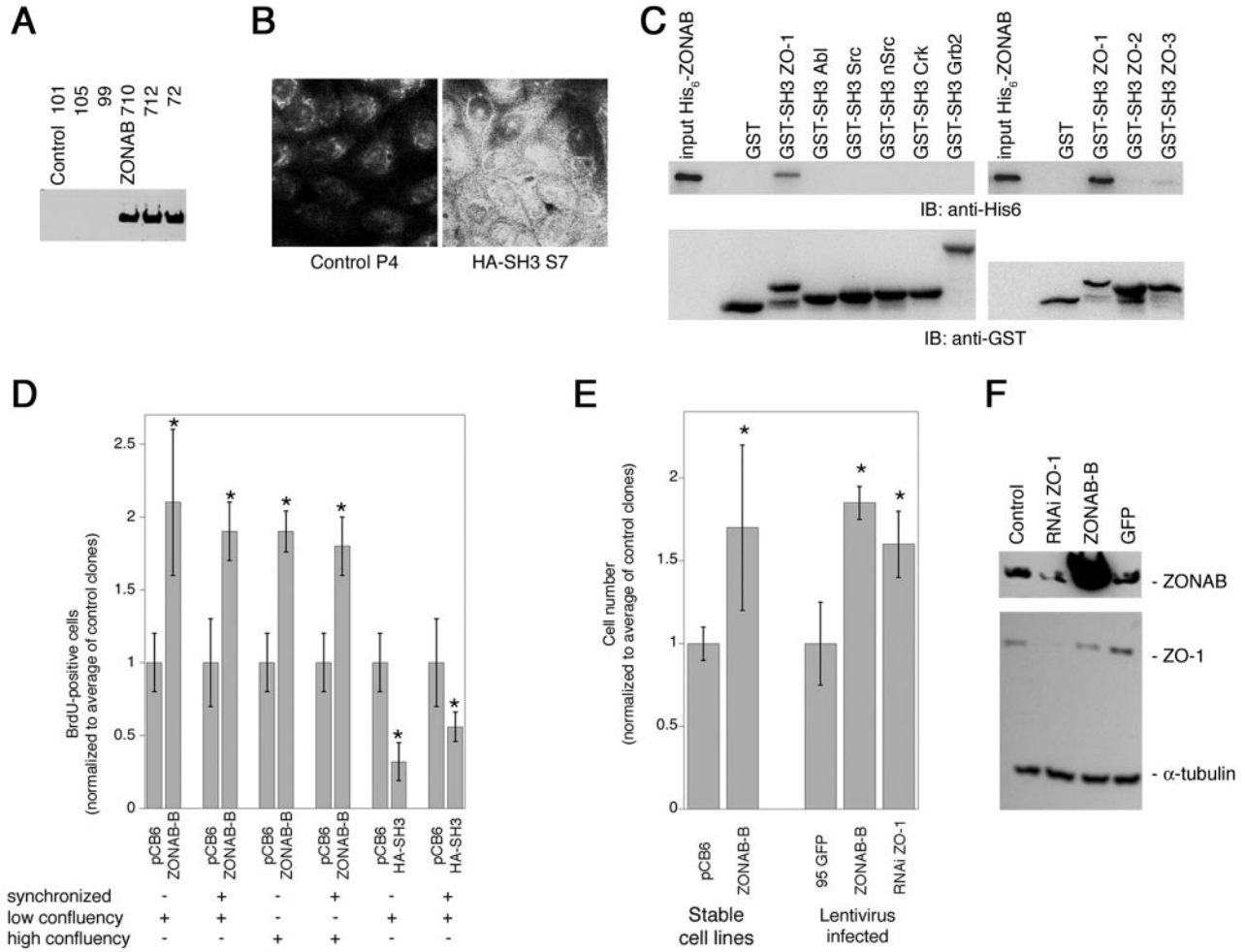

FIG. 1. ZONAB regulates cell proliferation and density of MCF-10A cells. (A) Equal amount of proteins of independent clones of MCF-10A cells transfected with empty vector or a plasmid that was driving ZONAB-B expression were analyzed by immunoblotting by using anti-ZONAB antibodies. Nontransfected cells express comparatively small amounts of ZONAB that cannot be seen on short exposures (see panel F for a comparison). (B) Expression of the HA-tagged SH3 domain of ZO-1 in MCF-10A cells was analyzed by immunofluorescence. Only clone S7 is shown, which is representative of the three clones used. (C) Pull-down assays using SH3 domains of the indicated proteins as glutathione *S*-transferase (GST) fusions were bound to glutathione-agarose and incubated with equal amounts of His_6-ZONAB. The pull downs were analyzed by immunoblotting (IB) by using either anti-His tag or anti-GST antibodies. (D) MCF-10A cells transfected with the empty vector, overexpressing ZONAB or expressing the HA-tagged SH3 domain of ZO-1 were grown for 4 days (low confluence) or 14 days (high confluence) in growth factor-containing medium. Where specified, cells had been synchronized and were then stimulated to enter S phase for 20 h. S-phase cells were detected by BrdU incorporation. Shown are means ± 1 standard deviation (error bars) of three clones for each transfection that were analyzed twice. ∗, samples with P values of <0.05 in t tests. (E) Cell density of clonal and lentivirus-transduced MCF-10A-95 cells were analyzed after culturing to full confluence for 14 days. Cells were then trypsinized and counted. Error bars indicate standard deviations. ∗, samples with P values of <0.05 in t tests. (F) The lentivirally transduced MCF-10A cells were analyzed for expression of ZONAB and ZO-1. Equal amounts of protein were loaded and confirmed by immunoblotting with antitubulin (α-tubulin) antibodies.

rescence for the SH3 domain (Fig. 1A and B). To test the specificity of ZONAB for the SH3 domain of ZO-1, we performed pull-down assays with recombinant proteins, including a range of different SH3 domains. Figure 1C shows that ZONAB does not bind to the SH3 domains of Abl, Src, Crk, and Grb2 or the homologue proteins ZO-2 and ZO-3. These results suggest that the binding of ZONAB to the SH3 domain of ZO-1 is a specific interaction.

We next tested whether ZONAB also regulates proliferation and G_1/S-phase progression for MCF-10A cells as it does for MDCK cells. Two days after plating, cells were either left in growth factor containing medium or synchronized in G_1 phase by culturing in low serum. After 2 days, fresh medium with growth factors was added for 20 h and the fraction of cells in S phase was measured by determining the number of cells that were incorporating bromodeoxyuridine (BrdU). Figure 1D shows that overexpression of ZONAB increased BrdU incorporation twofold, whereas the expression of the SH3 domain of ZO-1 reduced proliferation. The results were similar for continuously proliferating and synchronized cells. Moreover, stimulation of G_1/S-phase progression was also observed if the cells were cultured at a high density, suggesting that ZONAB overexpression reduced cell density-dependent inhibition of the proliferation of MCF-10A cells. Consequently, the overexpres-

sion of ZONAB also increased the cell numbers in MCF-10A monolayers (Fig. 1E).

To exclude the possibility that these phenotypic changes were caused by the selection process, we developed lentiviral vectors to overexpress ZONAB-B or to down-regulate ZO-1 by RNA interference. These viruses were then used to infect the MCF-10A-95 cell line. As expected, the resulting cell lines expressed either high levels of ZONAB or reduced levels of ZO-1 (Fig. 1F) and grew to larger cell numbers that were comparable to those of the above-described stable clones of ZONAB-overexpressing lines (Fig. 1E). The depletion of ZO-1 also increased the final cell density in MDCK cells (1). As MCF-10A cells that were infected with a virus inducing RNA interference of ZONAB stopped proliferating, we could not perform a quantitative analysis of the effect of ZONAB depletion.

To test whether the mammary epithelial MCF-10A cells are a suitable human epithelial cell line for studying the signaling pathways of ZO-1 and ZONAB, two proteins that are recruited to TJs in MDCK cells, we analyzed whether wild-type MCF-10A cells form TJs and express the two proteins. Immunofluorescence revealed that the analyzed MCF-10A cell line (MCF-10A-95) expresses the TJ membrane proteins claudin-4 and occludin and recruits ZO-1 and ZONAB to the junctional complex (see Fig. S1A in the supplemental material). In contrast to a previously described and widely used MCF-10A strain that does not form TJs (MCF-10A-JB) (19, 20, 22), TJ proteins exhibited an apparently continuous junctional distribution in the MCF-10A-95 cells used here, suggesting the presence of functional TJs. Indeed, values for transepithelial electrical resistance were comparable to those of MDCK-II cells (see Fig. S1B in the supplemental material). The depletion of ZO-1 and overexpression of ZONAB did not have strong effects on TJ formation under standard culture conditions; however, the depletion of ZO-1 led to a slight reduction in transepithelial electrical resistance (see Fig. S1C and D in the supplemental material), which is in agreement with previously published results obtained with other cell lines (1, 9, 66). Nevertheless, the data presented here indicate that ZONAB regulates the proliferation of mammary epithelial cells as it does that of MDCK cells.

ZONAB induces up-regulation of PCNA and cyclin D1. We next generated probes to hybridize cDNA arrays of human cancer genes (BD Atlas Clontech PT3547-3E arrays) from the stable MCF-10A cell lines that were transfected with either control vector or overexpressing ZONAB. Three different screenings were performed, which were always pooling RNA isolated from three independent clones for each type of probe.

Table 1 shows a summary of the quantification of these hybridizations. Shown are only cDNAs that gave sufficiently clear signals for analysis; these are arranged in groups corresponding to oncogenes, tumor suppressors, and proteins involved in cell cycle, DNA replication, DNA damage, energy metabolism, chromatin remodeling, and ribosomal function. In agreement with our previous observations in MDCK cells (11), ZONAB overexpression reduced mRNA levels of erbB-2 by two-thirds, indicating that it suppresses the expression of this gene as it does that in MDCK cells. Interestingly, erbB-3 mRNA was up-regulated. Future studies will address the relevance of these observations for signaling by erbB receptors. ZONAB overexpression also increased the expression levels of

TABLE 1. cDNA array analysis of cell cycle and cancer genes in MCF-10A cells[a]

Protein/gene	Ratio	Gene bank
Oncogenes and tumor suppressors		
c-*jun* proto-oncogene; transcription factor AP-1	1.5	J04111
myb proto-oncogene; c-*myb*	0.2	M15024
c-*myc* oncogene	1.7	V00568
c-*myc* binding protein MM-1	5.2	D89667
ERBB2 receptor protein-tyrosine kinase	0.3	M95667
ERBB-3 receptor protein-tyrosine kinase precursor	2.2	M29366
rhoC (H9); small GTPase (rhoC)	3.3	L25081
shb proto-oncogene	0.3	X75342
Transcription activators and repressors		
TIS11B protein; epidermal growth response factor 1 (ERF1)	1.0	X79067
Fuse binding protein 2 (FBP2)	1.1	U69126
Cell cycle proteins		
G_1/S-specific cyclin D1 (CCND1)	3.1	X59798
Cell cycle progression 2 protein (CPR2)	3.6	AF011792
Cell cycle protein P38-2G4 homolog; HG4-1	1.8	U59435
Cyclin-dependent kinase regulatory subunit (CKS2)	2.9	X54942
E2F-3	0.9	Y10479
G_1 to S phase transition protein 1 homolog	4.4	X17644
DNA replication		
Proliferating cyclic nuclear antigen	1.6	M15796
Activator 1 40-kDa subunit; RFC40	3.3	M87338
MCM2 DNA replication licensing factor	Up	D21063
MCM4 DNA replication licensing factor	1.2	X74794
DNA damage/repair signaling		
DNA repair protein XRCC1	0.7	M36089
HHR23A; UV excision repair protein protein RAD23A	3.5	D21235
Endonuclease III homolog 1 (HNTH1) (OCTS3)	1.1	U79718
Ubiquitin-conjugating enzyme E2 17 kDa (UBE2A)	2.8	M74524
Energy metabolism		
ADP/ATP carrier protein	2.0	J02683
Liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	1.2	X01677
L-lactate dehydrogenase H subunit (LDHB)	4.3	Y00711
L-lactate dehydrogenase M subunit (LDHA)	3.7	X02152
Ornithine decarboxylase	2.0	X16277
Chromatin proteins and histones		
High mobility group protein (HMG-I)	2.0	M23619
Histone H4	2.2	X67081
CENP-F kinetochore protein	2.0	U19769
Ribosomal proteins		
60S ribosomal protein L10; tumor suppressor QM	1.4	M73791
40S ribosomal protein S5	1.2	U14970
60S ribosomal protein L5	2.2	U14966
60S ribosomal protein L32	1.2	X03342
Ribosomal protein S21 (RPS21)	0.4	L04483
40S ribosomal protein S16	1.3	M60854
60S ribosomal protein L13A	0.9	X56932
40S ribosomal protein S9	1.0	U14971
Intermediate filament proteins and other cytoskeletal proteins		
Type II cytoskeletal 7 keratin (KRT7)	1.1	X03212
Type I cytoskeletal 10 keratin (K10)	1.1	M19156
Type II cytoskeletal 8 keratin (KRT8)	1.2	M34225
Type I cytoskeletal 13 keratin; cytokeratin 13 (K13; CK13)	0.7	X52426
Type I cytoskeletal 14 keratin; cytokeratin 14 (K14; CK14)	1.0	J00124
Type I cytoskeletal 18 keratin; cytokeratin 18 (K18)	2.0	M26326
Tubulin gamma subunit	0.8	M61764
Type II cytoskeletal 11 keratin (KRT11)	1.4	M98776
Type II cytoskeletal 2 epidermal keratin (KRT2E)	1.6	M99061
Brain-specific tubulin alpha 1 subunit (TUBA1)	1.2	K00558
Cytoplasmic beta-actin (ACTB)	0.8	X00351
Factors of translation		
Eukaryotic translation initiation factor 3 beta subunit	0.9	U78525
Eukaryotic translation initiation factor 4E 25-kDa subunit	1.3	M15353
Elongation factor 1 alpha (EF1 alpha)	1.0	M27364
Elongation factor 2 (EF2)	1.2	X51466

[a] RNA was extracted from clones that were overexpressing ZONAB or control transfections and labeled probes were generated as described in Materials and Methods. For each hybridization, RNAs from three independent clones were pooled. The shown results represent ratios of expression of ZONAB versus control transfections that represent averages of three hybridizations for each type of cell line.

other transcription factors, such as c-*jun* and c-*myc*. Importantly, several cell cycle regulators and genes coding for proteins involved in DNA synthesis and repair were up-regulated, suggesting that ZONAB might also stimulate cell cycle progression by regulating the expression of cell cycle regulators. Each group contains genes that were not affected by ZONAB expression, suggesting that the observed differences are specific.

Because ZONAB overexpression significantly increased G_1/S-phase progression of MCF-10A cells, we focused on proliferating cell nuclear antigen, a protein that participates in DNA replication and is required during different steps of the cell cycle, and cyclin D1, a key regulator of G_1/S-phase transition. The array analysis indicated that both genes were up-regulated at the mRNA level in ZONAB-overexpressing cells (Table 1). Both promoters contain potential Y-box factor binding sites (48, 69).

ZONAB induces PCNA protein expression. We next tested whether increased cell proliferation and mRNA levels of PCNA in ZONAB-overexpressing cells correlates with changes in protein expression. To rule out possible clonal variability, we also used cell lines generated with the lentiviral vector by using one of the control clones as a starting cell line. The cells were cultured at a low density for 4 days to assay expression during continuous proliferation or were synchronized in 0.2% serum and stimulated with growth factors for 20 h to trigger G_1/S-phase progression.

Figure 2A shows that PCNA protein expression was higher in ZONAB-overexpressing cells under both conditions (continuous growth and synchronized G_1/S-phase progression). Furthermore, the inhibition of ZONAB-function by the expression of HA-SH3, which inhibited proliferation (Fig. 1D), reduced the expression of PCNA protein (Fig. 2A). The stimulation of PCNA expression by ZONAB is not restricted to MCF-10A cells but was also observed in ARPE-19, a human retinal pigment epithelial cell line (Fig. 2B). These results show that the level of ZONAB activity affects the amount of PCNA protein that is expressed in MCF-10A cells.

ZONAB binds to the PCNA promoter in vivo. The PCNA promoter contains an inverted CCAAT box at nucleotides −90 to −86. Oligonucleotides containing this region bind to ZONAB in an electrophoretic mobility shift assay and are able to reduce the binding of an oligonucleotide containing the ZONAB-binding site of the erbB2 promoter (Fig. 2C) (11). To test whether ZONAB can regulate the PCNA promoter in vivo, we performed reporter gene assays by using the human promoter containing nucleotides −560 to +60 driving firefly luciferase expression (a kind gift from M. Mathews). We transfected this plasmid with a control reporter plasmid driving the expression of renilla luciferase and different concentrations of pCB6 expression vectors encoding ZONAB. The expression of renilla luciferase was then used to normalize the values obtained for firefly luciferase. Since we were not able to obtain sufficient transfection efficiencies in MCF-10A cells for this reporter promoter analysis, we used another human epithelial cell line derived from an ectodermal tissue, the human retinal pigment epithelial cell line ARPE-19, in which ZONAB also induces up-regulation of PCNA expression (Fig. 2B).

Figure 2D shows that cotransfection of expression vectors containing the ZONAB cDNA increased the activity of the PCNA promoter in a concentration-dependent manner. These results indicate that the manipulation of ZONAB affects the transcriptional activity of the human PCNA promoter in the same way, as it changes the levels of PCNA mRNA and protein expression.

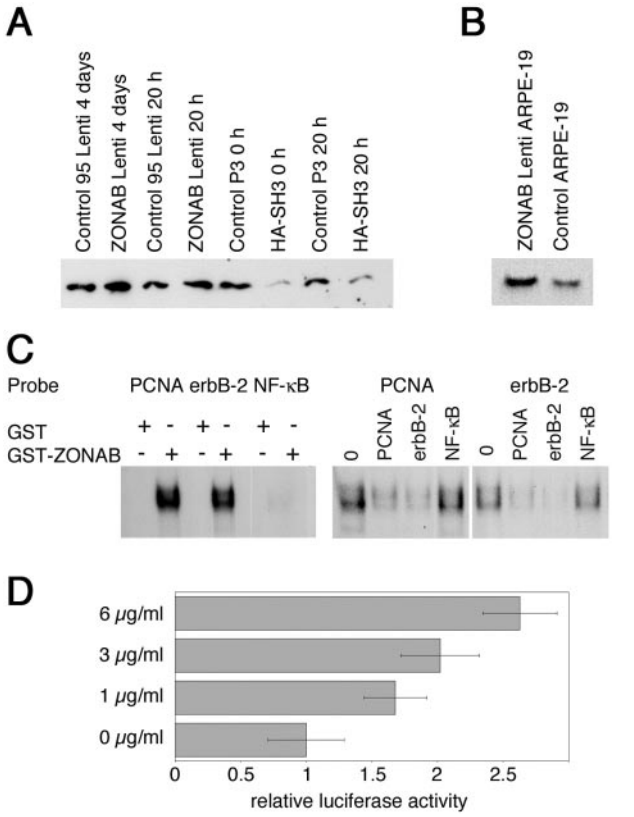

FIG. 2. Regulation of PCNA expression by ZONAB. (A) MCF-10A cells transduced with lentivirus (lenti) expressing ZONAB-B or stably expressing the HA-tagged SH3 domain of ZO-1 were either grown for four days in growth factor-containing medium or synchronized and then analyzed without or with stimulation with growth factors for 20 h. Equal amounts of proteins were analyzed for PCNA expression by immunoblotting using anti-PCNA monoclonal antibody. (B) Equal amounts of proteins of transduced ARPE-19 cells were analyzed for PCNA expression as described for panel A. lenti, lentivirus. (C) Double-stranded oligonucleotides from the PCNA, erbB-2, and NF-κB promoters were labeled with ^{32}P, incubated with GST or GST-ZONAB as indicated and analyzed by gel electrophoresis. For competition experiments, unlabeled oligonucleotides (none [0] or 500 pmols of PCNA, erbB2, or NF-κB) were used to quench binding of the PCNA or erbB-2 probe (5 pmols) as indicated. (D) Reporter gene assays were performed by cotransfecting ARPE-19 cells with a wild-type PCNA promoter reporter plasmid encoding firefly luciferase, a control renilla luciferase plasmid, and different concentrations of pCB6-ZONAB-B expression plasmid (DNA concentrations were equalized with empty expression plasmid). Expression of the luciferases was analyzed, and the values obtained for renilla luciferase were used for normalization. Shown are means ± 1 SD (error bars) of a typical experiment performed in triplicate.

To test whether the effect of ZONAB is due to the association with the promoter in the context of its local chromatin structure, we performed chromatin immunoprecipitation assays by using antibodies specific for ZONAB. Partially cross-linked, sheared chromatin was prepared from MCF-10A cells that were cultured for 4 days in growth factor-containing medium. After immunoprecipitation, PCR assays were performed

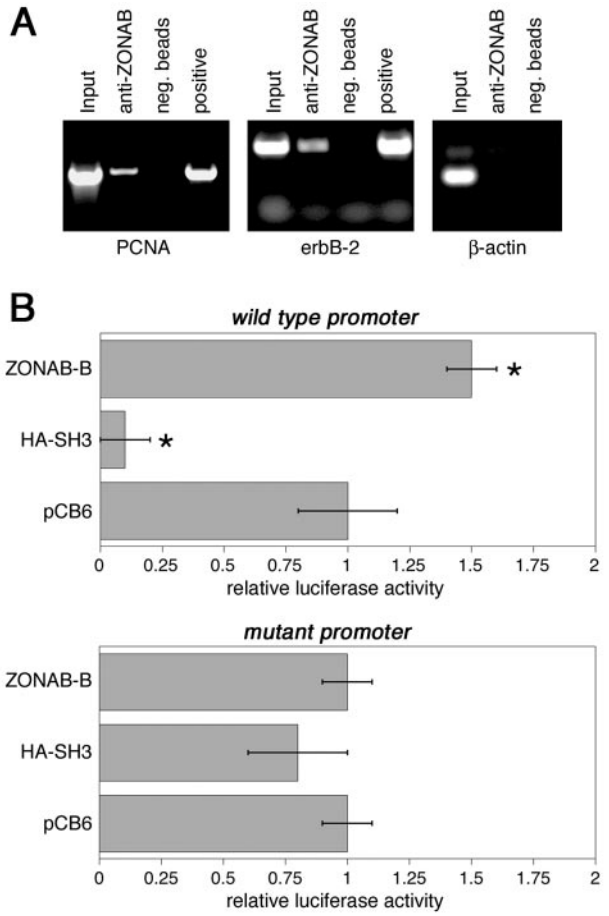

FIG. 3. ZONAB binds to the PCNA promoter in vivo. (A) MCF-10A cells were cross-linked, extracted, and immunoprecipitated with anti-ZONAB antibodies or negative control beads. The precipitated ZONAB-chromatin complexes were then analyzed by PCR for the presence of PCNA and erbB-2 promoter sequences. β-actin primers were used as a negative control. Input label PCRs in which total nuclear cell extracts were used as template. Plasmids containing the respective promoter sequences were used as positive controls. (B) Reporter gene assays were performed by cotransfecting ARPE-19 cells with a control renilla luciferase plasmid and pCB6-ZONAB-B, pCB6-HA-SH3, or empty pCB6, together with a firefly luciferase reporter plasmid containing the wild-type PCNA promoter or a promoter in which the inverted CCAAT box sequence had been substituted. Shown are means ± 1 standard deviation (error bars) of a typical experiment performed in triplicate. *, samples with P values of <0.05 in t tests.

to determine the presence of specific regions of chromatin that were immunoprecipitated with beads conjugated with antibodies against ZONAB or with negative beads.

Figure 3A shows that anti-ZONAB antibodies precipitated chromatin containing the region of the PCNA promoter that includes the inverted CCAAT box. Similarly, the region of the erbB-2 promoter, a previously identified ZONAB target gene that contains the ZONAB binding site, was also amplified. Precipitates with negative beads did not result in positive PCR. Similarly, PCR with primers that were specific for the β-actin gene did not result in detectable amounts of PCR product from ZONAB immunoprecipitations. These findings indicate that ZONAB associates with the endogenous PCNA promoter in vivo.

To determine whether the inverted CCAAT box is indeed part of the ZONAB binding site, we tested whether it is required for the regulation of the promoter by ZONAB. We constructed a new reporter plasmid, in which the inverted CCAAT box was substituted by AAAAA. We then repeated the reporter gene assays under the same conditions as described above. With the wild-type PCNA promoter, luciferase expression was stimulated by cotransfection of ZONAB and inhibited by the SH3 domain (Fig. 3B). In contrast, the mutated PCNA promoter, in which the inverted CCAAT box had been inactivated, did not respond to coexpression of ZONAB or the SH3 domain.

These experiments identify the PCNA gene as a target of ZONAB and indicate that the inverted CCAAT box of the PCNA promoter is essential for the transcriptional regulation by ZONAB.

ZONAB increases cyclin D1 expression. We next focused on the key cell cycle regulator cyclin D1, which was also found to be up-regulated upon ZONAB overexpression in the cDNA array analysis. Figure 4A shows that MCF-10A cells that were overexpressing ZONAB expressed more cyclin D1 if grown continuously for 4 days or if stimulated with growth factors for 20 h after synchronization in low-serum-containing medium. In agreement, MCF-10A cells stably expressing the HA-tagged SH3 domain of ZO-1 showed decreased cyclin D1 protein expression. Thus, ZONAB activity affects the protein level of cyclin D1, suggesting that this important cell cycle regulator might be another gene transcriptionally regulated by ZONAB.

To test whether the regulation of ZONAB expression can also be observed in an epithelial cell line derived from a different tissue and to determine whether normal ZONAB expression is required for cyclin D1 expression, we developed MDCK cells allowing the tetracycline-regulated depletion of ZONAB and ZO-1. Figure 5A shows that, when such conditional RNAi cell lines were cultured in the presence of tetracycline, the depletion of ZO-1 and ZONAB was observed. As previously reported for the constitutive depletion of ZONAB (9), tetracycline-induced RNA interference also inhibited the proliferation of MDCK cells (Fig. 5B). Both ZO-1- and ZONAB-depleted cell lines formed monolayers with functional junctions under normal culture conditions if given sufficient time (not shown). This is in agreement with previous studies in mouse mammary epithelial cells and MDCK cells (11, 66).

We next tested whether ZONAB affects the expression of cyclin D1 in MDCK cells that were synchronized in low-serum-containing medium as previously described (9). S-phase entry was then stimulated by serum addition for 12 and 24 h. Figure 5C shows that cyclin D1 expression was still low after 12 h in the wild type as well as in two clones of ZONAB-depleted cells. In contrast, ZO-1 depletion and ZONAB overexpression resulted in increased cyclin D1 expression. After 24 h, cyclin D1 expression increased in wild-type cells but remained low in ZONAB-depleted cells, suggesting that the depletion of ZONAB inhibits cyclin D1 expression. Without tetracycline, all RNAi cell lines expressed similar levels of cyclin D1 (not shown). These data show that ZONAB depletion inhibits the expression of cyclin D1 and that increasing ZONAB activity by overexpression of the transcription factor or depletion of ZO-1

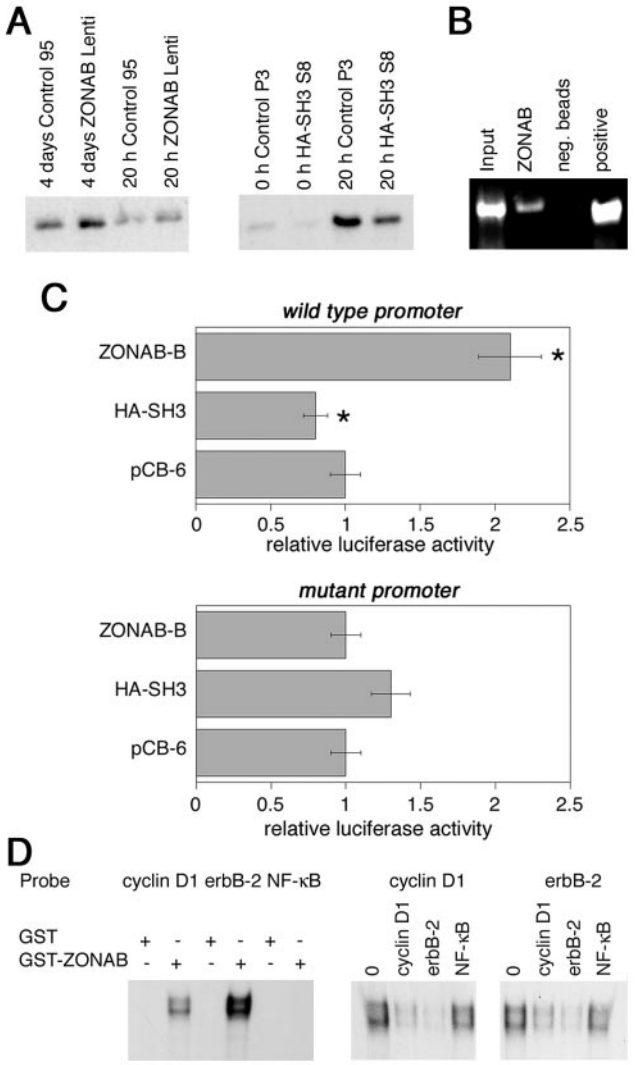

FIG. 4. Regulation of cyclin D1 expression by ZONAB. (A) Control cells, ZONAB-B-overexpressing cells, and HA-SH3-expressing MCF-10A cells were grown for 4 days in growth factor-containing medium or synchronized and then stimulated with growth factors for the indicated times. Equal amounts of protein were then analyzed by immunoblotting using an anti-cyclin D1 antibody. (B) Immunoprecipitated ZONAB-chromatin complexes were tested for the presence of cyclin D1 promoter sequences by using PCR. The used input and precipitates were derived from the same experiment as the one described in the legend for Fig. 3A. (C) Transcriptional regulation of the cyclin D1 promoter by ZONAB requires an inverted CCAAT box. Reporter gene assays were performed by cotransfection of ARPE-19 cells with a control renilla luciferase plasmid and either pCB6-ZONAB-B, pCB6-HA-SH3 or empty pCB6 plasmid and a firefly luciferase reporter plasmid containing the wild-type cyclin D1 promoter or a promoter in which the inverted CCAAT box had been substituted. Shown are means ± 1 standard deviation (error bars) of a typical experiment performed in triplicate. *, samples with P values of <0.05 in t tests. (D) Double-stranded oligonucleotides from the cyclin D1, erbB-2, and NF-κB promoters labeled with ^{32}P were incubated with GST or GST-ZONAB as indicated and analyzed by gel electrophoresis. For competition experiments, unlabeled oligonucleotides (500 pmols for cyclin D1, erbB2, or NF-κB) were used to quench binding of the cyclin D1 or erbB-2 probe (5 pmol) as indicated.

accelerates up-regulation of cyclin D1 during G_1/S-phase transition.

Regulation of cyclin D1 promoter by ZONAB depends on the inverted CCAAT sequence. Transcriptional regulation of cyclin D1 is driven by several *cis*-acting elements binding AP-1, Ets, E2F, NF-κB, Sp1, CRE, and TCF/LEF (3, 4, 30, 33, 40, 49, 60, 63, 68). It was therefore important to determine whether ZONAB interacted with the cyclin D1 promoter or whether the effect on expression was indirect. The promoter of cyclin D1 contains an inverted CCAAT sequence at positions −527 to −532. We therefore used the precipitates from the chromatin immunoprecipitation assays in Fig. 3A to detect coprecipitation of the cyclin D1 promoter by using primers flanking the inverted CCAAT box. Figure 4B shows that a cyclin D1 promoter product was amplified from ZONAB immunoprecipitations but not from negative beads. Thus, ZONAB associates with the cyclin D1 promoter in the context of its local chromatin structure in vivo.

We next performed luciferase reporter assays to determine the functional importance of the inverted CCAAT box for the transcriptional regulation of the cyclin D1 promoter by ZONAB. ARPE-19 cells were cotransfected with a control renilla luciferase plasmid, a reporter firefly luciferase plasmid containing the wild type, or a mutant cyclin D1 promoter in which the CCAAT box had been substituted as well as pCB6-ZONAB-B, pCB6-HA-SH3, or empty pCB6. Figure 4C shows that twofold increases in the transcription driven by the cyclin D1 promoter were observed when ZONAB was cotransfected, whereas a small inhibition was observed when HA-SH3 was coexpressed. In contrast, stimulation by ZONAB and inhibition by HA-SH3 were not observed when the mutant promoter was analyzed. Moreover, ZONAB was found to interact specifically with double-stranded oligonucleotides containing the inverted CCAAT box-containing sequence of the cyclin D1 promoter in gel shift assays (Fig. 4D). Thus, the inverted CCAAT box is required for the regulation of the cyclin D1 promoter by ZONAB in vivo and interacts with the Y-box factor in vitro.

ZONAB regulates differentiation of MDCK cells in 3-D cultures. We next used 3-D cultures to determine whether the manipulation of ZONAB influences the differentiation potential of epithelial cells. As we were unable to find conditions permitting the formation of cysts by MCF-10A-95 cells and the down-regulation of ZONAB-arrested MCF-10A proliferation, we used MDCK cells. The cultures were started by plating the same number of cells of the different MDCK lines in a mixture of collagen I and Matrigel. Conditional RNAi cell lines had been pretreated with or without tetracycline for 2 days. After 5 days of culture, the cells were fixed in 3% paraformaldehyde and were then fluorescently labeled using antibodies against beta-catenin, FITC-phalloidin to stain actin filaments, and Hoechst 33528 to visualize nuclei. Morphology of the formed structures was then assessed by fluorescence microscopy. Epifluorescence was used for quantification, and confocal microscopy was used for a more detailed morphological analysis of the obtained structures.

Figure 6A shows representative areas of the 3-D structures that were obtained with the different cell lines, and Fig. 6B shows a quantitative analysis for which the structures were classified as cell aggregates without a lumen, cysts with a single

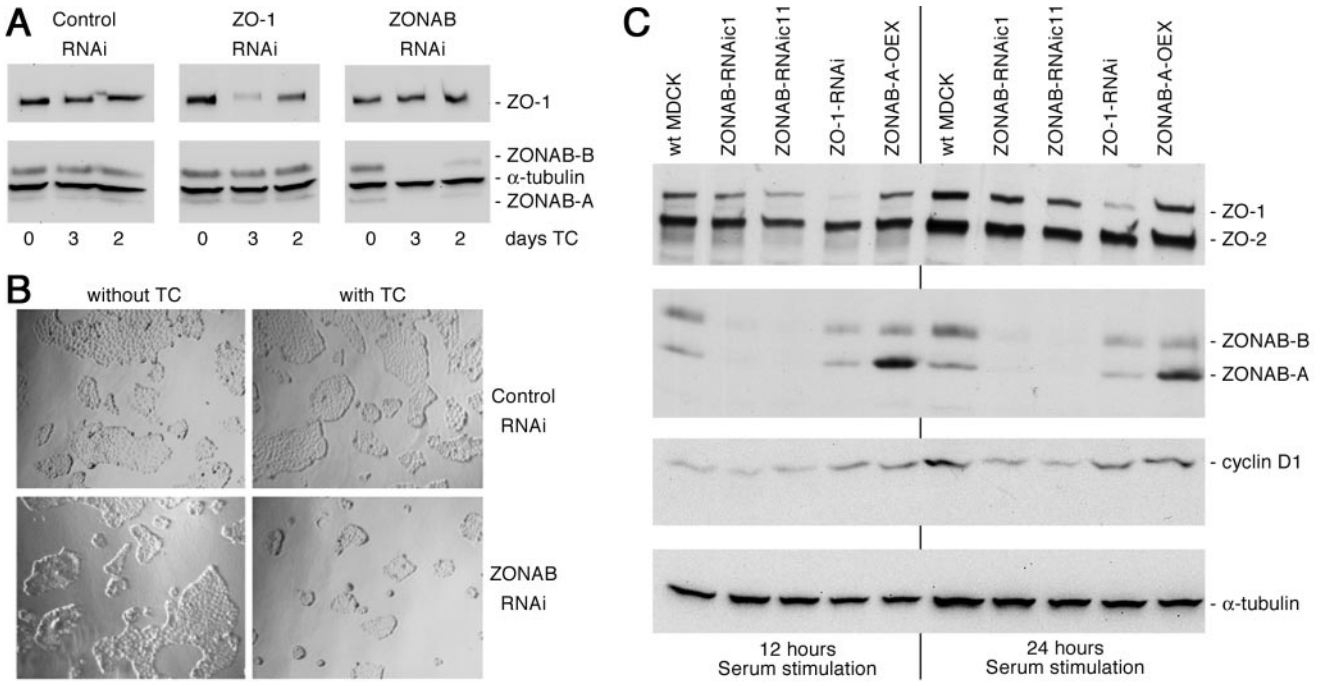

FIG. 5. ZONAB increases cyclin D1 expression in MDCK cells. (A) MDCK cells conditionally expressing either control, ZO-1 or ZONAB-directed RNA duplexes were plated and grown in the absence or presence of tetracycline (TC) for 3 or 2 days. Total cell extracts were then analyzed by immunoblotting with antibodies against ZO-1, ZONAB or tubulin (α-tubulin). (B) Equal numbers of conditional control or ZONAB RNAi MDCK cells were plated and then grown with or without tetracycline (TC) for 3 days, and phase-contrast pictures were taken to monitor proliferation. (C) MDCK wild-type cells, two independent clones of ZONAB RNAi cell lines, a ZO-1 RNAi line, and a cell line overexpressing ZONAB-A were plated at low confluence with tetracycline; after 36 h, they were synchronized by serum starvation. S-phase entry was then stimulated by adding 5% serum for 12 or 24 h. Total cell extracts were analyzed by immunoblotting with antibodies against ZO-1, ZO-2, ZONAB, cyclin D1 or tubulin (α-tubulin).

lumen, structures with multiple lumen, or other structures. The last class contains structures that did not represent clear spheres, cells that formed monolayer-like structures, and cells that formed aggregates with no clear organization.

This analysis revealed that about half of the control cultures and noninduced RNAi cell lines formed classical cysts with a single lumen. The multiple lumen structures were usually cysts with two apparently separate lumens but that appeared otherwise well organized. Together, cysts with one or two lumens accounted for more than two-thirds of all structures in such control cultures. In contrast, when ZONAB was down-regulated, almost no normal cysts developed and only small aggregates of cells were observed that often did not have any clearly polarized organization (Fig. 6A, panels a3 and a4). If ZO-1 was down-regulated, structures developed to a normal size but only a few developed a normal morphology with a single lumen (less than 10%); many structures were spheres without lumen or were disorganized (Fig. 6, panels b3 and b4). Similar to ZO-1 depletion, the overexpression of either one of the two ZONAB isoforms (Fig. 6A, panels d1 and d2) led to a large number of disorganized structures, suggesting that overexpression of the transcription factor has a consequence similar to that of the depletion of ZO-1, which functions as a ZONAB inhibitor. As such striking morphological defects in cells with reduced ZO-1 expression or increased ZONAB expression were observed in only 3-D cultures, these results suggest that the differentiation function of TJs depends on the extracellular environment.

DISCUSSION

We present evidence that ZONAB regulates G_1/S-phase progression in mammary epithelial cells and up-regulates the expression of PCNA, a crucial component of the DNA replication machinery, and cyclin D1, a key regulator of G_1/S-phase transition. ZONAB's effect on gene expression and cell proliferation correlates with a reduced differentiation potential of MDCK cells grown in 3-D cultures. Our data thus indicate that the ZO-1/ZONAB pathway regulates the G_1/S-phase transition of epithelial cells by two mechanisms (transcriptional regulation of cell cycle and DNA replication factors and, as we have previously demonstrated, modulation of the nuclear accumulation of CDK4, a kinase that regulates G_1/S-phase transition) and suggests that this junctional signaling pathway is important for the differentiation of epithelial cells.

Overexpression of ZONAB increases cell density but not proliferation in MDCK cells, whereas it increases cell density and proliferation in MCF-10A cells, suggesting that ZONAB function is cell context dependent. There are many differences between MDCK and MCF-10A cells that will guide future experiments in attempting to identify how ZONAB function is regulated in different cellular contexts. For example, genetic abnormalities, such as the deletion of the locus containing p16INK4a and p14ARF, might affect the function of the CDK4/ZONAB complex in MCF-10A cells (20, 62). It is also possible that the difference between the two cell lines is due to

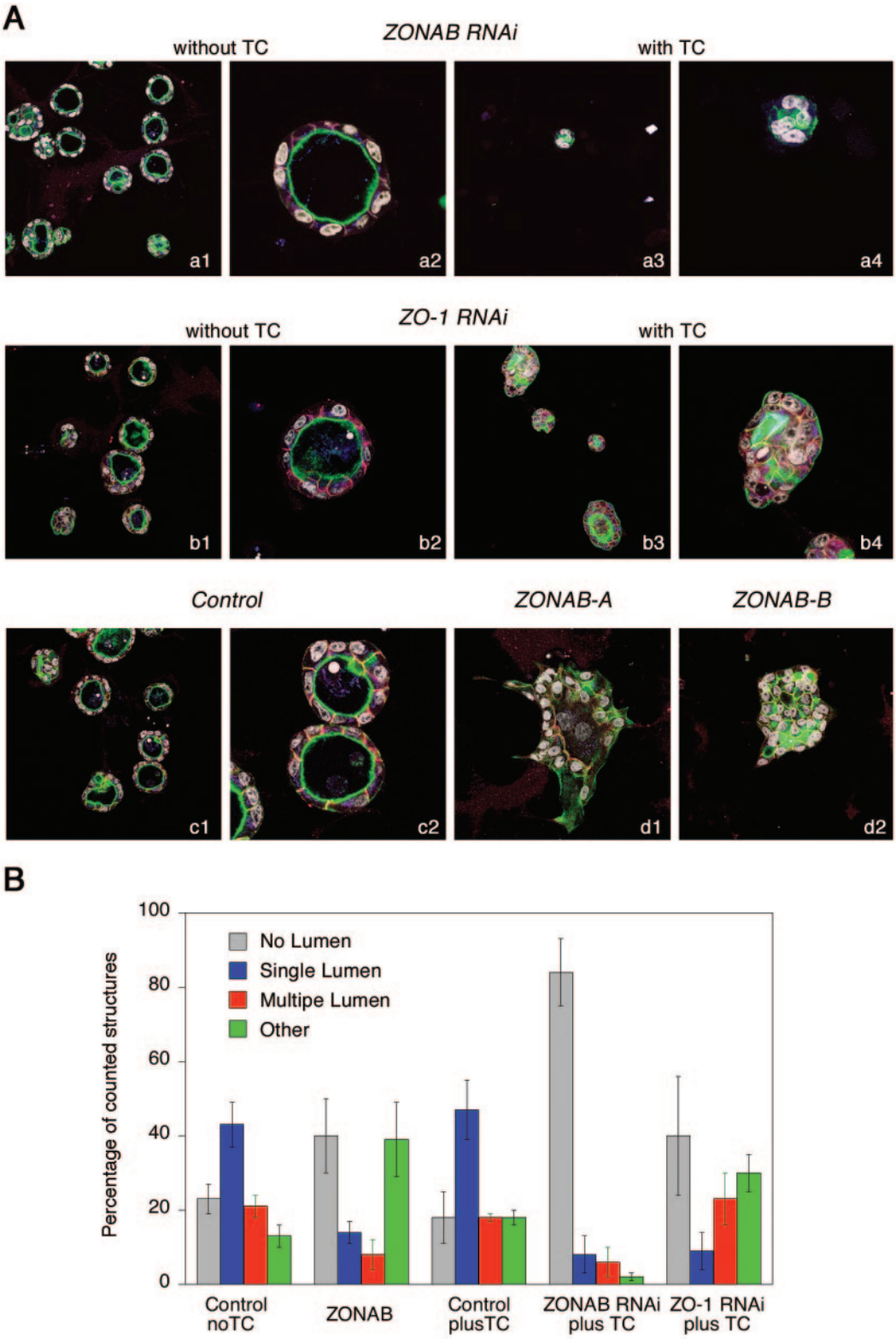

FIG. 6. ZONAB regulates cyst formation of MDCK cells grown in 3-D cultures. Control, ZONAB, and ZO-1 RNAi lines as well as ZONAB-A and B overexpressing MDCK lines were plated in the absence or presence of tetracycline (TC) in collagen-Matrigel cultures for 5 days. Cells were then fixed with 3% paraformaldehyde and processed for epifluorescence by using a Cy3-conjugated secondary antibody to detect beta-catenin (red), FITC-phalloidin (green) to detect actin, and Hoechst 33528 (white) to stain DNA. Wild-type and control RNAi cells formed normal cysts without or with tetracycline as the shown control cultures. Shown are overview images of larger fields (a1, a3, b1, b3, c1, d1, and d2) and larger magnifications of characteristic structures (a2, a4, b2, b4, and c2). (B) In two independent experiments, as in the one shown in panel A, pictures from 10 different areas were taken and quantified by counting the number and types of structures. The formed structures were divided into the following classes: spherical structures with no lumen as in a4, a single lumen as in a2, multiple lumens or structures that were not spherical and had a generally disorganized appearance as the ones shown in panels b4, d1, and d2.

differences in the endogenous expression levels of ZONAB and ZO-1 or to the degree of junctional organization. Nevertheless, the MCF-10A cells (MCF-10A-95) that are analyzed here express the ZONAB-binding partner ZO-1 as well as other TJ proteins, such as occludin and claudin-4, and develop a functional paracellular barrier.

ZO-1 knockout by homologous recombination in the mouse mammary epithelial cell line Eph4 has recently been shown to have a mild phenotype and no change in cell number (66). In contrast, we have observed increased cell densities in both MCF10-A and MDCK cells if ZO-1 was down-regulated, suggesting that proliferation arrest in EpH4 cells is differently regulated. Interestingly, oncogenic Ha-Ras induces increased cell proliferation in EpH4 mammary epithelial cells only if they were cultivated as organotypic structures in three-dimensional collagen-Matrigel matrices but not in standard two-dimensional cultures as in the one used by Umeda et al. (66), suggesting that proliferation is regulated by the culture condition (32). TJs appear to be closely linked to Ras effector pathways: the transmembrane protein occludin can suppress oncogenic Raf-1 signaling, and RalA binds to ZONAB (24, 41). It is thus striking that modulation of the ZO-1/ZONAB pathway only significantly affects the differentiation of MDCK cells in 3-D cultures and not when grown as standard 2-D cultures. It will thus be necessary to test whether ZO-1 knockout in Eph4 cells will have a stronger phenotype when cultivated in three-dimensional collagen-Matrigel matrices.

PCNA is an essential eukaryotic DNA replication factor that also plays a role in DNA repair (45, 53). The cDNA array analysis suggested that ZONAB overexpression results in the up-regulation of several components that either participate in replication (e.g., RFC40 and replication licensing factors) or in chromatin remodeling (histone H4 and HMG-I) or are part of the cellular machinery for DNA repair (e.g., Rad23A and UBE2A). It thus seems that ZONAB promotes S-phase progression by up-regulating the expression of proteins that are required for DNA replication. Additionally, it is possible that ZONAB might participate in stress-related signaling responses by up-regulating DNA repair genes.

The PCNA promoter can be transcriptionally regulated by various oncoproteins and transcription factors, such as adenovirus oncoprotein E1A, cAMP response element-binding protein (CREB), RFX1 transcription factors, the coactivator CREB-binding protein (CBP), tumor suppressor p107, p53, and E2F (15, 34, 37–39, 42, 43, 51, 59). The ZONAB binding site identified here is between the p53 (−237), E2F (−84), and ATF-1 (−52) sites. As it is currently unknown how ZONAB-binding to a promoter can cause up- or down-regulation of the transcriptional activity, it will be interesting to determine whether these binding sites and the proteins that bind to them affect each other.

As ZONAB regulates G_1/S-phase progression, the identification of cyclin D1 as a ZONAB target gene is of particular importance. Cyclin D1 plays a central role in cell cycle control, and its expression is regulated at multiple levels, including transcription, mRNA stability, translation, and protein turnover (16, 25). Transcriptional regulation of the cyclin D1 promoter is driven by several *cis*-acting elements that are controlled by signaling pathways regulated by cell-extracellular matrix and cell-cell adhesion and involves integrin-linked kinase, focal adhesion kinase, and β-catenin (5, 10, 17, 60, 63, 75, 76). We identified a novel regulatory binding site in the cyclin D1 promoter, an inverted CCAAT sequence that is essential for the stimulation of transcription by ZONAB. Thus, the regulation of cyclin D1 expression by ZONAB suggests that TJs can cross talk with cell-extracellular matrix and cell-cell adhesion pathways to regulate cell proliferation.

Cyclin D1 activates CDK4 and CDK6 and thereby promotes G_1/S-phase transition. ZONAB also binds CDK4, suggesting that the nuclear translocation of the ZONAB/CDK4 complex results in the up-regulation of cyclin D1 expression and, hence, further stimulation of the kinase. Cyclin D1 is also involved in novel functions, such as histone acetylation and chromatin remodeling, cellular metabolism, fat cell differentiation, and cellular migration (25). Because other up-regulated genes that were identified in the cDNA array analysis encode chromatin binding proteins and enzymes involved in energy metabolism, the regulation of cyclin D1 expression by ZONAB may contribute to one of these new cyclin D1 functions.

The increased expression of Y-box binding proteins in somatic cells is associated with cell proliferation and transformation. DbpA, the human homologue of ZONAB, was suggested to be a cellular recombinogenic protein that leads to genomic instability and inflammation-mediated hepatocarcinogenesis, and mutations in the DbpA promoter have been proposed to accelerate hepatocarcinogenesis (29). Moreover, DbpA is regulated by E2F1, suggesting that one of the effects of E2F1 on cell proliferation is the up-regulation of the Y-box factor (6). Similarly, the Y-box factor DbpB/YB-1, which does not localize to TJs, is also up-regulated in many cancers (23, 28). YB-1 binds preferentially to cisplatin-modified DNA and interacts with the PCNA protein (31), suggesting that different Y-box factors regulate PCNA expression and function. The overexpression of ZONAB or down-regulation of ZO-1 decreases the differentiation potential of MDCK cells in 3-D cultures and results in structures with a transformed appearance. On the other hand, the down-regulation of ZONAB significantly decreases cell proliferation, which also interferes with the formation of cysts in 3-D cultures. Thus, the levels of expression of ZONAB and its activation must be tightly regulated during epithelial differentiation. Although the target genes identified now (erbB-2, cyclin D1, and PCNA genes) might be sufficient to explain this phenotype, the cDNA array data suggest that a larger group of genes might be regulated by ZONAB during epithelial differentiation and, possibly, transformation.

By regulating the transcription of the PCNA and cyclin D1 genes, ZONAB regulates the expression of two proteins that are known to be functionally associated. Oncogenesis and chemical carcinogenesis disrupt the stoichiometry of different cyclins, CDK, PCNA, and p21 complexes that are needed for normal linkage of the cell cycle to DNA replication and repair (27, 44, 72, 73). Cyclin D1 is thought to regulate the function of PCNA in DNA repair and replication (52). Thus, the up-regulation of PCNA and cyclin D1 by ZONAB suggests that TJs might play a role in the coordination of the cell cycle with DNA replication and repair.

ZO-1 and ZONAB regulate cell proliferation by at least two mechanisms, sequestration of CDK4 (9) and, as we show here, transcriptional regulation of PCNA and cyclin D1. ZONAB and ZO-1 are up-regulated by serum (11) and DbpA/ZONAB

is transcriptionally up-regulated by E2F1 (6). Thus, the target genes of ZONAB that are identified here integrate this transcription factor into a pathway by which well-known regulators of proliferation can stimulate the expression of genes that are required for cell cycle progression. The G_1 phase is central to the integration of signals that regulate exit from the cell division cycle to differentiation and the reactivation of cell proliferation; factors involved in these pathways play a role in regulating cell size and number and organogenesis. The most studied regulatory networks of the G_1 phase are the retinoblastoma/E2F pathway and the complexes initiating DNA replication. ZONAB regulates both networks, the retinoblastoma/E2F pathway by transcriptional regulation of cyclin D1 and modulation of the nuclear accumulation of CDK4 and the initiation of DNA replication by transcriptional regulation of replication factors, such as PCNA.

As DbpA/ZONAB up-regulation has been observed in carcinomas of the liver and the pancreas (29, 50), two types of cancers that are difficult to treat, it will be important to study in more detail the role of DbpA/ZONAB in tissue biogenesis and maintenance as well as the molecular mechanisms of ZONAB regulation to identify molecular targets and strategies that could be used for pharmacological intervention in cancer cells.

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