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Taxol Resistance in Breast Cancer Cells Is Mediated by the Hippo Pathway Component TAZ and Its Downstream Transcriptional Targets *Cyr61* and *CTGF*

Dulcie Lai, King Ching Ho, Yawei Hao, and Xiaolong Yang

Abstract

Taxol (paclitaxel) resistance represents a major challenge in breast cancer treatment. The *TAZ* (transcriptional co-activator with PDZ-binding motif) oncogene is a major component of the novel Hippo–LATS signaling pathway and a transcriptional coactivator that interacts with and activates multiple transcription factors to regulate various biological processes. Here, we report that elevated levels of TAZ found in human breast cancer cells are responsible for their resistance to Taxol. DNA microarray analysis identified the oncogenes *Cyr61* and *CTGF* as downstream transcriptional targets of TAZ. Short hairpin RNA–mediated knockdown of both *Cyr61* and *CTGF* reversed TAZ-induced Taxol resistance in breast cancer cells. Interaction of TAZ with the TEAD family of transcription factors was essential for TAZ to activate the *Cyr61/CTGF* promoters and to induce Taxol resistance. Our findings define the TAZ-TEAD-Cyr61/CTGF signaling pathway as an important modifier of the Taxol response in breast cancer cells, as well as highlighting it as a novel therapeutic target to treat drug-resistant breast cancers that arise commonly at advanced stages of disease. *Cancer Res; 71(7); 2728–38. ©2011 AACR.*

Introduction

Breast cancer is the most frequently diagnosed cancer and second leading cause of cancer-related deaths in women worldwide (1). However, because of the multifactor and multistage nature of mammary tumorigenesis (2), the successful diagnosis and treatment of breast cancer remain a difficult task for oncologists. One of the major obstacles for successful breast cancer therapy is that, although mammary tumors can sometimes be suppressed by therapeutic drugs, they often recur and acquire resistance to therapy. Therefore, identification of proteins or biomarkers responsible for drug resistance is critical for planning and designing alternative breast cancer treatment strategies.

TAZ (transcriptional co-activator with PDZ-binding motif) is a WW domain–containing protein that functions as a coactivator of many transcription factors that are critical for the development of various tissues (3–7). Recently, TAZ was identified as a component of the emerging Hippo–LATS tumor suppressor pathway, which plays important roles in regulating cell proliferation and apoptosis in both *Drosophila* and mammals (8–10). The LATS tumor suppressor can phosphorylate and inhibit TAZ function in mammary cells (11). In addition, TAZ is overexpressed in about 70% of breast cancer cell lines and 20% of invasive breast cancer tissues (12). Overexpression of TAZ has been shown to cause enhanced cell proliferation and cell migration, transformation, and epithelial-to-mesenchymal transition (EMT) in immortalized mammary epithelial cells (11, 12), suggesting that *TAZ* may function as an oncogene in breast cancer. In this study, we provided the first evidence that TAZ is also involved in drug resistance in breast cancer.

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Experimental Procedures

Plasmid construction and site-directed mutagenesis

The DNA of *Cyr61* [nucleotide (nt) position −163 to +57] and *CTGF* (nt −250 to −1) promoters were amplified by PCR from genomic DNA extracted from MCF10A mammary cells (Supplementary Table 1S) and subsequently cloned into pGL3-basic luciferase reporter vector (Promega). Site-directed mutagenesis was done using the QuickChange Mutagenesis Kit (Stratagene) according to the manufacture's protocol.

Cell culture, transfection, and trypan blue exclusion assay for cell death

The general methods for cell culture and cell transfection are as described (13, 14). Human mammary epithelial cells (HMEC) and Hs574-Mg mammary epithelial cells were cultured in Mammary Epithelial Growth Media (MEGM; Clonetics). Trypan blue exclusion assay for Taxol (paclitaxel)-induced cell death was done as described previously (15).

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Antibodies, GST pull-down assay, coimmunoprecipitation, and Western blot

Mouse monoclonal antibodies to FLAG and HA were M2 (Sigma) and 12C5 (Santa Cruz), respectively. Rabbit polyclonal antibody to TAZ and mouse monoclonal antibody to E-cadherin were purchased from Cell Signaling and Transduction Lab, respectively, whereas rabbit polyclonal antibody to Cyr61, mouse monoclonal antibody to vimentin (V9), and goat polyclonal antibody to CTGF were purchased from Santa Cruz. Mammalian cell protein extraction, glutathione *S*-transferase (GST) pull-down assay, coimmunoprecipitation (Co-IP), and Western blot are as described (13, 14).

Lentiviral production, infection, and establishment of stable cell lines overexpressing or knocking down cellular genes

Lentiviral production, titration, and infection are as described (14). Two MDA-MB231 cell lines stably expressing short hairpin RNAs [shRNA; shTAZ1: 5′-GCGATGAATCAGCCTCTGAAT-3′ (sense); shTAZ2: 5′-GCCCTTTCTAACCTGGCTGTA-3′ (sense); Open Biosystems] with the best TAZ knockdown were established and subsequently used for further experiments. Similar to TAZ knockdown, a set of pGIPZ lentiviral vector expressing shRNAs against different regions of *Cyr61* or *CTGF* mRNAs (Open Biosystems) was also used for screening of Cyr61 or CTGF knockdown in MCF10A-TAZ cells. MCF10A-TAZ cells expressing shRNAs with the best knockdown for Cyr61 [5′-GGCAGACCCTGTGAATATA-3′ (sense)] or CTGF [5′-CCCAGACCCAACTATGATT-3′ (sense)] or Cyr61/CTGF were expanded and used for further experiments.

RNA isolation, microarray analysis, and qRT-PCR

RNA extraction, microarray, and quantitative reverse transcriptase PCR (qRT-PCR) analysis was done as described (14). The RNAs were labeled, amplified, and used for the following microarrays: array 1: WPI/Cy3-CTP, TAZ-1/Cy5-CTP; array 2: TAZ-1/Cy3-CTP, WPI/Cy5-CTP (flip color); array 3: WPI/Cy3-CTP, TAZ-2/Cy5-CTP (biological replicate).

Luciferase assay

Triplicates of 5×10^4 SK-BR3 cells in a 12-well plate were transfected with Cyr61-luc or CTGF-luc or their mutants (0.1 μg) alone or together with TAZ (0.2 μg) or TAZ (0.2 μg) plus TEAD (0.2 μg), using Lipofectamine 2000 (Invitrogen). As an internal transfection control, 10 ng of *Renilla* luciferase vector (pRL-TK) was also cotransfected in each sample. Luciferase activity was measured 2 days after transfection, using the Dual Luciferase Reporter Assay System (Promega) and the Turner Biosystems 20/20 Luminometer.

Chromatin immunoprecipitation assay

A ChIP-IT Express kit (active motif) was used for chromatin immunoprecipitation (ChIP) analysis of TAZ and *Cyr61* promoter interaction. In brief, MCF10A cells expressing WPI vector or TAZ-HA were treated with 1% formaldehyde, lysed, and homogenized using a Dounce homogenizer. DNA was sheared by sonication and the sheared chromatin was incubated with 2 μg of mouse IgG (Sigma) or anti-HA (F7) monoclonal antibody (Santa Cruz Biotechnology), followed by PCR by using the primers used for amplification of the *Cyr61* promoter. The PCR products were run on a 3% ethidium bromide–agarose gel and visualized under UV.

Results

Levels of TAZ in mammary epithelial cells are negatively correlated with their sensitivity to Taxol

To explore the roles of TAZ in drug resistance of breast cancer cells, we first examined the level of TAZ in normal, immortalized, and tumorigenic mammary cells representing multistage tumorigenesis (Fig. 1A). Although TAZ levels were low in both normal and immortalized mammary epithelial cells, they were significantly higher in most of the breast cancer cell lines examined (Fig. 1A). We subsequently examined the responses of 2 mammary cell lines MCF10A and MDA-MB231, representing low and high TAZ levels, respectively, to increasing concentrations of Taxol, a chemotherapeutic drug commonly used for the treatment of breast cancer and other cancers (16). Compared with TAZ-low MCF10A, TAZ-high MDA-MB231 were significantly resistant to apoptosis induced by Taxol (Fig. 1B), suggesting that the higher level of TAZ in mammary cells may be responsible for their resistance to Taxol. To confirm that overexpression of TAZ is directly responsible for Taxol resistance in mammary cancer cells, we carried out the following 2 experiments. First, we stably overexpressed TAZ in TAZ-low/drug-sensitive MCF10A cells (Fig. 1A), using lentivirus expressing HA-tagged TAZ. Significantly, compared with MCF10A-WPI control, overexpression of TAZ in MCF10A (MCF10A-TAZ) causes significant resistance to apoptosis induced by Taxol (Fig. 1C). Second, we knocked down TAZ in TAZ-high/drug-resistant MDA-MB231 by using lentiviruses expressing shRNAs against different regions of TAZ mRNA (Fig. 1D). Significantly, knockdown of TAZ by both shTAZs in MDA-MB231 cells sensitizes their response to Taxol treatments (Fig. 1D). In summary, our findings strongly suggest that TAZ is a novel biomarker for the resistance of breast cancer cells to Taxol.

Identification of *Cyr61* and *CTGF* as major downstream targets of TAZ

To understand the molecular mechanism underlying TAZ-induced drug resistance in mammary epithelial cells, we aimed to identify the downstream targets of TAZ. Because TAZ is a transcriptional coactivator (4), we used a whole human genome microarray to identify the transcriptional targets of TAZ. HA-tagged TAZ was stably expressed in TAZ-low MCF10A cells (Fig. 2A). Overexpression of TAZ in MCF10A causes EMT (Fig. 2B) with reduced levels of the epithelial marker E-cadherin and increased levels of the mesenchymal marker vimentin (Fig. 2C). Gene expression profiles were subsequently compared between MCF10A-WPI and the 2 lines of MCF10A-TAZ (Fig. 2A), using 44K whole human genome microarray analysis. After statistical analysis, 389 cellular genes were transcriptionally activated after overexpression of TAZ (Supplementary Table 2S). We further confirmed some of the transcriptional

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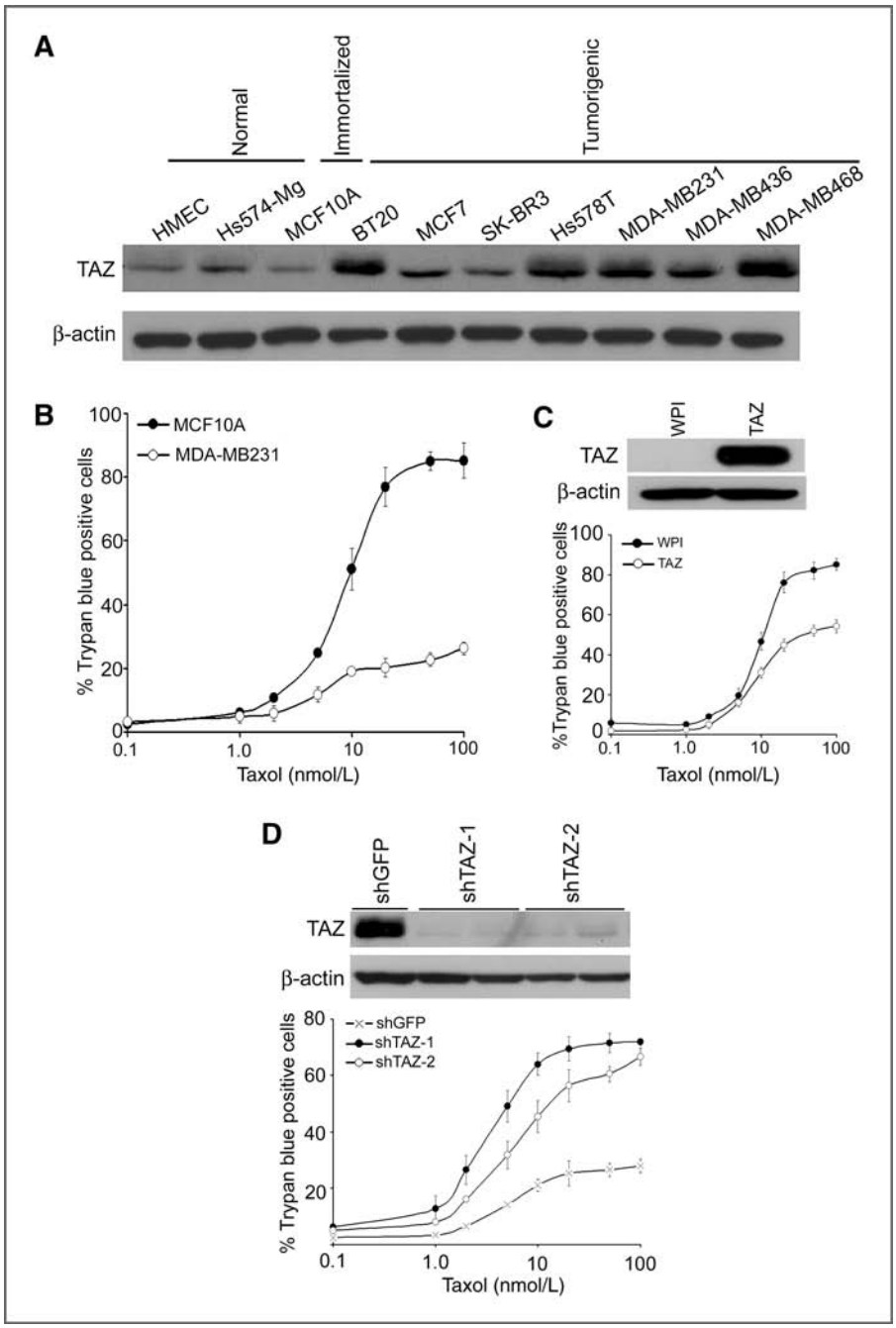

Figure 1. Correlation of TAZ expression levels with response of breast cancer cells to chemotherapeutic drug treatments. A, Western blot analysis of TAZ protein levels in normal (HMEC and Hs574-Mg), immortalized (MCF10A), and tumorigenic (BT20, MCF7, SK-BR3, Hs578T, MDA-MB231, MDA-MB436, and MDA-MB468) mammary cells. About 10 μg of cell lysate extracted from mammary cells by using lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1.0% NP40, and 1 × protease inhibitor) were subjected to Western blot by using rabbit polyclonal anti-TAZ antibody (Cell Signaling). β-Actin was used as an internal protein loading control. B, trypan blue exclusion analysis of apoptosis induced by Taxol in MCF10A and MDA-MB231 cells. About 2.5×10^4 cells were seeded into each well of 24-well plate, followed by treatment with increasing amounts of Taxol (0, 0.1, 1, 2, 5, 10, 25, 50, 100 nmol/L) for 72 hours. Because Taxol induces apoptotic cell death in breast cancer cells (16), the trypan blue exclusion assay is used for measuring apoptosis. Dead cells were stained with 0.2% trypan blue, and the percentage of trypan blue–positive (dead) cells was calculated. Taxol concentrations are shown in $\log_{10}$ scale. The mean and standard error of 3 independent experiments for each cell line are shown. C, overexpression of TAZ caused Taxol resistance in MCF10A cells. Protein lysate extracted from MCF10A cells stably expressing WPI lentiviral vector or TAZ were subjected to Western blot by using anti-TAZ antibody (top). Cell death assay (bottom) was done as described in (B). D, enhanced sensitivity of MDA-MB231 cells to Taxol after TAZ knockdown. shRNAs against different regions of TAZ mRNA (shTAZ-1 and shTAZ-2) were stably expressed in MDA-MB231 cells. Duplicate protein lysate extracted from MDA-MB231 cells stably expressing shTAZ were subjected to Western blotting by using anti-TAZ antibody (top). Protein lysate from MDA-MB231 cells stably expressing shGFP was used as a nontargeting negative control. Procedures and conditions for cell death assay (bottom) are described as in (B).

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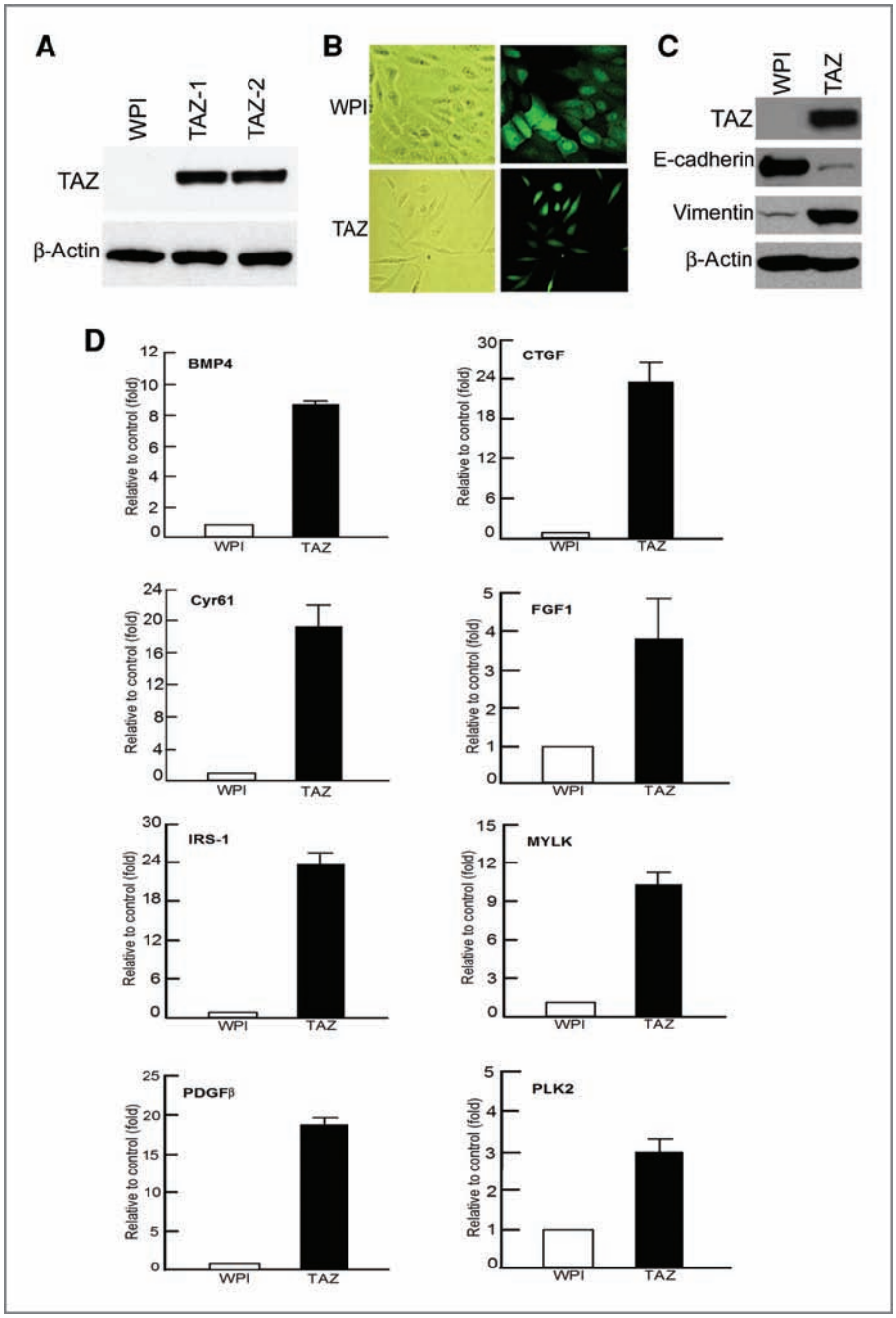

Figure 2. Identification of transcriptional targets of TAZ by microarray. A, expression of TAZ in MCF10A cells. Duplicate MCF10A cells were individually infected with lentivirus expressing TAZ (TAZ-1 and TAZ-2). MCF10A infected with lentivirus expressing WPI vector control was used as a negative control. B, cell morphology. Cell morphology was examined using white light (left) or fluorescent light (right) at 10× magnification. Green fluorescent protein expressed from the lentiviral vector was used as a marker for infection efficiency. C, Western blot analysis of EMT markers. D, qRT-PCR analysis of cellular genes upregulated by TAZ. The fold increase at the mRNA level in MCF10A-TAZ cells relative to those in MCF10A-WPI (control) cells are presented here. The mean and SD were calculated from C_t values of triplicate real-time RT-PCR reactions for each RNA sample.

targets, which have been previously shown to play important roles in mammary tumorigenesis, by real-time qRT-PCR using the RNAs extracted from MCF10A-WPI and MCF10A-TAZ. Significantly, 8 cellular genes identified have higher levels of mRNA after TAZ overexpression (Fig. 2D).

Next, we further characterized 2 of the most highly activated genes, *Cyr61* and *CTGF* (Fig. 2D). Cyr61 and CTGF are 2 members of the connective tissue factor CCN (Cyr61, CTGF, Nov) family, which are secreted as cysteine-rich extracellular matrix proteins that function as ligands of the integrins to regulate cell proliferation, apoptosis, cell migration/adhesion, and angiogenesis (17). Similar to *TAZ*, *Cyr61* and *CTGF* have previously been identified as oncogenes that are activated in breast cancers and cause resistance to apoptosis induced by Taxol or hypoxia when overexpressed in mammary epithelial cells (18–28). Recently, *CTGF* has been identified as a transcriptional target of TAZ (29). However, the functional significance of its activation by TAZ is unknown. Moreover, *Cyr61* has not been identified as a transcriptional target of TAZ. To confirm that both *Cyr61* and *CTGF* are indeed bona fide downstream targets of TAZ, we carried out the following experiments. First, we used western blot analysis to show that

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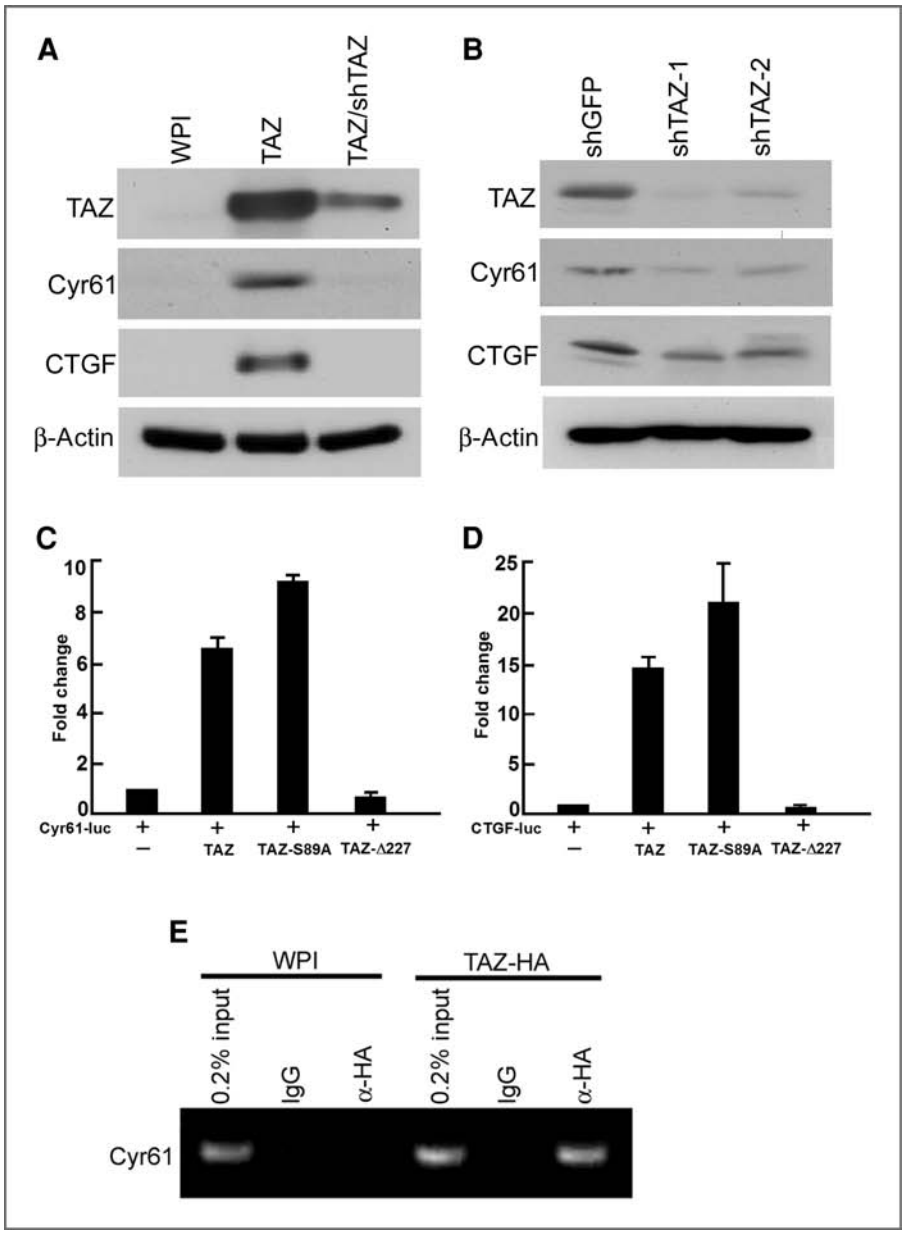

Figure 3. Activation of *Cyr61* and *CTGF* by TAZ. A, expression of Cyr61 and CTGF after TAZ overexpression and knockdown. Protein lysate extracted from MCF10A cells expressing WPI vector, TAZ, or TAZ together with shTAZ (TAZ/shTAZ) were subjected to Western blot analysis (see legend of Fig. 1A for details). B, reduced expression of Cyr61 and CTGF in MDA-MB231 cells after TAZ knockdown. MDA-MB231 cells were stably infected with lentivirus expressing shGFP, shTAZ-1, or shTAZ-2. Protein lysate extracted from these cell lines were subjected to Western blotting. C and D, activation of *Cyr61* and *CTGF* promoters by TAZ. SK-BR3 cells were transfected with luciferase reporter plasmid expressing *Cyr61* (C) or *CTGF* (D) promoters (Cyr61-luc or CTGF-luc) together with plasmids expressing vector, TAZ, TAZ-S89A (constitutively active TAZ), or TAZ-Δ227 (TAZ-lacking transactivating domain). Luciferase assays were carried out using a Dual Luciferase Assay Kit. The fold changes in luciferase activity were calculated by normalizing SK-BR3 cells transfected with Cyr61-luc or CTGF-luc together with TAZ, TAZ-S89A, or TAZ-Δ227 to those transfected with Cyr61-luc or CTGF-luc alone. E, ChIP analysis of TAZ interaction with the *Cyr61* promoter *in vivo*. DNA and protein were cross-linked by treatment of MCF10A-WPI or MCF10A-TAZ-HA cells with 1% formaldehyde. After purification of chromatins and DNA-binding proteins by homogenization, sonication, and centrifugation, they were subjected to immunoprecipitation by using rabbit IgG (negative control) or rabbit polyclonal anti-HA (F7) antibody, followed by PCR and agarose electrophoresis. About 0.2% input chromatin (1 μL) extracted from MCF10A-WPI or MCF10A-TAZ cells were used as positive PCR controls.

both Cyr61 and CTGF protein levels were much higher in MCF10A-TAZ than those in MCF10A-WPI (Fig. 3A). Most interestingly, enhanced Cyr61 and CTGF protein were reduced back to their original levels when overexpressed TAZ in MCF10A-TAZ was knocked down by shTAZ (Fig. 3A). Second, we knocked down TAZ in TAZ-high MDA-MB231 cells to assess whether endogenous TAZ is essential for maintaining enhanced levels of Cyr61 and CTGF in these cells. Contrary to

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TAZ overexpression, knockdown of TAZ by 2 shRNAs (shTAZ-1 and shTAZ-2) reduced the expression of both Cyr61 and CTGF in MDA-MB231 cells (Fig. 3B). Third, we found that TAZ significantly activated the luciferase activity of both *Cyr61* and *CTGF* promoters (Cyr61-luc or CTGF-luc) 6.8- and 14.3-fold, respectively (Fig. 3C and D). Cotransfection of a constitutively active TAZ, TAZ-S89A (a mutant lacking LATS phosphorylation site), further enhanced *Cyr61* and *CTGF* promoter activity to 9- and 20.7-fold, respectively, whereas cotransfection of TAZ-Δ227, a mutant lacking the transactivating domain, abolished activation of the *Cyr61* and *CTGF* promoters (Fig. 3C and D), suggesting that the transactivating function of TAZ is essential for its activation of *Cyr61* and *CTGF*. Finally, our ChIP assay showed that TAZ could be coimmunoprecipitated with the *Cyr61* promoter DNA *in vivo* (Fig. 3E). Collectively, these studies strongly suggest that *Cyr61* and *CTGF* are indeed bona fide transcriptional targets of TAZ.

TAZ activates *Cyr61* through interaction with TEADs

Because TAZ is a transcriptional coactivator, it should activate downstream genes through interaction with transcription factors. Recently, the TEAD family of proteins including TEAD1, 2, 3, and 4 (or TEADs) have been identified as the transcription factors mediating TAZ functions in cell growth, oncogenic transformation, and EMT (6, 29, 30). In addition, TEADs have also been shown to enhance TAZ-induced transcriptional activation of *CTGF* (29). To examine whether TEADs mediate TAZ-induced activation of *Cyr61*, we transfected Cyr61-luc alone, along with each of the TEADs, or with both TAZ/TEADs into SK-BR3 cells. Although transfection of any member of the TEAD family alone has no effect on *Cyr61* promoter activity (data not shown), cotransfection of TEAD with TAZ significantly enhanced TAZ-induced activation of the *Cyr61* promoter. On the other hand, cotransfection of Runx2, another TAZ-interacting transcription factor, has no effect (Fig. 4A), suggesting that TAZ may directly interact with the TEADs to activate the *Cyr61* promoter.

Next, we further examined whether the interaction of TEAD with TAZ is essential for TAZ-induced transcriptional activation of the *Cyr61* promoter. Because TEAD4 has been shown to be the most important TEAD in TAZ-induced tumorigenesis (29, 30), we chose to use TEAD4 in the following experiments. First, our Co-IP assay clearly showed that, although TEAD4-FLAG interacts strongly with TAZ-HA *in vivo*, a single amino acid mutation in TEAD4, TEAD4-Y429H corresponding to TEAD1-Y421H (31), abolished binding to TAZ (Fig. 4B). In addition, the TEAD4-Y429H mutant also failed to enhance TAZ-induced activation of the *Cyr61* promoter (Fig. 4C), suggesting that the interaction of TAZ with TEAD is critical for its effect on activation of *Cyr61* transcription.

Identification of the TEAD response element in the *Cyr61* promoter

Previous studies have shown that TEAD activates CTGF through binding to 3 TEAD response elements (TREs) in the *CTGF* promoter (32). Therefore, we wanted to determine whether TAZ activates the *Cyr61* promoter through TEAD interaction with the TREs. We have also identified 2 putative TREs in the *Cyr61* promoter region (Fig. 4D) and mutated the 2 TREs individually (TRE1M or TRE2M) or in combination (TRE1/2M; Fig. 4E). Our luciferase assay showed that mutation of TRE1, rather than TRE2, completely abolished TAZ/TEAD-induced activation of the *Cyr61* promoter (Fig. 4F), suggesting that TAZ/TEAD may bind TRE1 to activate *Cyr61* transcription.

TAZ and TEAD interaction is essential for its effects on the *Cyr61* and *CTGF* promoter and drug response in mammary cells

Although TEADs have been identified as the critical transcription factors mediating TAZ-induced transformation, it is still unknown whether TEADs are essential for TAZ-induced drug resistance in breast cancer cells. To answer this question, we carried out the following experiments. First, our GST pull-down assay showed that while equal amounts of fusion protein were used (Ponceau S staining for proteins), wild-type TAZ-GST rather than TAZΔ72-GST mutant [corresponding to TAZ paralogue YAP mutant lacking interaction with TEADs (33)] could interact with TEAD4-FLAG *in vitro* (Fig. 5A). In addition, although overexpression of wild-type TAZ increased the levels of *Cyr61* and *CTGF* mRNA and protein, overexpression of TAZΔ72 had no effects (Fig. 5B and E). Moreover, TAZΔ72 also failed to activate *Cyr61* and *CTGF* promoter activity (Fig. 5C and D) and its overexpression in MCF10A failed to induce resistance to Taxol in these cells (Fig. 5F).

Knockdown of *Cyr61* and *CTGF* reverses the drug-resistant phenotypes caused by TAZ

Because enhanced levels of Cyr61 and CTGF have also been shown to cause drug resistance in breast cancers (17, 23, 25, 34), we wanted to confirm that Cyr61 and CTGF are the major downstream transcriptional targets mediating TAZ-induced Taxol resistance. We produced lentiviruses expressing shRNA against different regions of *Cyr61* (shCyr61) or *CTGF* (shCTGF) mRNA, which were used for single (TAZ/shCyr61 or TAZ/shCTGF) or double knockdown (TAZ/shCyr61/shCTGF) in MCF10A-TAZ cells (Fig. 6A and B). As shown in Figure 6C, whereas knockdown of Cyr61 or CTGF alone partially, but significantly, sensitized MCF10A-TAZ cells to Taxol, knockdown of both Cyr61 and CTGF completely blocks TAZ-induced resistance to Taxol. Together, these results strongly suggest that Cyr61 and CTGF are 2 of the major downstream proteins mediating TAZ-induced Taxol resistance in breast cancer cells.

Discussion

TAZ as a novel gene inducing drug resistance

Although tremendous progress has been made toward understanding the molecular mechanism underlying breast cancer development and treatment, the overall survival of breast cancer patients is still significantly hindered because of the evolution of drug-resistant tumors. Therefore, the identification of proteins responsible for drug resistance is critical for successful breast cancer treatment. In this study, we have for the first time identified *TAZ* as a novel gene responsible for drug resistance in breast cancer. By

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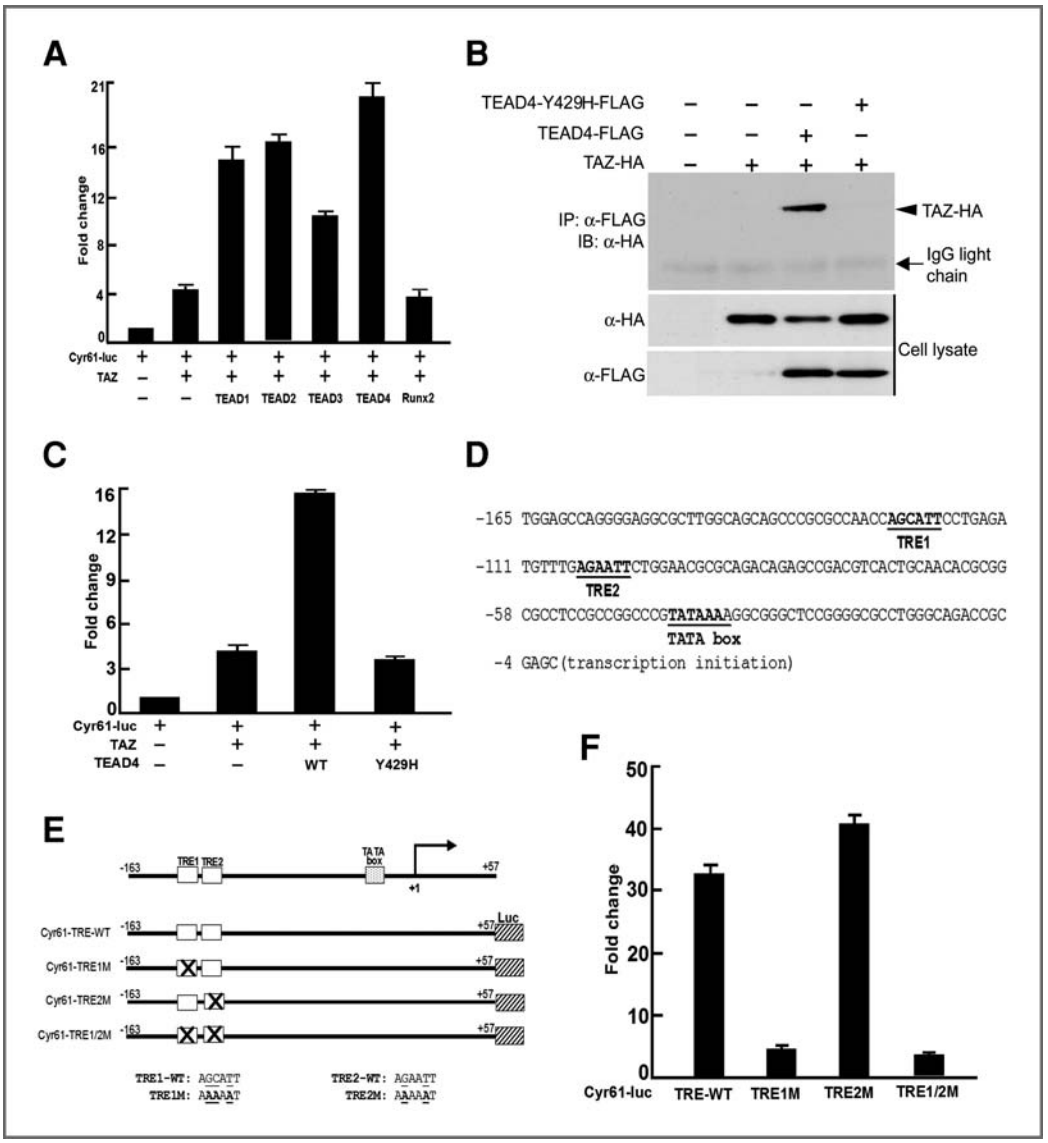

Figure 4. TAZ activates *Cyr61* through interaction with TEADs. A, luciferase assay. Procedures for measurement and calculation are as described in Figure 3C and D. B, Co-IP analysis of interaction of TAZ and TEAD4 *in vivo*. Cos-7 cells were mock transfected or transfected with TAZ-HA alone or together with TEAD4-FLAG (wild-type) or TEAD4-Y429H-FLAG. About 500 μg of protein lysate extracted from transfected cells were precipitated using mouse anti-FLAG (M2) monoclonal antibody. The precipitated proteins were subjected to Western blotting by using rabbit anti-HA (A14) polyclonal antibody (first) and horseradish peroxidase–conjugated light-chain specific anti-rabbit IgG (second). Expression of TAZ-HA and TEAD4-FLAG/TEAD4-Y429H-FLAG were detected by western blotting using cell lysate from transfected cells and anti-HA and anti-FLAG antibodies, respectively. C, luciferase assays. Procedures and labels are as described in (A). D, sequence of the *Cyr61* promoter. Two TREs (TRE1 and TRE2) were identified (bold/underlined). E, constructs used for luciferase assay as in (F). The structures of Cyr61-luc with wild-type or mutated (TRE1M, TRE2M, TRE1/2M) TREs are shown. Mutated nucleotides in TRE1 or TRE2 are bolded and underlined. F, the constructs (as shown in E) were transfected together with TAZ/TEAD into SK-BR3 cells. Luciferase assays were carried out as described in Figure 3C and D.

overexpressing TAZ in TAZ-low/drug-sensitive MCF10A mammary epithelial cells, we have shown that enhanced levels of TAZ can directly cause resistance of mammary epithelial cells to apoptosis induced by the chemotherapeutic drug Taxol. On the other hand, knockdown of endogenous TAZ in TAZ-high/drug-resistant MDA-MB231 breast cancer cells sensitized these cells to Taxol. We are currently examining whether TAZ can be used as a biomarker in predicting chemotherapeutic drug response in breast cancer in clinical setting by using tissue microarray samples obtained from breast cancer patients treated with chemotherapeutic drugs such as Taxol. If TAZ levels are indeed correlated with the drug resistance of breast cancer, targeting TAZ in TAZ-overexpressed, drug-resistant patients will be a novel therapeutic strategy for successful breast cancer treatment.

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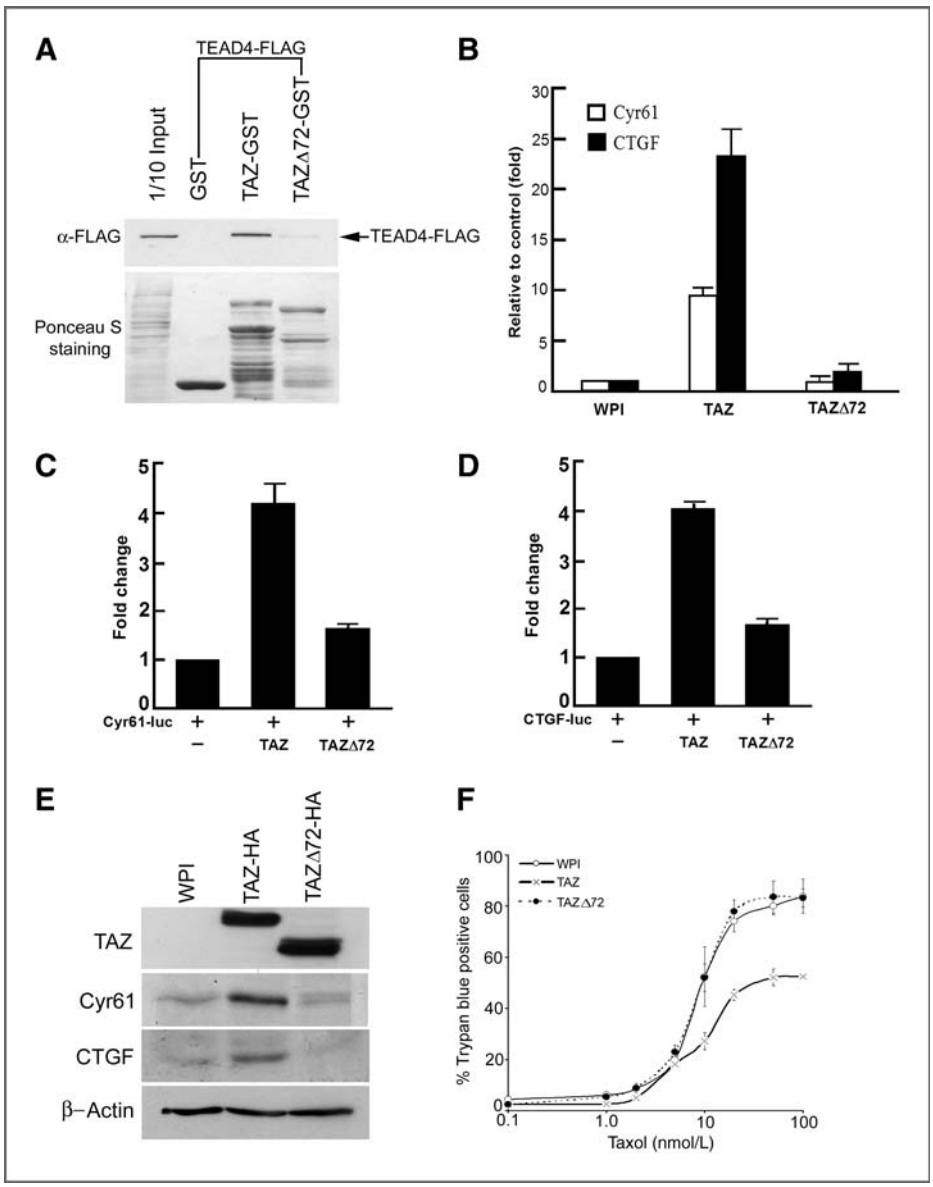

Figure 5. Interaction of TAZ with TEAD is essential for its activation of *Cyr61/CTGF* and induction of Taxol resistance. A, GST pull-down analysis of the functional domain of TAZ interacting with TEAD. About 10 μg of GST (control), TAZ-GST, or TAZΔ72-GST (lacking TEAD-interacting domain) fusion proteins were incubated with 100 μg of cell lysate extracted from cells expressing TEAD4-FLAG, washed with lysis buffer, and subjected to Western blotting along with one tenth of the amount of cell lysate used for GST pull-down assays (1/10 input) by using mouse anti-FLAG monoclonal antibody. The membrane was stained with 1% Ponceau S to visualize the GST fusion proteins used in the GST pull-down assays. B, loss of TAZ and TEAD interaction abolishes activation of *Cyr61 and CTGF* transcription. qRT-PCR analysis of *Cyr61* and *CTGF* mRNA levels were done using mRNAs extracted from MCF10A cells expressing WPI (vector), wild-type TAZ, or TAZΔ72. C and D, loss of activation of *Cyr61* (C) and *CTGF* (D) promoters by TAZΔ72. Luciferase assays were carried out as described in Figure 3C and D. E, loss of Cyr61 and CTGF protein expression by TAZΔ72. F, loss of resistance to Taxol in MCF10A-TAZΔ72 cells. Conditions and procedures for the experiment are as described in Figure 1B.

Recently, TAZ and its paralogue YAP have been identified as 2 major proteins mediating the effects of the novel Hippo–LATS tumor suppressor pathway (10, 35). Consistent with our finding that TAZ is a novel protein responsible for drug resistance of breast cancer, some of the proteins in the Hippo–LATS pathway have also been shown to be involved in the drug resistance seen in human cancers. For example, we have recently reported that loss of *LATS1* and *LATS2* tumor suppressor genes, 2 negative regulators of TAZ (11), induced resistance of cervical cancer cells to Taxol (15). On the other hand, overexpression of YAP, a TAZ paralogue, also caused resistance of breast cancer cells to multiple chemotherapeutic drug treatments (36). In addition, loss of Mst or RASSF1A, which are upstream activators of LATS, have also been shown to cause drug resistance in prostate cancer and hepatocellular carcinoma (37, 38). Altogether, these studies suggest that the emerging Hippo–LATS tumor suppressor pathway, which has been shown to play important roles in tumorigenesis, may also have significant roles in drug resistance of human cancers. Therefore, it will be very interesting to examine whether and how TAZ and other components of the Hippo–LATS pathway interact to induce drug resistance in breast cancer.

Identification of *Cyr61* and *CTGF* as transcriptional targets of TAZ

Although TAZ has been shown to be involved in regulating various biological functions, the downstream transcriptional targets mediating TAZ function remain largely unknown. By using a 44K whole genome microarray and real-time qRT-PCR, we have identified and confirmed many important cellular genes as novel downstream transcriptional targets of TAZ. Most interestingly, many of these genes including *BMP4*,

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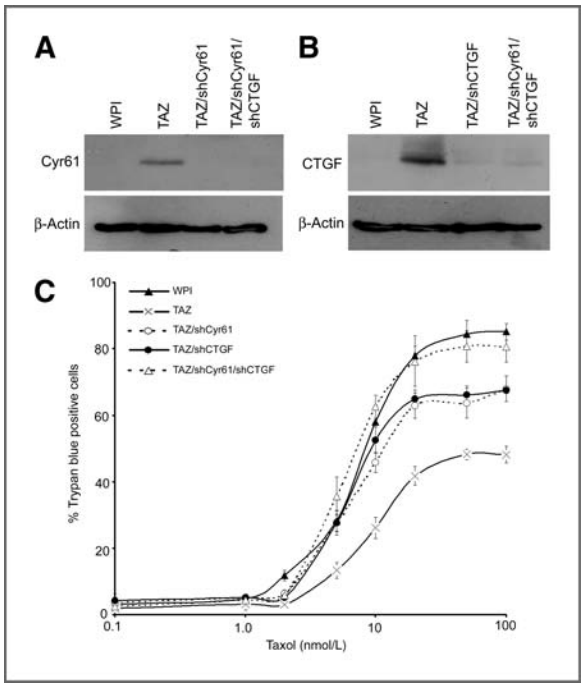

Figure 6. Knockdown of Cyr61 and CTGF reverses TAZ-induced, drug-resistant phenotype. A and B, Western blot analysis of Cyr61 (A) or CTGF (B) expression after shRNA knockdown of Cyr61 (TAZ/shCyr61), CTGF (TAZ/shCTGF), or both (TAZ/shCyr61/shCTGF) in MCF10A-TAZ cells. C, cell death analysis after treatment of cells with increasing concentration of Taxol. Conditions and procedures are as described in Figure 1B.

Cyr61, *CTGF*, *FGF1*, and *PDGFβ* are growth factors secreted from the cells to promote cell proliferation, cell migration/adhesion, and tumorigenesis of mammary epithelial cells (17, 27, 39–42). Other TAZ downstream genes identified such as *IRS-1*, *MYLK/MLCK*, and *PLK2* are also important genes in tumor development and metastasis (43, 44). Therefore, it will be very interesting to examine whether these genes mediate TAZ-induced functions in mammary cells.

In this study, we have characterized and confirmed *Cyr61* and *CTGF* as 2 major bona fide transcriptional targets of TAZ by the following experiments: First, we have shown that overexpression of TAZ in MCF10A mammary epithelial cells caused increased mRNA and protein levels of *Cyr61* and *CTGF*. Second, we have also shown that knockdown of TAZ in these TAZ-overexpressing MCF10A reduced the increased Cyr61 and CTGF protein back to normal levels, whereas reduction of endogenous TAZ by shTAZ in TAZ-high MDA-MB231 reduces Cyr61 and CTGF levels in these cells. Third, we have shown that TAZ activates *Cyr61* and *CTGF* transcription by binding to and activating *Cyr61* and *CTGF* promoters.

After confirming that *Cyr61* and *CTGF* are indeed downstream transcriptional targets of TAZ, we then examined whether Cyr61 and CTGF mediate TAZ-induced drug resistance in human mammary epithelial cells. Significantly, we found that individual knockdown of Cyr61 or CTGF by shRNA partially but double Cyr61/CTGF knockdown completely reversed TAZ-induced Taxol resistance. These studies suggest that *Cyr61* and *CTGF* are 2 critical TAZ transcriptional targets mediating TAZ-induced drug resistance in breast cancer cells.

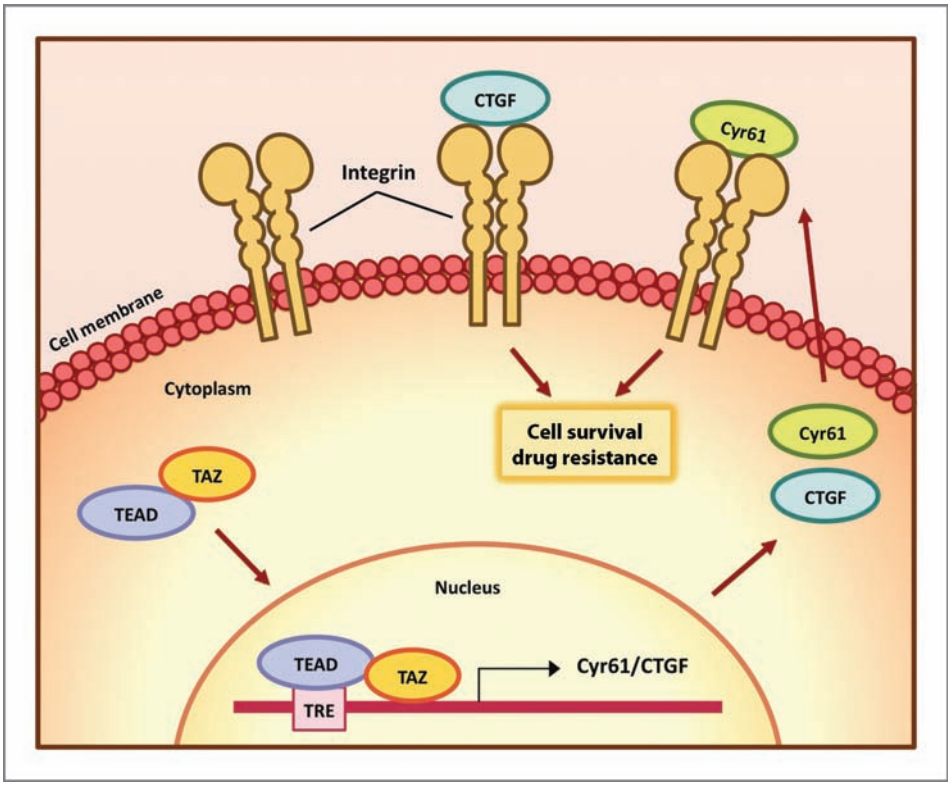

Figure 7. A proposed model for TAZ-induced drug resistance in breast cancer cells. In this model, TAZ and TEAD form a complex which translocates to the nucleus and binds to the TRE on the promoter regions of *Cyr61* or *CTGF* that leads to their transcriptional activation. After proteolytic processing, Cyr61 and CTGF are subsequently secreted out of the cells and activate integrin heterodimers on the cell membrane, consequently resulting in enhanced cell survival and resistance to chemotherapeutic drug-induced apoptosis.

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Together, our studies provided the first biological evidence that overexpression of the *TAZ* oncogene can cause drug resistance of breast cancer cells through direct activation of 2 secreted and matrix-associated proteins Cyr61 and CTGF.

Consistent with our findings, previous studies have also shown that *Cyr61* and *CTGF* are indeed oncogenes that caused resistance of breast cancers to various chemotherapeutic drugs (17, 23, 25, 34). Further studies also showed that both Cyr61 and CTGF caused drug resistance by activating integrin $\alpha_v\beta3$ (Fig. 7), which subsequently activates both MAPK–ERK and PI3K–Akt signaling pathways or by upregulation of the antiapoptotic proteins Bcl-X_L and cIAP1/XIAP (23, 25, 34). We have also found that overexpression of TAZ activated both ERK and PI3K pathways (Lai and Yang, unpublished results). Therefore, further characterization of how integrin and MAPK/PI3K pathways mediate TAZ-induced drug resistance will enhance our understanding of this signaling pathway in mediating TAZ-induced drug resistance in breast cancer cells.

Identification of TEADs as transcription factors mediating TAZ-induced drug resistance

In this study, we have identified the TEAD family of transcription factors as the critical mediators of TAZ-induced *Cyr61* and *CTGF* transcriptional activation and drug resistance. The interaction between TAZ and TEAD is essential for its effect on *Cyr61* and *CTGF* transcription and TAZ-induced, drug-resistant phenotypes. Although the roles of TEADs in breast cancer has not been reported, TEAD1 and TEAD2 have been shown to increase both cell proliferation and survival (45) whereas TEAD4 was found to be the major TEAD mediating TAZ-induced transformation (29, 30). However, it remains unknown whether TEADs are also directly involved in the response of breast cancer cells to chemotherapeutic drug treatments. Therefore, further characterization of TEADs in breast cancer development and drug response will be invaluable for our understanding of the roles of TAZ in mammary tumorigenesis and drug resistance.

In conclusion, we have provided convincing evidence that *TAZ* is a novel gene causing Taxol drug resistance through activation of *Cyr61* and *CTGF* in breast cancer cells as illustrated in our proposed model (Fig. 7). Further confirmation of our findings using an *in vivo* mouse model and clinical breast cancer patient samples will greatly facilitate our efforts in the treatment of drug-resistant breast cancers in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Taxol Resistance in Breast Cancer Cells Is Mediated by the Hippo Pathway Component TAZ and Its Downstream Transcriptional Targets *Cyr61* and *CTGF*

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