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Hippo component TAZ functions as a co-repressor and negatively regulates ΔNp63 transcription through TEAD

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*Running title: TAZ suppresses ΔNp63 transcription as a co-repressor

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Background: TAZ functions as a co-activator by upregulating downstream transcriptional targets as a co-activator
Results: TAZ can negatively regulate transcription of many genes such as ΔNp63 through TEAD transcription factor
Conclusion: TAZ can also function as a transcriptional co-repressor
Significance: Open a new avenue for TAZ function in cancer

ABSTRACT

TAZ is a WW domain-containing transcriptional co-activator and a core component of an emerging Hippo signaling pathway that regulates organ size, tumorigenesis, metastasis, and drug resistance. TAZ regulates these biological functions by up-regulating downstream cellular genes through trans-activation of transcription factors such as TEAD and TTF-1. To understand the molecular mechanism underlying TAZ-induced tumorigenesis, we have recently performed a gene expression profile analysis by overexpressing TAZ in mammary cells. In addition to the TAZ-upregulated genes that were confirmed in our previous studies, we identified a large number of cellular genes that were downregulated by TAZ. In this study, we have confirmed these down-regulated genes (including cytokines, chemokines and p53 gene family members) as *bona fide* downstream transcriptional targets of TAZ. By using human breast and lung epithelial cells, we have further characterized ΔNp63, a p53 gene family member, and shown that TAZ suppresses ΔNp63 mRNA, protein expression and promoter activity through interaction with the transcription factor TEAD. We also show that TEAD can inhibit ΔNp63 promoter activity and TAZ can directly interact with ΔNp63 promoter-containing TEAD binding sites. Finally, we provide functional evidence that downregulation of ΔNp63 by TAZ may play a role in regulating cell migration. Together, this study provides novel evidence that the Hippo component TAZ can function as a co-repressor and regulate biological functions by negatively regulating downstream cellular genes.

The Hippo pathway was originally discovered in *Drosophila* as an evolutionarily conserved tumor suppressor pathway that acts as a key regulator of organ size control (1, 2). This signaling pathway has been shown to control many biological functions such as cell proliferation, apoptosis, cell-cell contact inhibition, stem cell self-renewal, and tissue regeneration (2-10). In mammals, cell-cell contact or increased actin polymerization can activate MST1/2 (mammalian sterile-20 like kinase 1/2), which subsequently activates adaptor proteins Mob1A/1B and scaffold protein Sav1 (salvador) to promote the phosphorylation and activation of LATS1/2 (large tumor suppressor 1/2) kinases. In turn, LATS1/2 phosphorylates downstream transcriptional co-

activators TAZ (transcriptional co- activator with a PDZ-binding domain) and its paralog YAP (yes-associated protein) to promote their cytoplasmic retention and subsequent degradation (11-14). Conversely, dephosphorylated YAP and TAZ are able to enter the nucleus where they interact with multiple transcription factors and exert high transactivation activity.

TAZ is a widely characterized oncogene that is overexpressed or dysregulated in several cancer types including breast (15, 16), lung (17, 18), colorectal (19), and thyroid (20). It is proposed as a major regulator of cell proliferation, cell migration and invasion, epithelial-mesenchymal transition (EMT), human embryonic stem cell (ESC) renewal, and drug resistance (21-27). Within the N-terminus of TAZ lies a TEAD binding domain (TBD) responsible for the interaction with the TEAD family of transcription factors. Mounting evidence over the years has supported TEAD family members as one of the most common binding partners of TAZ, which play crucial roles in mediating many TAZ functions, including cellular growth, proliferation and oncogenic transformation (28-31). The mechanisms underlying TAZ-mediated transcriptional activation of downstream genes through its interaction with transcription factors has been often studied and observed by many research groups. However, there has been little interest in elucidating novel targets negatively regulated by TAZ, as well as addressing their molecular mechanisms and functional implications in tumorigenesis.

In this study, we have identified ΔNp63, a member of the p53 tumor suppressor family, as a significant downregulated target in TAZ overexpressing breast and lung epithelial cells. Moreover, we show that TAZ-induced repression of ΔNp63 transcription is mediated by the TEAD family of transcription factors, and re-introduction of ΔNp63 into TAZ overexpressing cells partially rescues TAZ- induced cell migration. Together, our findings provide the first evidence that TAZ can directly negatively regulate cellular gene transcription by interacting with TEAD transcription factor.

EXPERIMENTAL PROCEDURES

Plasmid construction and Site-directed Mutagenesis

The promoter region of ΔNp63 [nucleotide (nt.) position -1500 to +40] was amplified by PCR from genomic DNA extracted from MCF10A human immortalized mammary cells using the following primers: ΔNp63-pr sense primer: 5'-ATGGTACCTATGTGTGAAGAAATGAATGT TTTGTCTG-3' (Kpn I site is underlined) and ΔNp63-pr antisense primer: 5'-AATCTCGAGAAGATAACAGAACTCAAGTC CCTCTCTCTC-3' (Xho I is underlined). The PCR products were digested with KpnI/XhoI and subsequently cloned into the KpnI/XhoI sites of the pGL3 basic luciferase reporter vector (Promega). Human ΔNp63 cDNA (Addgene) was cloned into the XhoI/MluI sites of the doxycycline (Dox)-inducible pTRIPZ lentiviral vector (Open Biosystems). Mutation of TAZ-F52/53A (F, phenylalanine; A, alanine) was performed by overlapping PCR using TAZ-mutagenic primers. TAZ-S89A-F52/53A mutant was created using overlapping PCR and subsequently cloned into the XhoI/MluI sites of the pTRIPZ lentiviral vector.

Cell culture

MCF10A (human immortalized epithelial breast) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F12 Ham (Sigma-Aldrich) supplemented with 5% horse serum (HS), 1% penicillin-streptomycin, 2.5 mM L-glutamine, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, and 20 ng/mL hEGF. SK-BR-3 (human breast cancer) cells were cultured in McCoy's 5A Modified Medium (Sigma-Aldrich) supplemented with 2.2 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. SK-Luci-6 (human anaplastic lung cancer), HEK293T (human embryonic kidney), COS7 (monkey fibroblast-like kidney), A549 (lung adenocarcinoma), and HCC38 (human ductal breast carcinoma) cells were cultured in RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C with 5% CO_2.

Lentiviral production, infection and establishment of cell lines with stable overexpression of cellular genes

Lentiviral production, purification, titration and infection of overexpressing constructs were performed as described (11). Generation of

ΔNp63-overexpressing stable cell lines was performed by infecting TAZ-overexpressing MCF10A cells with lentivirus expressing Dox-inducible ΔNp63 (pTRIPZ vector) at a multiplicity of infection (MOI) of 2. Generation of stable cell lines with overexpression of TEAD-binding mutants of TAZ was performed by infecting TAZ-low MCF10A cells with lentivirus expressing TAZ-F52/53A-HA (WPI vector) or Dox-inducible TAZS89A-F52/53A-HA at a MOI of 2. Cells were selected 48 hours post-infection using 1μg/mL puromycin.

Microarray and data analysis

Gene expression profile analysis by microarray and data analysis were as described (32).

Transient knockdown of gene expression by small interfering RNA (siRNA)

To knock down TEAD1/3/4, 5×10^4 SK-BR-3 cells were transfected with 50nM of TEAD1/3/4 siRNA [5'-CACAAGACGU CAAGCCUUU-3' (sense)/5'-UUGUGGAUGA AGUUGAUCAUU-3' (anti-sense)] (GE Healthcare) using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer's protocol. Efficiency of knockdown was assessed by western blot 48 hours post-transfection.

Reagents, antibodies, western blot, and Co-immunoprecipitation (Co-IP)

Trichostatin A (TSA) and UNC0631 were purchased from Sigma. The mouse monoclonal antibodies used in this study were obtained from the following companies: anti-TAZ from BD Pharmingen, anti-p63 (4A4), anti-VGLL4, and anti-FLAG (M2) mouse monoclonal antibodies from Sigma-Aldrich, anti-TEAD (TEF-1) from Abcam, and anti-HA (F7) from Santa Cruz Biotechnology. Anti-histone acetylation component antibodies were obtained from Cell Signaling. Protein extraction, western blot analyses, and Co-IP were performed as described (11).

RNA isolation and quantitative reverse transcriptase PCR (qRT-PCR)

Cells were grown to about 70-90% confluency. RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA quantitation and quality was assessed by spectrophotometry and RNAase-free gel electrophoresis. qRT-PCR analyses were performed in duplicates of 200 ng/μL of RNA per sample per reaction, 200 nM gene-specific forward and reverse primers (Table 1), the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen), and ran on the Applied Biosystems ViiA 7 Real-Time PCR System. 18S ribosomal RNA (rRNA) expression was used as an internal control. mRNA expression levels were calculated as described (11) and shown as fold change.

Dual luciferase assay

Triplicate of $5x10^4$ cells/well of SK-BR-3 or SK-LuCi-6 cells were seeded in 12-well plates and transfected with ΔNp63-luc or its mutants (0.1 μg) alone or in combination with TAZ (0.2 μg), TAZ (0.2 μg) plus TEAD (0.1 μg), or their respective mutants using PolyJet reagent (SignaGen). 10 ng/well of renilla luciferase vector (pRL-TK) was used as an internal transfection control. Luciferase activity was assessed 48 hours post-transfection using the Turner Biosystem 20/20 luminometer and the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP) assay

A ChIP-IT Express Enzymatic kit (Active Motif) was used for ChIP analysis of TAZ-S89A and ΔNp63 promoter interaction. MCF10A cells expressing WPI or TAZ-S89A were grown to 70-80% confluency on 150mm dishes. Cells were treated with 1% formaldehyde, lysed, harvested and homogenized using a dounce homogenizer according to the manufacturer's protocol. DNA was enzymatically sheared and the fragmented chromatin was incubated with 2μg of mouse anti-HA (F7) monoclonal antibody. Chromatin was eluted, reverse cross-linked and treated with Proteinase K. Amplification of the ΔNp63 promoter was performed by PCR using the following primers: [5'-ATGGTACCGTCTGTCT CCTGGGTTTG-3' (sense) and 5'-GTGCACT TTCTTATGAAAGAGAC-3'(anti-sense)]. The PCR products were ran on a 3% ethidium bromide agarose gel and visualized under UV light using the Gel Doc system.

Wound healing cell migration assay

MCF10A-WPI, MCF10A-TAZ and MCF10A-TAZ-ΔNp63 cells were grown to 80% confluence, serum-starved overnight in 2% horse serum (HS), and scratch-wounded 24 hours later using a P20 pipette tip. Cell migration was monitored and pictures were taken at 0, 20 or 40 hours under white light at 10× magnification using the Nikon Eclipse TE-2000U Inverted Microscope and Nikon Coolpix 990 camera. Distance migrated (pixels) was measured with Adobe Photoshop software. MCF10A-TAZ-S89A, MCF10A-TAZ-S89A-F52/F53A, MCF10A-TAZ-S89A-WWm, and HBE135-TAZ-S89A cells were untreated (-) or treated (+) with Dox (1 μg/ml), and A549 and HCC38 cells infected with siTAZ, and subsequently plated and scratched-wounded as previously above. Pictures were taken at 24 or 48 h.

Statistic analysis

Significant differences were analyzed by student *t*-test, and difference on mRNA levels between MCF10A-TAZ and its mutants, as well as promoter activities were calculated using ANOVA tests. P-value <0.05 was regarded as statistically significant.

RESULTS

Identification and validation of target genes negatively regulated by TAZ

To identify downstream genes mediating TAZ function, we performed a 44K whole human genome microarray profiling (32) using RNAs from MCF10A stably expressing WPI empty vector control (MCF10A-WPI) or wild-type TAZ (MCF10A-TAZ). Enhanced TAZ mRNA and protein expression levels were confirmed by qRT-PCR and western blot (Fig. 1A and 1B). Although 390 genes were upregulated by TAZ (32), surprisingly, about 328 cellular genes were also found to be downregulated by TAZ (Fig. 1C; Table S1). Based on their functional relevance in tumorigenesis, we confirmed several target genes from our DNA microarray results using real-time qRT-PCR. Interestingly, we identified several pro-inflammatory cytokines and chemokines, including IL-1α/β, IL-6, IL8, CXCL1/2/3 and BMP2, as well as p63 isoforms ΔNp63 and TAp63 as significantly downregulated（1.5-10±0-0.05 fold) targets in MCF10A-TAZ cells (Fig. 1D). Amongst these, pro-inflammatory cytokines IL-1α and IL-1β showed the most significant decrease in their relative mRNA expression, suggesting a possible involvement of TAZ in immune and inflammatory responses. Surprisingly as well, the p53 family members TAp63 and its N-terminal truncated form ΔNp63 showed an important TAZ-induced suppression in mRNA expression levels. Since p63 has been previously suggested as a marker of epithelial breast carcinoma and plays important roles in tumorigenesis and metastasis (33, 34), we sought to elucidate the molecular mechanisms and functional implications of TAZ-mediated repression of p63.

ΔNp63 is a novel downregulated target of TAZ

To further confirm TAZ-mediated suppression of p63 in MCF10A cells, we performed protein analysis using western blotting. Protein lysates from MCF10A-WPI and MCF10-TAZ cells were analyzed together with MCF-7 p63 negative control and Cos7 cells transfected with TAp63-Flag and ΔNp63-Flag plasmids. We identified ΔNp63 rather than TAp63 as the p63 protein isoform predominantly suppressed by TAZ in MCF10A-TAZ cells (Fig. 2A). Similarly, ΔNp63 protein expression was highly decreased in MCF10A cells after transient induction of wild-type TAZ (TAZ-WT) or constitutively active TAZ [TAZ-S89A, serine 89 (S89) is mutated into alanine (A)] by Dox (Fig. 2B), suggesting that suppression of ΔNp63 by TAZ is not caused by viral infection or puromycin selection during establishment of stable lines. As expected, inducible expression of TAZ-S89A displayed a stronger effect on ΔNp63 repression compared to TAZ-WT. To characterize p63 as a *bona fide* downregulated target gene of TAZ in other cell type, we analyzed ΔNp63 protein expression levels in HBE135 human bronchial epithelial lung cells containing inducible expression of TAZ-S89A (Fig. 2C). Interestingly, ΔNp63 expression was also repressed in HBE135 cells after TAZ induction, similar to the effect observed in MCF10A cells. Furthermore, we performed transient TAZ knockdown using siRNAs (siTAZ) in HCC38 human epithelial breast cancer cells and A549 human lung adenocarcinoma cells that expressed high levels of TAZ (35)(Yang, unpublished). ΔNp63 protein levels were significantly increased after TAZ was knocked down in both cell lines (Fig. 2D). Finally, we sought to elucidate the

effects of TAZ overexpression on ΔNp63 promoter activity using dual luciferase assay. The promoter region of ΔNp63 was cloned from MCF10A genomic DNA into a luciferase reporter vector (ΔNp63-luc), which was transiently transfected into TAZ-low SK-BR-3 breast cancer cells alone or in combination with increasing dosages of TAZ (Fig. 2E). Concordant with our previous results, TAZ showed a dosage-dependent suppression of ΔNp63-luc activity, suggesting that TAZ causes reduced ΔNp63 expression by suppressing its promoter activity. Together, these results strongly suggest ΔNp63 as a *bona fide* downstream target negatively regulated by TAZ in several breast and lung cell lines.

TEAD binding domain (TBD) is necessary for TAZ-induced negative regulation of ΔNp63

TAZ was originally identified as a transcriptional co-activator that lacks a DNA-binding domain. Thus, in order to modulate transcription of downstream cellular genes, TAZ requires interactions with transcription factors through its TBD or WW (W, tryptophan) domain. Previous studies have shown that TAZ interacts with members of the TEAD family of transcription factors (TEAD1-4) through 7 conserved residues in the TBD of TAZ (23, 28). Among these, two phenylalanine (F) residues located at positions 52 and 53 (F52/53) were shown to be critical for TAZ-TEAD binding. Therefore, we examined whether the effect of TAZ on ΔNp63 repression could be abolished in TBD mutants of TAZ. We generated a missense mutation in TAZ F52 and F53 residues (TAZ-F52/53A) to abolish TEAD interaction with TAZ. We then established MCF10A cells stably expressing TAZ-F52/53A and examined the effect of this TAZ mutant on mRNA and protein expression of ΔNp63. qRT-PCR and western blot analysis showed that loss of interaction with TEAD in TAZ-F52/53A mutant completely abolished TAZ-induced suppression of both protein and mRNA of ΔNp63 (Fig. 3A and 3B). The TAZ-F52/F53A mutant also showed inability to suppress ΔNp63 promoter activity (Fig. 3C). Interestingly, loss of interaction with TEAD in TAZ-F52/F53A had a dominant-negative effect and activates transcription (1-28±0-1 fold) of many downregulated genes including ΔNp63, BMP2, CXCL2, CXCL3, IL1α, and IL-1β (Fig. 3B and 3D). On the other hand, overexpression of TAZ-F52/53A can still suppress TAp63 mRNA, suggesting that TAZ regulates TAp63 and ΔNp63 differently and that TAZ may down-regulate TAp63 independently of TEAD. Next, we sought to explore whether TAZ's TBD was the only domain responsible for p63 suppression. Since TAZ WW domain has been shown critical for gene transcription regulation through interaction with L/PPxY (L, lysine; P, proline; x, any amino acid; Y, tyrosine) motif-containing transcription factors such as TTF1 and Pax8 (36-38). We tested whether a WW domain TAZ mutant (TAZ-WWm) containing two residue mutations, W152A and P155A, had any effect on TAZ-induced suppression of ΔNp63. MCF10A cells stably expressing TAZ-S89A-WWm were established by infecting MCF10A cells with lentivirus expressing inducible TAZ-S89A-WWm (MCF10A-TAZ-S89A-WWm). Similarly to the effect observed in MCF10A-TAZ-S89A, in the presence of Dox, the WW domain mutant TAZ-WWm, effectively suppressed ΔNp63 expression (Fig. 3A). Together, these findings suggest that the TBD, but not the WW domain, of TAZ, is essential for TAZ-induced repression of ΔNp63.

TEAD is essential for TAZ-induced suppression of ΔNp63

To directly confirm whether TAZ suppresses ΔNp63 through TEAD, we performed transient knockdown of TEAD using a previously used siRNA simultaneously targeting TEAD1, TEAD3, and TEAD4 (siTEAD) in MCF10A cells with inducible expression of constitutively active TAZ-S89A. While ΔNp63 protein and mRNA was significantly repressed by TAZ-S89A in the presence of Dox in cells expressing a siRNA negative control (siCtrl), TAZ-S89A-mediated repression of ΔNp63 seemed to be abolished in MCF10A cells with TEAD knockdown (Fig. 4A and 4B). In addition, TEAD knockdown also diminished TAZ-induced suppression of ΔNp63 promoter (Fig. 4C). Moreover, while TAZ can suppress ΔNp63-luc activity in TEAD-positive SK-BR3 cells, its suppression on ΔNp63 promoter is abolished in a TEAD-negative SK-Luci-6 lung cancer cells (Fig. 4D and 4E). Together, these studies strongly suggest that TEAD is essential for TAZ-induced suppression of ΔNp63.

TAZ suppresses ΔNp63 by directly binding to the ΔNp63 promoter through TEAD

Next, we tested whether TAZ suppresses ΔNp63 transcription by directly interacting with ΔNp63 promoter through TEAD. Since TEAD is required for TAZ-induced suppression of ΔNp63 transcription, we first tested whether TEAD can directly suppress ΔNp63 promoter activity by transfecting ΔNp63-luc reporter alone or together with TEAD1, TEAD2, TEAD3, or TEAD4 into SK-BR-3 cells. Significantly, all TEADs showed ΔNp63 promoter repression (Fig. 5A), although TEAD1/3/4 exerted a more dramatic effect than TEAD2 in ΔNp63 repression with over 3.5±1.2 fold decrease in the promoter activity, suggesting that TEADs are involved in ΔNp63 repression. To confirm that TAZ-TEAD indeed directly binds to ΔNp63 promoter**,** we performed a ChIP assay in MCF10A cells expressing WPI vector or TAZ-S89A-HA using anti-HA antibody and primers flanking a TEAD response element (TRE; Fig. 5C, TRE1). Interestingly, our ChIP assay showed that TAZ-S89A could indeed be co-immunoprecipitated with the ΔNp63 promoter DNA *in vivo* (Fig. 5B). After further examination of ΔNp63 promoter sequences, we identified 3 potential TREs (TRE1, TRE2, and TRE3) in the ΔNp63 promoter and mutated them individually (TRE1M, TRE2M or TRE3M) or in combination (TRE1/2; Fig. 5C)**.** While mutation of TRE1 or TRE2 rather than TRE3 partially blocked, combined mutations of both TRE1 and TRE2 (TRE1/2M) completely abolished TAZ-induced suppression of ΔNp63 promoter (Fig. 5C), thus suggesting that TAZ/TEAD complex binds to TRE1 and TRE2 to suppress ΔNp63 transcription.

Modulation of deacetylation is critical for TAZ-TEAD-induced suppression of *ΔNp63*

Two recent studies suggest that TEAD or its *Drosophila* homolog Scallop can suppress cellular gene transcription through interaction with transcription cofactor vestigial-like protein 4 (VGLL4) or Tgi, respectively (39, 40). However, VGLL4 knockdown in MCF10A cells could not block TAZ-induced suppression of *ΔNp63* (Fig. 6A and 6B), suggesting that VGLL4 is not involved in TAZ-TEAD-induced transcriptional repression of *ΔNp63*.

Previous studies also suggest that transcriptional suppression of some genes may depend on DNA methylation or histone deacetylation of chromatin of their promoter regions (41-43). To explore whether TAZ-TEAD-induced *ΔNp63* transcriptional repression is due to chromosome methylation/acetylation, we treated breast cancer cells with inhibitors of histone modification. Significantly, treatment of cells with HDAC (histone deacetylase) inhibitor TSA rather than HMT (histone methyltransferase) inhibitor UNC0631, partially rescued TAZ-induced suppression of ΔNp63 transcription (Fig. 6C). Moreover, we have further shown that TAZ directly interacts *in vivo* with some components (CHD4, MTA1, and RBP46) of the HDAC complex (Fig. 6D).

Functional implications of TAZ/TEAD interactions and ΔNp63 suppression

Our results have strongly elucidated the role of TEAD and its co-activator TAZ in p63 transcriptional repression, particularly ΔNp63, in breast and lung epithelial cells. Since TAZ overexpression has been previously correlated with enhanced cell migration and invasion (14, 15, 44), and ΔNp63 knockdown causes EMT, increased cell migration and metastasis in MCF10A cells or breast cancer (45-47), we sought to elucidate the functional consequences of TAZ/TEAD-mediated repression of ΔNp63 in breast cell migration. Firstly, we reintroduced ΔNp63 expression in MCF10A-TAZ cells by using lentiviral infection. Assessment of similar TAZ and ΔNp63 protein expression levels was performed by western blot (Fig. 7A). Next, we sought to examine the functional implications of ΔNp63 suppression on cell migration by performing a wound-healing assay in MCF10A cells expressing WPI vector control, TAZ or TAZ plus ΔNp63. Cell migration was compared at different time points after wound induction. Compared to the WPI control (MCF10A-WPI), TAZ overexpressing cells (MCF10A-TAZ) increased cell migration 9.0±1.5 and 8.8±3.5 fold at 20 and 40 h, respectively. However, this effect was partially abolished in TAZ-ΔNp63 expressing cells (MCF10A-TAZ-ΔNp63)(Fig. 7B and 7C).

Interestingly, further examination of TAZ and its two domain mutants showed that while Dox-induction of TAZ-S89A (+Dox) caused increased cell migration, TBD mutant TAZ-F52/53A completely abolished TAZ-induced

increased cell migration in MCF10A cells (Fig. 3A, 7D). On the other hand, WW domain mutant TAZ-WWm can still cause increased cell migration (Fig. 7D), suggesting that TEAD-binding domain, rather than WW domain, is essential for TAZ-induced increased cell migration. Furthermore, enhanced cell migration is also observed when TAZ-S89A is overexpressed in the presence of Dox (Dox+) in HBE135 cells (Fig. 2C, Fig. 7E). Moreover, down-regulation of TAZ in A549 and HCC38 cells inhibits cell migration (Fig. 7F). Finally, we have further shown that overexpression of TAZ-S89A in both breast MCF10A and lung HBE135 cells causes epithelial-mesenchymal transition (EMT), thus suggesting that this TAZ-induced increased cell migration is due to loss of cell-cell adhesion (Fig. 7G and 7H). In summary, our results suggest that TAZ interacts with TEAD to promote ΔNp63 suppression and reduced cell-cell adhesion, thus increasing the migratory capacity of cells, which can further lead to metastatic progression in breast and lung cancer cells.

DISCUSSION

TAZ is a dual regulator of gene transcription

Studies have widely characterized TAZ as a transcriptional co-activator of gene expression. Its ability to interact with a wide range of transcription factors accounts for TAZ's multi-functional effects in tumor development and progression. Moreover, TAZ-induced activation of pro-tumorigenic genes, such as Cyr61, CTGF, BMP4 (32, 44) and many others, has been often reported in the literature. However, our DNA microarray data have uncovered a whole new perspective on TAZ transcriptional regulation. Besides its well-studied role as a transcriptional co-activator, our results have suggested a novel function of TAZ in transcriptional repression. This transcriptional duality of TAZ has been previously questioned after observing that TAZ-induced activation of RUNX2 and repression of PPAR-γ's transcriptional activity was critical for mesenchymal stem cell differentiation (48). Recently, phosphorylation of Y316 of TAZ has been shown to promote TAZ interaction and repression of NFAT5's transcriptional activity in response to hyperosmotic stress (49). Although these studies have shed light on the transcriptional repressing potential of TAZ, they have failed to elucidate the molecular mechanisms and oncogenic functions underlying TAZ-induced suppression. The fact that our microarray results have shown that transcription of over 320 cellular genes can be suppressed by TAZ has uncovered a new layer of the signaling complexity of TAZ and the Hippo pathway. Furthermore, validation analysis of several target genes including p63 and pro-inflammatory cytokines and chemokines, has indeed confirmed that TAZ expression is also critical for transcriptional inhibition. Moreover, the strong repression of IL-1α/β exerted by TAZ has suggested a novel role in modulating the inflammatory response and tumor microenvironment. In fact, TAZ was found to indeed repress IL-1β promoter in the same manner as shown for ΔNp63 (data not shown). Collectively, our results have elucidated an underrated role of TAZ as a negative regulator of transcription in breast and lung epithelial cells. Moreover, we have uncovered TAZ's duality in gene transcription regulation, a trait that has been previously reported for other transcriptional co-factors, such as CCAAT-enhancer binding protein (C/EBP) or CREB-binding protein (CBP) and its paralog p300 (50, 51).

ΔNp63 is a novel downstream target negatively regulated by TAZ

Despite the progress made towards elucidating the molecular mechanisms involved in breast cancer development and progression, metastatic cancer cells remain a major obstacle for successful breast cancer treatments. In this context, TAZ has been highly associated with cell acquisition of EMT phenotypes and subsequent metastatic dissemination of breast cells (14, 15, 32, 52). By regulating gene expression, TAZ has shown to modulate oncogenic traits in cells. However, the transcriptional downstream targets mediating these TAZ-induced phenotypes remain mostly unexplored. By using a DNA microarray and real-time qRT-PCR, we identified p63 isoforms TAp63 and ΔNp63 as transcriptional targets negatively regulated by TAZ in mammary tumorigenesis. Of these, ΔNp63 showed the most significant repression and was shown to be the predominant isoform expressed in MCF10A cells. Therefore, we characterized ΔNp63 as a *bona fide* negative transcriptional target of TAZ involved in cell migration. Firstly, we have shown that TAZ overexpression in MCF10A non-tumorigenic

breast cells causes a significant decrease in ΔNp63 mRNA expression levels (Fig. 1D). Next, we have shown that overexpression of both wild-type TAZ and its constitutively active mutant TAZ-S89A suppresses ΔNp63 protein expression in MCF10A cells, whereas TAZ knockdown caused increased ΔNp63 in breast and lung cancer cells (Fig. 2A, 2B, and 2D). Finally, we have confirmed that TAZ physically interacts with ΔNp63 promoter and causes ΔNp63 transcriptional repression through interaction with TEAD and TRE (Fig. 2E).

After confirming that ΔNp63 is indeed a real downstream target negatively regulated by TAZ, we examined the functional implications of ΔNp63 down-regulation in mammary epithelial cells. After re-introducing ΔNp63 protein expression into TAZ-overexpressing MCF10A cells, we found that ΔNp63 could partially reverse TAZ-mediated cell migration (Fig. 7A-7C). Specifically, assessment of the migration distance of MCF10A cells expressing TAZ plus ΔNp63, showed a significantly slower wound closure rate that was more similar to the one displayed by MCF10A-WPI control than to MCF10A-TAZ cells. Since multiple genes may regulate breast cancer cell migration, ΔNp63 re-expression could not completely reverse this TAZ-mediated phenotype. Nonetheless, our studies strongly suggest that ΔNp63 is one of the genes involved in TAZ-induced cell migration. These results are consistent with studies showing ΔNp63 as an inhibitor of cell migration, invasion and metastatic progression in breast cells (45, 46). Moreover, p63 and particularly ΔNp63 expression has been specifically observed in normal myoepithelial breast cells, has been proposed as a marker for cell differentiation and is shown to be down-regulated in non-metaplastic invasive breast carcinomas (33, 53-55). Importantly, TAZ overexpression is highly correlated with breast cancer invasiveness and dissemination (32). Thus, it is likely that TAZ-induced suppression of ΔNp63 enhances its metastatic potential by promoting EMT and breast cell migration. It will be interesting to further investigate the correlation between the levels of TAZ and ΔNp63, and whether these could be used as prognostic biomarkers for clinical metastatic breast cancer. Until then, our results have provided a better understanding of the molecular mechanisms of TAZ-induced metastatic dissemination that might be critical for future development of effective targeted therapies for breast and lung cancer patients.

TEADs are critical binding partners of TAZ that suppresses ΔNp63 transcription

Our results have identified the members of TEAD/TEF family as major transcription factors mediating TAZ-induced transcriptional repression of downstream genes particularly *ΔNp63*. We showed that TAZ could no longer suppress ΔNp63 promoter activity in TEAD-null SK-luci-6 cells or MCF10A cells with TEAD knockdown (Fig. 4D and 4E). This TAZ-TEAD-mediated suppression seems to be direct since our results have shown that TEAD itself can suppress ΔNp63 promoter activity and that TAZ can suppresses ΔNp63 promoter activity by directly interacting with two TREs on the ΔNp63 promoter (Fig. 5B and 5C). Consistent to our findings, through ChIP-sequencing, a recent study also suggests that TEAD2 can regulate EMT-relevant genes by acting as a transcriptional activator or repressor, mainly by directly binding to the promoters containing TREs (56). It is still unclear why the same TAZ/TEAD complex activates transcription of some cellular genes such as *CTGF* and *Cyr61* but suppresses transcription of other genes such as *ΔNp63*. Most interestingly, several recent studies have identified components of the chromatin/chromatin-remodeling complexes (BRM, MED mediator complexes, and SWI/SNF complexes) and histone methyltransferase (Ncoa6) complexes as binding partners of TAZ or its *Drosophila* homolog Yki (57-60). Since these complexes control transcriptional status (activation or inactivation) of a specific gene, the methylation or acetylation status of chromatin on the promoter regions of a specific gene (soil) may determine the transcriptional activation or suppression functions of the TAZ-TEAD complex. Indeed, our data further showed that TAZ can directly interact with histone deacetylation complex (Fig. 6D) and inhibition of HDAC partially releases TAZ-induced suppression of ΔNp63 transcription by directly interacting with histone deacetylation complex (Fig. 6C), suggesting that TAZ may suppresses gene transcription by activating histone deacetylation and HDAC-mediated chromatin tightening.

Besides TEAD-dependent transcriptional suppression by TAZ, we also observed TEAD-

independent suppression of some downstream genes such as TAp63 and GJA1 (Fig. 3D). In addition, a recent study reported a ZEB2-dependent suppression of *ΔNp63* promoter by YAP (TAZ paralog) during squamous transdifferentiation of lung epithelial cells (61). These studies suggest that TAZ, like YAP, may also suppress cellular gene transcription through interacting with other transcription factors. Nevertheless, our findings have shed light on the unanticipated complexity of the mechanisms underlying TAZ and TEAD interaction for gene transcriptional regulation, and provide convincing evidence that TAZ can exert its function by negatively regulating transcription of downstream cellular genes, such as *ΔNp63*, through interaction with TEAD transcription factor.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. Validation of target genes negatively regulated by TAZ using real-time qRT-PCR. *A*, Western blot analysis of TAZ expression in MCF10A cells. MCF10A cells were stably infected with lentivirus expressing WPI vector control or TAZ-HA. Western blot analysis was performed by using anti-TAZ antibody. β-actin was used as an internal loading control. *B* and *D*. qRT-PCR analysis of TAZ (*B*) and its down-regulated cellular gene mRNA expression (*D*). Total RNAs were extracted from MCF10A-WPI and MCF10A-TAZ cells. mRNA levels were measured by real-time qRT-PCR using gene-specific primers (Table 1). Relative expression levels of mRNA in MCF10A-TAZ (black bars) were compared to MCF10A-WPI control cells (white bars). Data is represented as relative fold decrease. The experiment was performed in duplicate and error bars represent standard deviation from each set of duplicate. Statistic difference on mRNA levels between MCF10A-WPI and MCF10A-TAZ cells were analyzed by student *t*-test. *Statically significant difference ($P<0.05$). *C*. Heat map for down-regulated genes by TAZ.

Figure 2. Validation of *ΔNp63* as a downstream transcriptional target of TAZ. *A*, p63 protein expression levels were assessed in MCF10A cells overexpressing WPI control or TAZ using anti-p63 antibody. MCF-7 protein cell lysate was used as a negative control for p63 staining. Cos7 cells transfected with TAp63-Flag or ΔNp63-Flag expression were used as positive controls. β-actin was used as an internal loading control. *B*, ΔNp63 and TAZ protein levels were assessed in MCF10A cells in the presence (+) or absence (-) of doxycycline (Dox)-mediated inducible expression of wild-type (TAZ-WT) or constitutively active TAZ (TAZ-S89A). *C*, Expression of ΔNp63 in MCF10A mammary and HBE135 lung epithelial cells. TAZ expression was induced with Dox (+) in MCF10A-TAZ or HBE135-TAZ-S89A cells. *D*, Knockdown of TAZ in breast and lung cancer cells caused enhanced protein expression of *ΔNp63*. HCC38 breast and A549 lung cancer cells were transiently transfected with control siRNA (siCtrl) or siRNA against TAZ (siTAZ). Three days after transfection, cells were subjected to protein extraction and western blot analysis using anti-TAZ or anti-p63 antibody. *E*, TAZ suppresses ΔNp63 promoter activity. SK-BR3 cells grown in 12-well plate were transfected with ΔNp63-luc alone (0.1 μg) or in combination with increasing amount (0, 0.1, 0.4 μg) of TAZ, followed by dual luciferase assay. Fold changes were calculated by normalizing SK-BR3 cells transfected with ΔNp63-luc alone to those transfected with TAZ. The experiment was performed in triplicate. *Statistically significant difference ($P<0.05$).

Figure 3. TEAD-binding domain is essential for TAZ-induced transcriptional repression of ΔNp63. *A,* TEAD binding domain is critical for ΔNp63 repression. Constitutively active TAZ-S89A with mutations on TEAD binding (S89A-F52/53A) or WW (S89A-WWm) domains were induced with Dox in MCF10A cells. ΔNp63 and TAZ protein expression was assessed and compared to MCF10A cells with inducible expression of TAZ-S89A (S89A). β-actin was used as an internal loading control. *B,* qRT-PCR analysis of ΔNp63 mRNA in MCF10A cells expressing WPI, TAZ, or TAZ-F52/53A. *C,* TEAD-binding mutant of TAZ abolishes TAZ-mediated suppression of ΔNp63 promoter. SK-BR-3 cells were transfected with ΔNp63-luc alone or in combination with wild-type TAZ (TAZ) or its TEAD binding mutant (TAZ-F52/53A). Promoter activity was measured as described in Fig. 2E. The experiment was performed in triplicate. *D*, qRT-PCR analysis of TAZ-down-regulated genes in MCF10A-WPI, TAZ, or TAZ-F52/F53A cells. Procedures and data analyses were performed as described in Fig. 1B. *Statistically significant difference ($P<0.05$).

Figure 4. TEAD-dependent suppression of ΔNp63 by TAZ. *A,* TEAD knockdown diminishes TAZ-induced repression of ΔNp63 protein**.** Transient siRNA knockdown of TEAD1/3/4 (siTEAD) was performed in MCF10A cells with inducible expression of TAZ-S89A. A siRNA targeting a non-specific sequence was used as a negative control (siCtrl). Twenty-four hours post-transfection, cells were induced (+) or not (-) with Dox. Protein was extracted 48 hours post-induction and ΔNp63 expression was assessed in cells with and without TAZ-S89A expression. β-actin was used as an internal loading control. *B,* Knockdown of TEAD abolishes TAZ-induced suppression of ΔNp63 mRNA. qRT-PCR analysis of

ΔNp63 mRNA. Cell lines and treatment conditions were as described in *A*. *C,* Knockdown of TEAD by siRNA partially blocks TAZ-induced suppression of ΔNp63 promoter activity. *D,* Expression of TEAD in MCF10A-WPI, MCF10A-TAZ, SK-Luci-6 and SK-BR-3 cells. *E*, TAZ fails to inhibit TAZ promoter in TEAD-negative SK-Luci6 cells. Luciferase analysis was performed as described in Fig. 2E. *Statistically significant difference (P<0.05).

Figure 5. TAZ and TEAD are recruited on *ΔNp63* promoter through TREs to directly suppress *ΔNp63* transcription. *A*. TEADs repress *ΔNp63* promoter activity. SK-BR-3 cells were transfected with ΔNp63-luc alone or in combination with TEAD1, TEAD2, TEAD3 or TEAD4, and luciferase assay was performed as described in Fig. 2E. *B*. ChIP analysis of TAZ interaction with the ΔNp63 promoter. DNA and protein were cross-linked after treating MCF10A-WPI and MCF10A-TAZ-S89A-HA cells with 1% formaldehyde. Chromatin and DNA-binding protein were subjected to immunoprecipitation using mouse monoclonal α-HA (F7) antibody, followed by PCR and electrophoresis on a 3% agarose gel. 0.2% input chromatin extracted from MCF10A-WPI or MCF10A-TAZ-S89A-HA cells were used as positive PCR controls. *C*. Mapping the TRE in the *ΔNp63* promoter. Three potential TREs [TRE1 (GGAAT), TRE2 (CATGCC), and TRE3 (GGTAT)] in the *ΔNp63* promoter were mutated [TRE1M (AAAAA), TRE2M (AAAAAA), and TRE3M (AAAAA),] alone or in combination (TRE1/2M). ΔNp63-luc containing WT, TRE1M, TRE2M, TRE3M, or TRE1/2M were transfected alone (-TAZ control) or together with TAZ (+TAZ) into SK-BR3 cells, followed by dual luciferase assay. Promoter activity is shown as relative to control and calculated as the ratio of relative luciferase unit (RLU) of +TAZ to –TAZ. The mean and SD of three experiments are shown. *Statistically significant difference (P<0.05) between RLU of +TAZ and -TAZ.

Figure 6. Suppression of *ΔNp63* transcription by TAZ through modulation of chromatin acetylation rather than VGLL4. *A*. Knockdown of VGLL4 by siRNA. MCF10A-TAZ-S89A cells were transfected with control siRNA (siCtrl) or siRNA against VGLL4 (siVGLL4), followed by incubation in the absence (-) or presence (+) of Dox for 1 d. Cells were subjected to protein extraction and western blot analysis using anti-p63 and anti-VGLL4 antibodies. *B*. qRT-PCR analysis of ΔNp63 mRNA. Experimental procedures were as described in *A*. *C*. Levels of *ΔNp63* mRNA after treatment of cells with HDAC and HMT inhibitors. MCF10A-TAZ-S89A cells were untreated or treated with TSA (300 nM) or UNC0631 (20 μM) in the absence (-) or presence (+) of Dox for 1 day, followed by qRT-PCR analysis. Data analysis were described in Fig. 1B. *D*. Interaction of TAZ with histone deacetylase complex. Co-IP analysis was performed by immunoprecipitation of TAZ-HA in 250 μg of protein lysates extracted from MCF10A-WPI and MCF10A-TAZ-S89A-HA cells using anti-HA antibody, followed by western blot using each specific antibody against each protein of the histone deacetylase complex. The membrane was stripped and re-probed with anti-HA antibody to see whether TAZ-S89A-HA was pulled down from MCF10A-TAZ-S89A-HA rather than WPI cells. About 1/100 of input protein lysate (2.5 μg) was also subjected to western blot. *Statistically significant difference (P<0.05).

Figure 7. Reintroduction of ΔNp6 partially recues TAZ-mediated cell migration. *A*. Western blot analysis of ΔNp63 expression. MCF10A-TAZ cells were infected with lentivirus expressing ΔNp63 (MCF10A-TAZ-ΔNp63). Protein was extracted from these cells and ΔNp63 expression was compared to MCF10A-WPI and MCF10A-TAZ cells. β-actin was used as an internal loading control. *B*. ΔNp63 reintroduction in TAZ overexpressing cells partially rescues TAZ-induced increased cell migration. MCF10A-WPI, MCF10A-TAZ and MCF10A-TAZ-ΔNp63 were plated to confluency and starved in 2% HS overnight. Wound healing assay was performed and cell migration was analyzed between cells at different time points (0, 20, 40h). *C.* Quantification of cell migration. Cell migration distance (pixels) was quantified in all cells as described in *B*. *Statistically significant difference (P<0.05) between MCF10A-TAZ and MCF10A-WPI or MCF10A-TAZ-ΔNp63. *D*. TEAD-dependent increased cell migration by TAZ. Cell migration analyses were performed using the established cell lines and conditions described in Fig. 3A. *E*. Overexpression of TAZ-S89A causes increased cell migration in HBE135 cells. Wound-

healing analyses were performed in cell lines described in Fig. 2C. *F*. Knockdown of TAZ in HCC38 and A549 cells decreases cell migration. *G. and H*. Overexpression of TAZ-S89A induces EMT in both MCF10A (*G*) and HBE135 (H) cells.

Table 1. Primers used for qRT-PCR

TAP63	F	GGACTGTATCCGCATGCA
	R	GACCTGGGCTGTGCGTAG
Np63	F	GAGTTCTGTTATCTTCTTAAG
	R	TGTTCTGCGCGTGGTCTG
BMP2	F	TGCGCATGCTTCCACCATGAAG
	R	TCTGCTGSGGTGATAAACTCC
CXL1	F	AGTCATAGCCACACTCAAGAATGG
	R	GATGCAGGATTGAGGCAAGC
CXL2	F	CGCCCAAACCGAAGTCATAG
	R	AGACAAGCTTTCTGCCCATTCT
CXL3	F	TCCCCCATGGTTCAGAAAATC
	R	GGTGCTCCCCTTGTTCAGTAT
IL1A	F	TGTGACTGCCCAAGATGAAG
	R	CTTAGCGCCGTGAGTTTCCC
IL1B	F	GAAGCTGATGGCCCTAAACAG
	R	GAAGCCCTTTGCTGTAGTGGT
IL6	F	TCCTCGACGGGCATCTCAGCC
	R	ATCTTTGGAAGGTTCAGGTTG
IL8	F	CGGAAGGAACCATCTCACTG
	R	AGCACTCCTTGGCAAAACTG
GJA1	F	ACA CCT TCC CTC CAG CAG TT
	R	GGA GTT CAA TCA CTT GGC GT

Figure 1

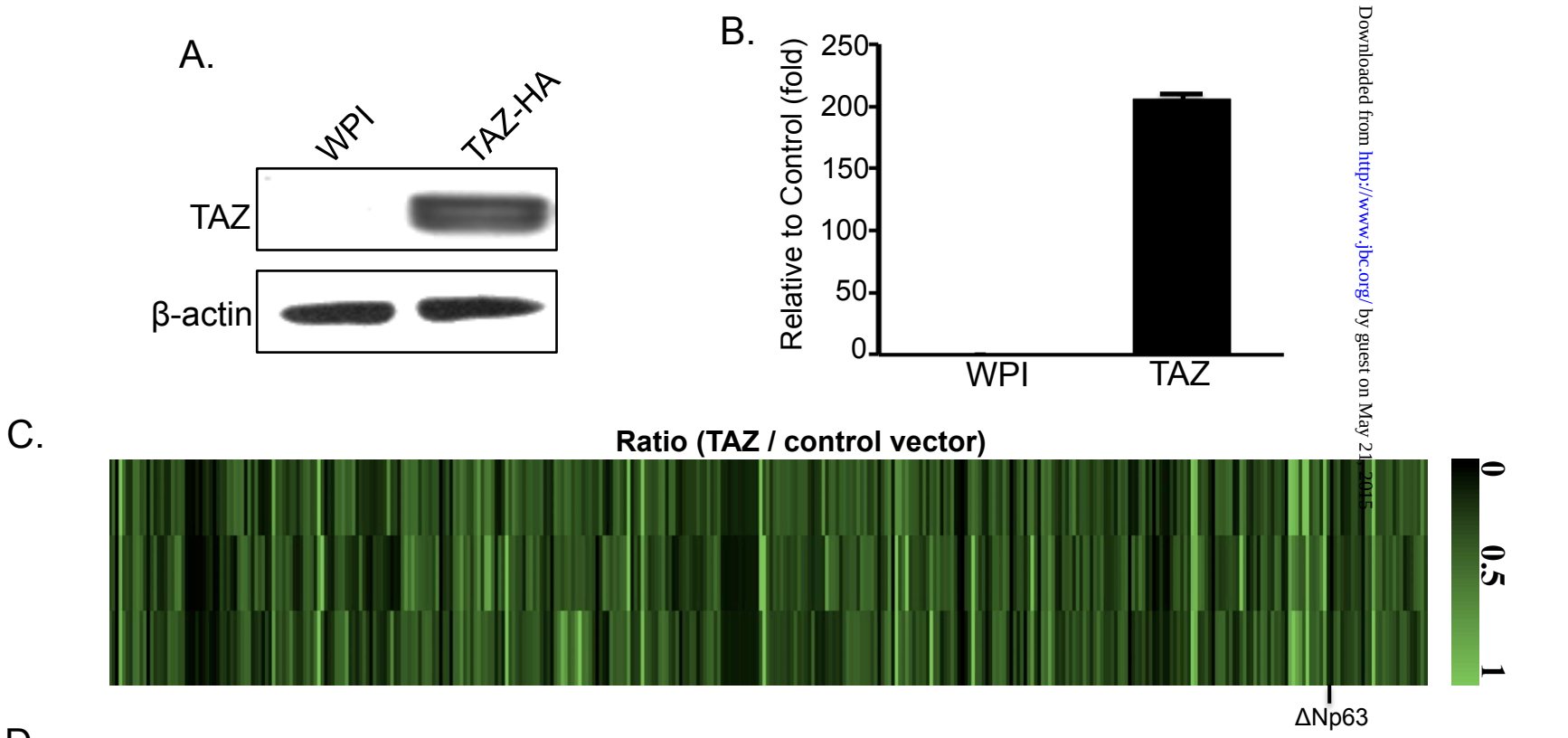


Figure 2

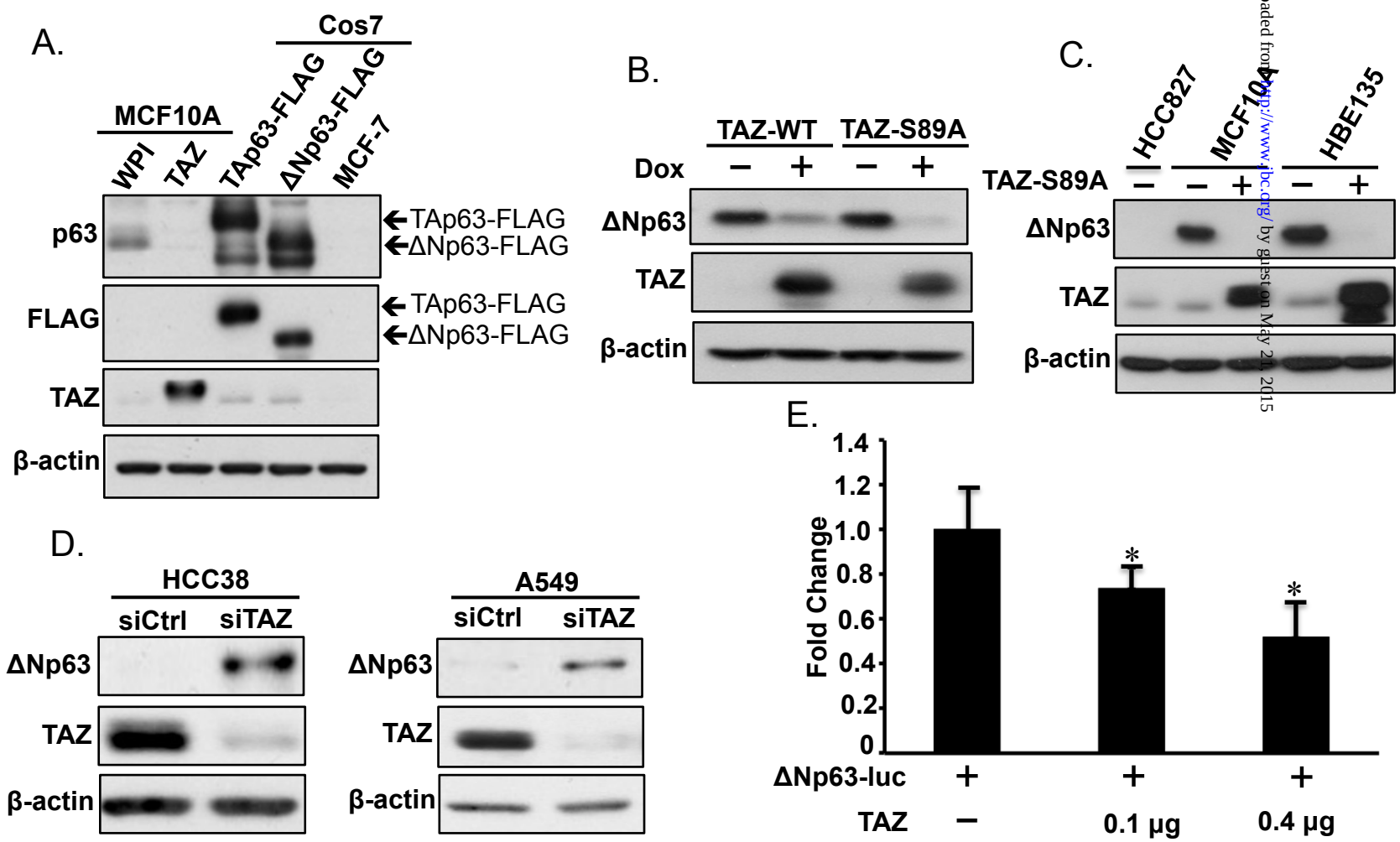
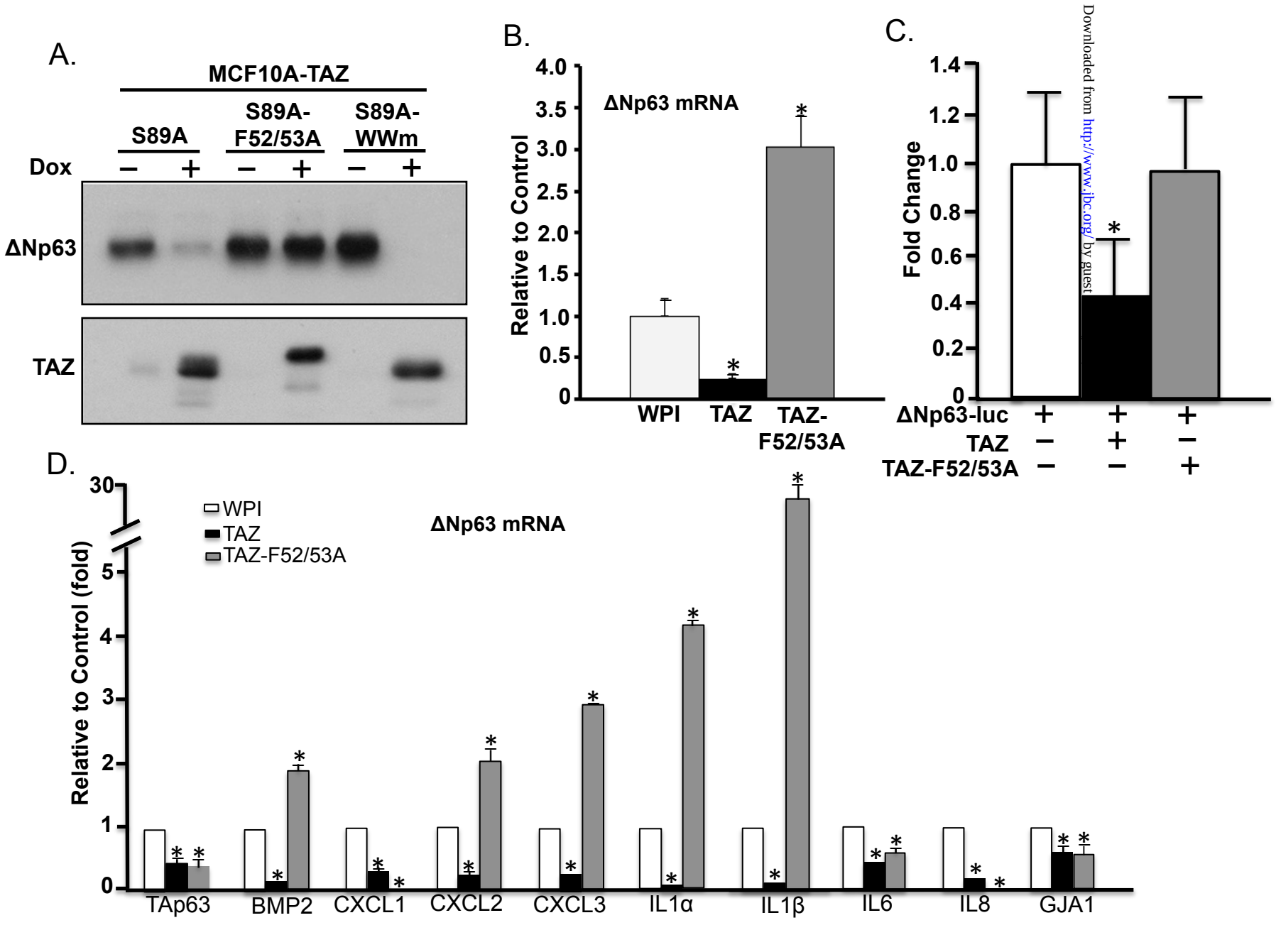


Figure 3



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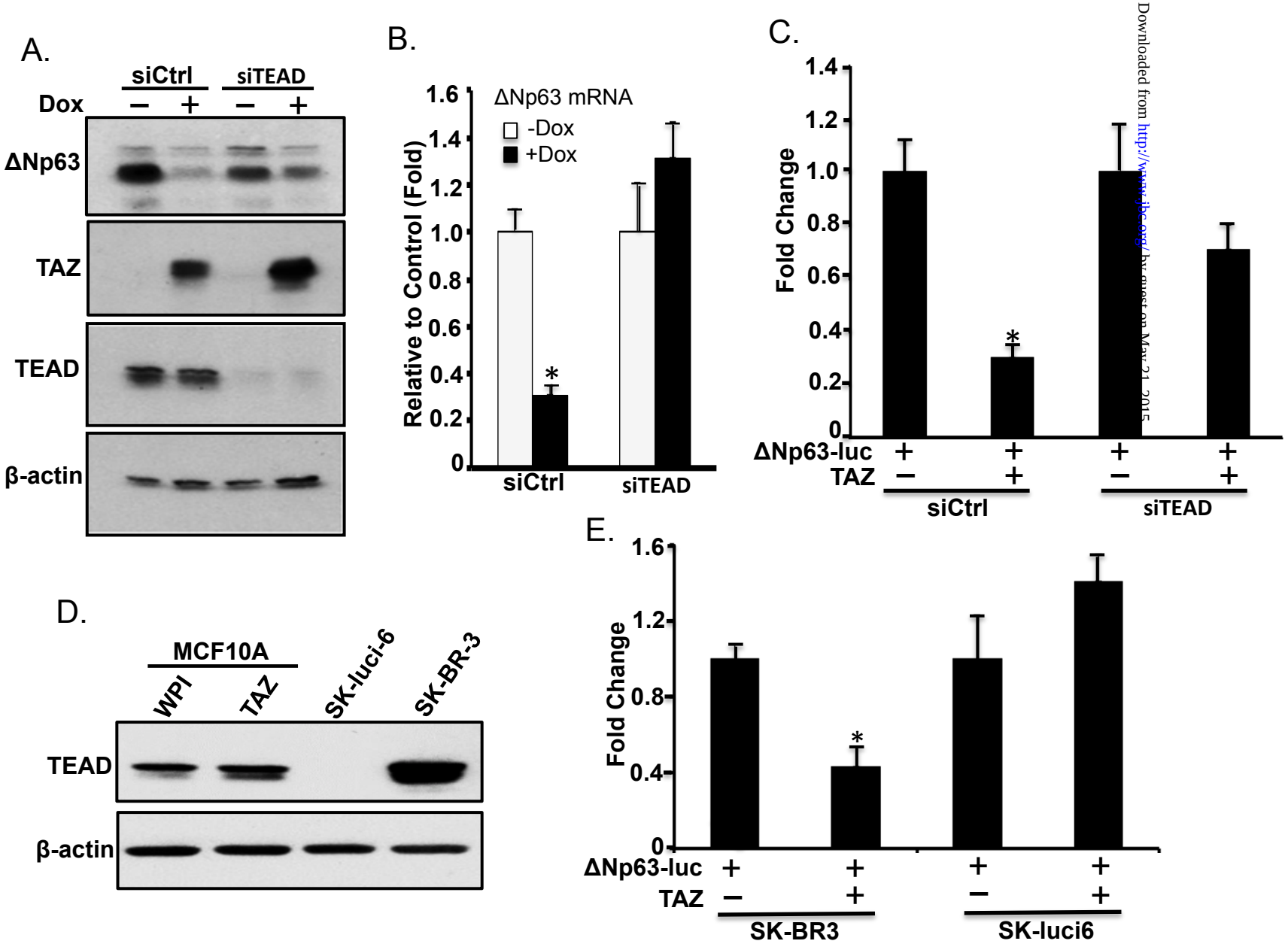
A.
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Dox
– + – +
ΔNp63
TAZ
TEAD
β-actin
B.
ΔNp63 mRNA
-Dox
+Dox
Relative to Control (Fold)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
siCtrl
siTEAD
C.
Fold Change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
ΔNp63-luc + + + +
TAZ – + – +
siCtrl
siTEAD
Downloaded from http://www.jbc.org/ by guest on May 21, 2015
D.
MCF10A
WPI
TAZ
SK-luci-6
SK-BR-3
TEAD
β-actin
E.
Fold Change
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1.2
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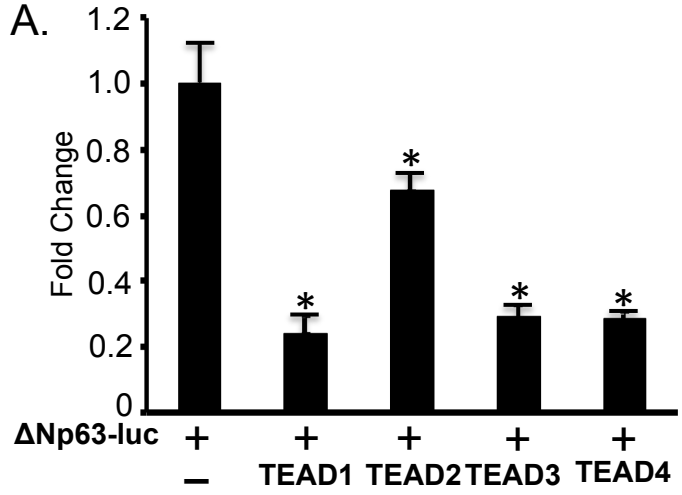
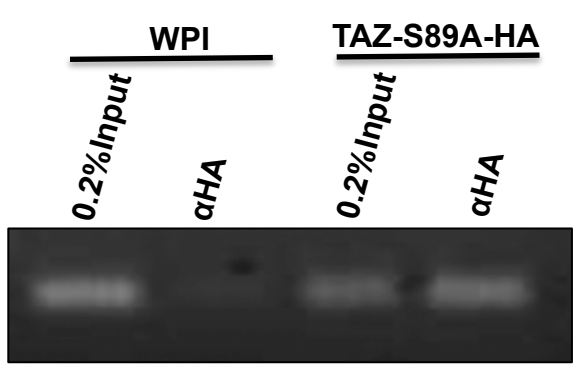
0.4
0
*
ΔNp63-luc + + + +
TAZ – + – +
SK-BR3
SK-luci6

Figure 5





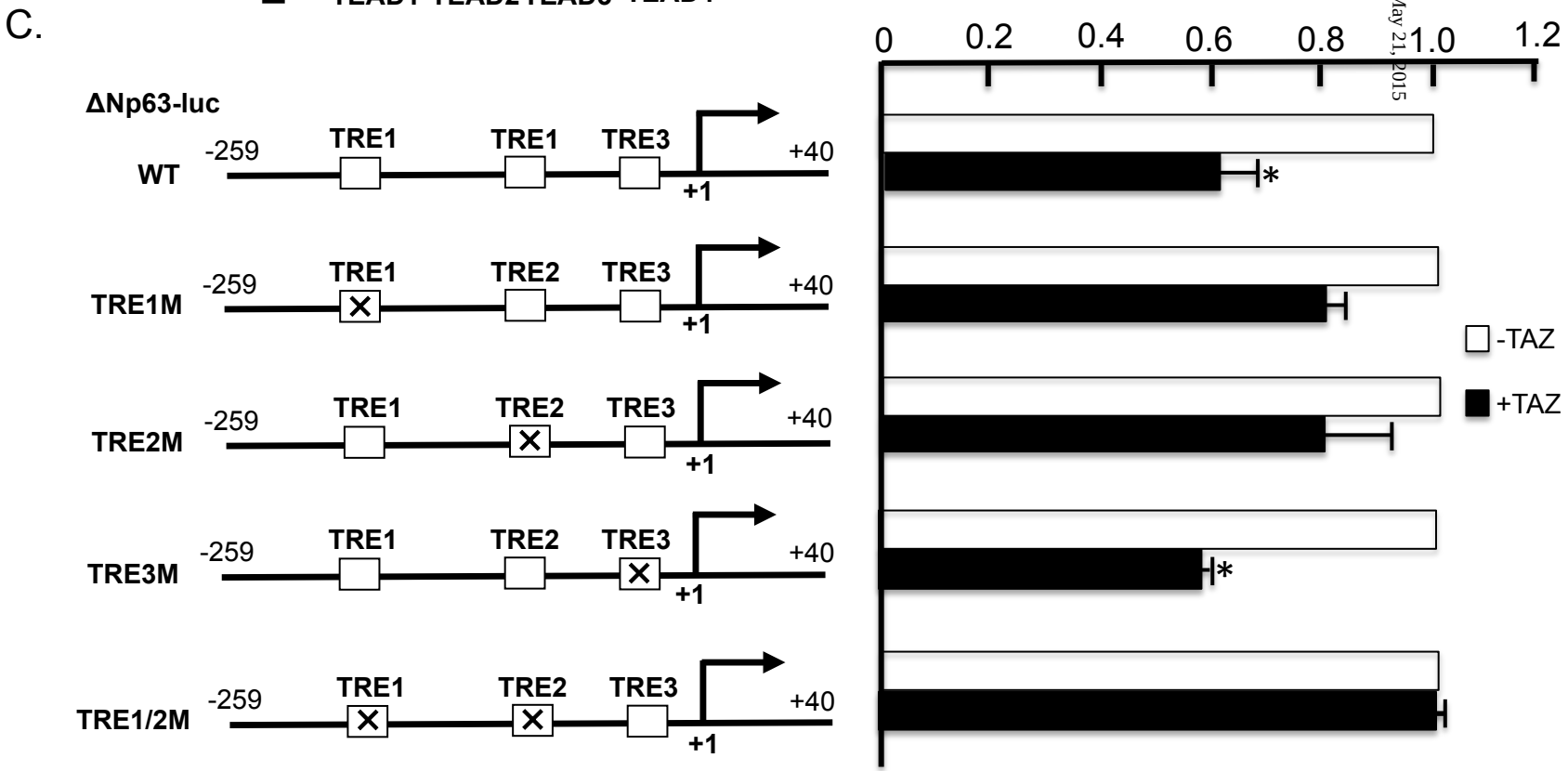


Figure 6

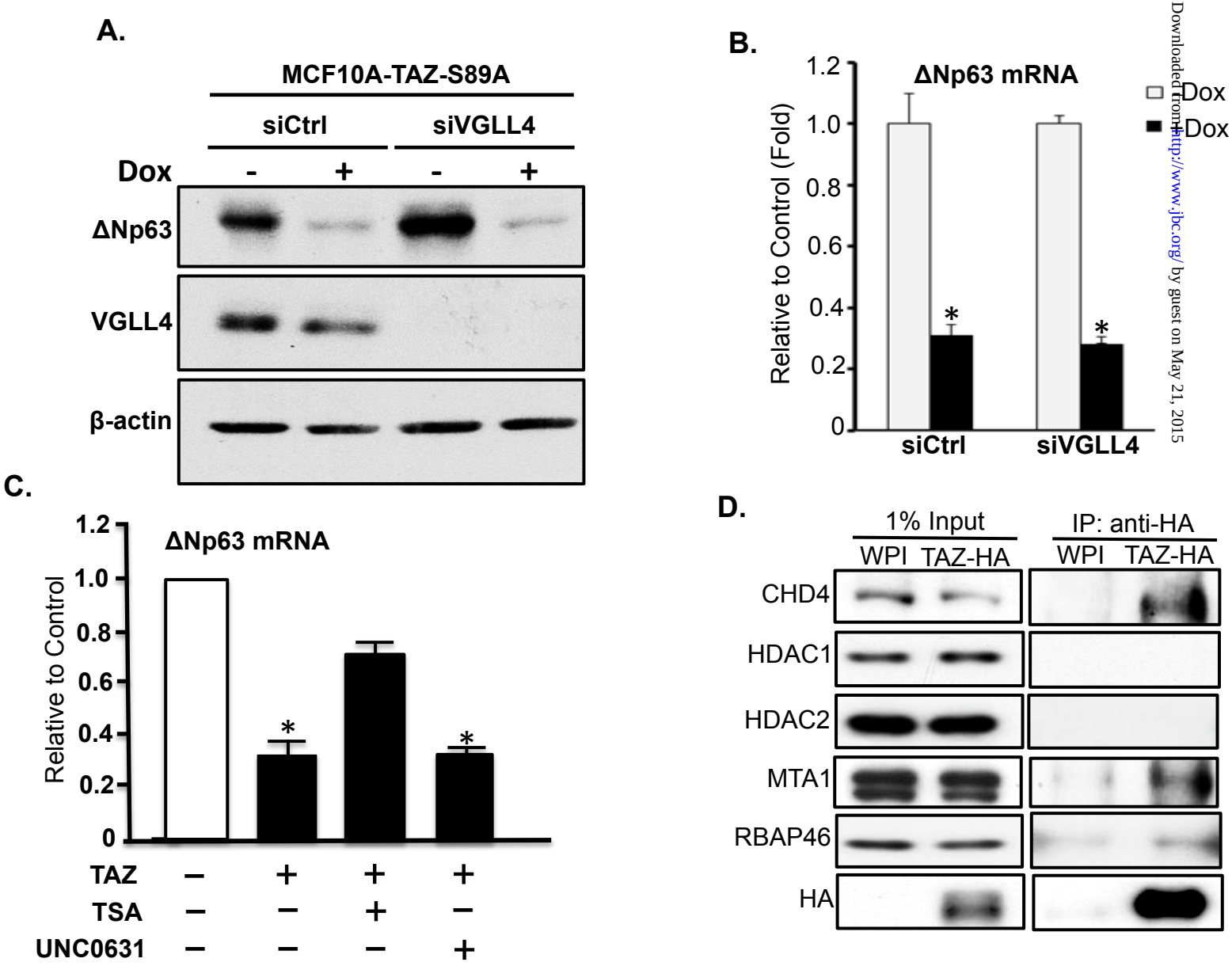
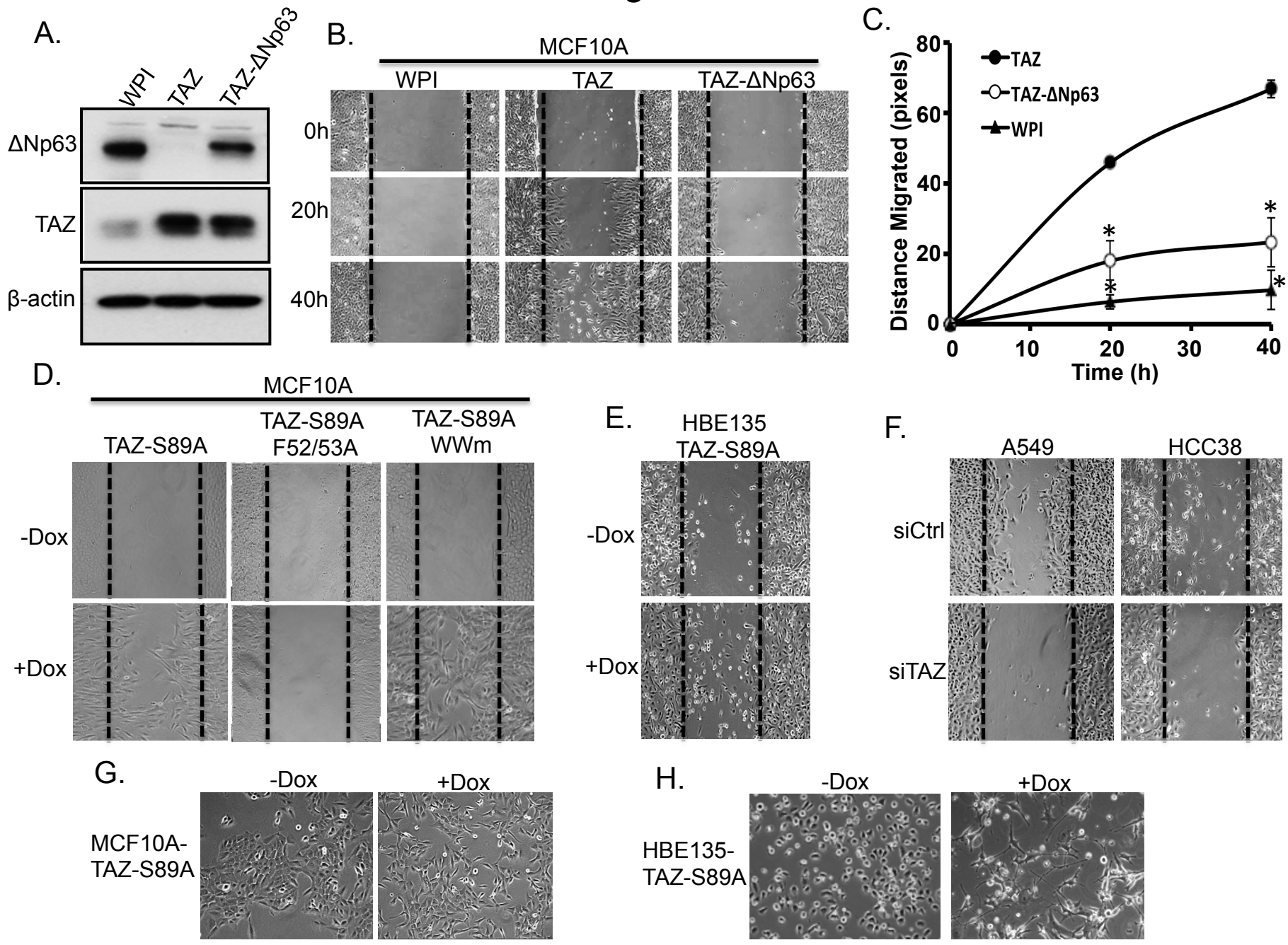


Figure 7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WPI
TAZ
TAZ-ΔNp63
ΔNp63
TAZ
β-actin
B.
MCF10A
WPI
TAZ
TAZ-ΔNp63
0h
20h
40h
C.
TAZ
TAZ-ΔNp63
WPI
Distance Migrated (pixels)
Time (h)
0
10
20
30
40
0
20
40
60
80
D.
MCF10A
TAZ-S89A
TAZ-S89A F52/53A
TAZ-S89A WWm
-Dox
+Dox
E.
HBE135 TAZ-S89A
-Dox
+Dox
F.
A549
HCC38
siCtrl
siTAZ
G.
-Dox
+Dox
MCF10A-TAZ-S89A
H.
-Dox
+Dox
HBE135-TAZ-S89A

Signal Transduction:

Hippo component TAZ functions as a co-repressor and negatively regulates ΔNp63 transcription through TEAD

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