TEADs Mediate Nuclear Retention of TAZ to Promote Oncogenic Transformation*

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The transcriptional coactivators YAP and TAZ are downstream targets inhibited by the Hippo tumor suppressor pathway. The expression level of TAZ is recently shown to be elevated in invasive breast cancer cells and some primary breast cancers. TAZ is important for breast cancer cell migration, invasion, and tumorigenesis, but the underlying mechanism is not defined. In this study, we show that TAZ interacts with TEAD transcriptional factors. Knockdown of TEADs suppresses TAZ-mediated oncogenic transformation of MCF10A cells. Uncoupling TAZ from Hippo regulation by S89A mutation enhances results reveal a novel mechanism for TEADs to regulate nuclear retention and thus the transforming ability of TAZ.

Genetic studies performed in Drosophila melanogaster have uncovered the Hippo tumor suppressor pathway consisting of Hippo (Hpo), Salvador (Sav), Warts (Wts), and Mats as the core components (1–3). Hippo and Salvador form a protein kinase complex whose activity is regulated by cell-cell contact and organ size. Upon activation, Hippo-Salvador complex phosphorylates and activates another protein kinase complex formed by Warts and Mats. The activated Warts-Mats complex then phosphorylates transcriptional coactivator Yorke at Ser residues (primarily Ser168) of the HXRRXS consensus sites, leading to cytoplasmic sequestration through interaction with 14–3–3 proteins and thus inactivation of Yorke. Dissociation of Yorke from 14–3–3 proteins allows it to be translocated into the nucleus to activate the transcriptional program that promotes cell proliferation and suppresses apoptosis. Cell cycle regulator cyclin E, anti-apoptotic dIAP1, and Bantam microRNA are among the best characterized downstream targets of Yorke (4–6). Recent studies suggest that Yorke interacts with transcriptional factor Scalloped to mediate the transcriptional programming (7–9). Mutation of core components of the Hippo pathway or overexpression of Yorkie leads to tissue overgrowth caused by increased numbers of cells (3).

The Hippo tumor suppressor pathway is evolutionally conserved in the mammals. The fly Hippo, Salvador, Warts, and Mats are homologous to human Mst1/2, WW45, LATS1/2, and Mob1, respectively. Mammalian LATS1, Mst2, and Mob1 can functionally rescue corresponding fly mutants in vivo (1, 2, 10). YAP (Yes-associated protein) is homologous to fly Yorkie. Tissue atrophy caused by Yorkie-inactivating mutations in the fly can be functionally rescued by YAP, suggesting that YAP is a functional counterpart of Yorkie. Consistent with a pro-proliferative and anti-apoptotic role for YAP, recent studies suggest that YAP is a candidate oncogene in mammalian cells because the chromosome region containing YAP is amplified in mouse liver and mammary tumors as well as several human cancers (11–13). YAP protein is also overexpressed in several human cancers such as hepatocellular carcinoma (14). Overexpression of YAP in nontransformed human MCF10A mammary epithelial cells causes epithelial-mesenchymal transition, suppression of apoptosis, growth factor-independent proliferation, and anchorage-independent growth in soft agar (11) and transgenic overexpression of YAP in mouse liver causes tissue enlargement and eventually hepatocellular carcinoma (15). Consistently, knock-out of the YAP gene in mice leads to early embryonic lethality at approximately embryonic day 8.5 caused by defect in yolk sac vasculogenesis, chorioallantoic attachment, and embryonic axis elongation (16). The mammalian Hippo core components are activated upon cell-cell contact. In sparsely growing cells, YAP is primarily present within the nucleus where it functions as a transcriptional coactivator. Upon confluence and cell-cell contact, the Hippo pathway is activated to phosphorylate YAP at Ser residues (primarily Ser127) of the HXRRXS motifs, leading to cytoplasmic sequestration of phosphorylated YAP through interaction with 14–3–3 proteins (17). TEAD transcriptional factors (TEAD1–4), the mammalian homologues of fly Scalloped, have been shown to interact with YAP to mediate the pro-proliferative and anti-apoptotic transcriptional program (18).

TAZ (transcriptional coactivator with PDZ-binding motif) (also called WWTR1 for WW domain containing transcription regulator 1) was initially identified as an interacting protein for 14–3–3 proteins (19). TAZ is homologous to YAP as well as to fly Yorkie. A recent study has established that TAZ is also a downstream target regulated by the Hippo core components, and phosphorylation of Ser residues (particularly Ser89) at the consensus HXRRXS sites by LATS1/2-Mob1 complex leads to 14–3–3 protein-mediated cytoplasmic sequestration (20). Unlike YAP, knock-out of TAZ gene in mice does not overtly

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affect mouse development or fertility but selectively affects the structure and function of the kidney and lung (21–23), suggesting that TAZ may play distinct cellular and/or developmental functions, although TAZ and YAP are both targeted by the Hippo pathway. We have recently shown that TAZ protein is overexpressed in a significant fraction of primary breast cancers, and that the expression levels of TAZ but not YAP in breast cancer cell lines correlate with the invasiveness of the cells (24). Consistently, expression profiling has also shown that TAZ transcript levels are increased in basal (or triple negative) type of breast cancer (25, 26). Functionally, TAZ promotes cell migration, invasion, and tumorigenesis. However, the underlying mechanism responsible for TAZ action in these oncogenic processes remains to be defined. Many proteins (especially several transcriptional factors) other than 14-3-3 proteins have been shown to interact with TAZ (27), and these proteins include NHERF-2 to mediate link of TAZ to plasma membrane proteins such as ion channels and receptors (19), TTF-1 to regulate the expression of surfactant protein-C in lung epithelial cells (28), Cbfa1/Runx2 to coactivate Runx2-dependent gene transcription (29, 30), polyomavirus T antigens to potentially regulate viral DNA replication (31), TEAD1/TEF-1 to coactivate the transcriptional activity (32, 33), TBX5, which is important for in cardiac and limb development, to enhance its transcriptional activity (34), peroxisome proliferator-activated receptor γ to repress peroxisome proliferator-activated receptor γ-dependent gene transcription (30, 35), Pax3 to enhance its transcriptional activation (36), and Pax 8, which together with TTF-1 is involved in thyroid differentiation, as a transcriptional coactivator (33). A recent study suggests that TAZ interacts with SMAD2/3–4 complexes to regulate their nucleocytoplasmic shuttling and self-renewal of embryonic stem cells (37). The identification of a myriad of TAZ-interacting transcriptional factors participating in various cellular and developmental processes raises an important question as to which protein(s) is most relevant to the role of TAZ in oncogenesis of breast cancer cells and what is the underlying molecular mechanism. We here present evidence supporting a novel mechanism for TEADs to mediate nuclear accumulation of TAZ to promote oncogenic transformation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Materials**—MCF10A and MCF7 cells were purchased from American Type Culture Collection. MCF10A cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum, 20 ng/ml of epidermal growth factor, 0.5 μg/ml of hydrocortisone, 100 ng/ml of cholera toxin, and 10 μg/ml of insulin, and penicillin/streptomycin. MCF7 cells were cultured in RPMI supplemented with 10% fetal bovine serum. Leptomycin B was from Sigma.

**Plasmids**—FLAG-TAZ was previously described (24). Single or double amino acid mutations were introduced into TAZ coding region to create TAZ-S89A and S89A-M1–9 mutants by PCR. TEAD1–4 cDNAs were retrieved from the MegaMan Human Transcriptome Library (Stratagene) using PCR and cloned into pBABEpuro retroviral vector. PCR-amplified cDNA fragments coding for the C termini of TEADs were subcloned into pGEX4T (Amersham Biosciences). GST fusion proteins were prepared as described previously (24). S89A-M4-TEAD4 and S89A-M9-TEAD4 were constructed by fusing the full-length of mutant TAZ coding region to the second codon of TEAD4. GFP-TAZ3 and GFP-TAZ-M9 were subcloned into pLPC vector from Scott Lowe (Cold Spring Harbor Laboratory).

**Antibodies**—TAZ antibody was purchased from ImageNeX. TEAD4 antibody was purchased from Aviva Systems Biology. Anti-FLAG and anti-FLAG M2-peroxidase (anti-FLAG-horse radish peroxidase) mouse antibodies were from Sigma. Anti-HA rabbit antibody was from Upstate Biotechnology. Anti-HA-peroxidase (anti-HA-horseradish peroxidase) antibody was from Roche Applied Science.

**Retrovirus Generation and Infection**—The amphotropic Phoenix packaging cells (Nolan Lab) were transfected with the indicated retroviral vectors using Lipofectamine according to the manufacturer’s instructions (Invitrogen). After 48 h, the retroviral supernatants were collected, filtered (0.45 μm; Millipore), and added onto the target cells in the presence of 5 μg/ml of polybrene (Sigma-Aldrich) for 6–8 h. Infection was done twice. After infection, the cells were selected with puromycin (1 μg/ml) for a week before being analyzed for protein expression by immunoblotting, followed by soft agar assays. If two constructs were expressed in MCF10A cells, the transductions were done sequentially, and the cells were selected with two different antibiotics.

**Anchorage-independent Growth in Soft Agar**—1.5 ml of 0.5% agar (electrograde ultra pure; Invitrogen) supplemented with culture media for MCF10A was plated in six-well plates as the bottom agar. Twenty thousand cells were mixed with 1.5 ml of 0.35% agar-supplemented MCF10A medium and plated on the solidified bottom agar. 1 ml of medium was added on top of the solidified agar layers, and the colonies were allowed to grow in incubator at 37 °C, 5% CO2 for 1 month. The colonies were stained with thiazolyl blue tetrazolium bromide and scanned. The colonies were quantified using the Image J program.

**shRNA-mediated Knockdown of TEADs**—The sequences of the shRNA against human TEADs were designed as described (18), except that the shRNAs were cloned into retroviral vector pSuper.Retro.puro vector (Oligoengine). The relative amount of transcripts for TEAD1–4 was assessed by real time PCR using reagents from Applied Biosystems.

**Immunofluorescence**—The cells were cultured on coverslips a day before the experiments. The second day, the cells were fixed with 3% paraformaldehyde in PBS containing 1 mM CaCl2 and 1 mM MgCl2 for 30 min and then permeabilized the cell with 0.1% Triton-100 in PBS. The permeabilized cells were incubated with the indicated antibodies in blocking buffer (10% bovine serum in PBS) for 1 h. The slides were washed with PBS and incubated either with fluorescein isothiocyanate-conjugated or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and then mounted with Vectashield mounting medium with 4’,6-diamidino-2-phenylindole from Vector Laboratories Inc.

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3 The abbreviations used are: GFP, green fluorescent protein; HA, hemagglutinin; shRNA, short hairpin RNA; PBS, phosphate-buffered saline; GST, glutathione S-transferase; FRAP, fluorescence recovery after photobleaching.
Fluorescence Recovery after Photobleaching—MCF7 cells were transiently transfected with GFP-TAZ or GFP-TAZ-M9 using FuGENE 6 (Roche Applied Science). The experiments were done 24–36 h later. The live images were taken at 10-s interval using the Olympus Fluoview 1000 confocal microscope equipped with a 37 °C live cell chamber. After obtaining a stable base line, the fluorescence in one area of the cell was photobleached repeatedly. Each bleaching protocol consisted of bleaching a selected region of interest with a 488-nm laser for 5 s at maximum laser intensity. The normalized fluorescence intensity curves were plotted with the following formula: 

\[ I_{\text{frap norm}}(t) = \frac{I_{\text{Adj whole}}(0)(I_{\text{Adj whole}}(t) - I_{\text{Bgd}}(t))}{(I_{\text{frap}}(t) - I_{\text{Bgd}}(t))} \]

where \( I_{\text{frap norm}}(t) \) is the normalized fluorescence intensity, \( I_{\text{frap}}(t) \) is the fluorescence intensity of the adjacent whole cell, which was used for correcting the acquisition bleaching effects, and \( I_{\text{Adj whole}}(t) \) is the fluorescence of the region of interest set outside the cell.

RESULTS

TAZ Interacts with TEADs—Because TAZ interacts with many different proteins (especially transcriptional factors) to mediate various cellular and developmental processes, it is important to identify which protein(s) is most relevant to the role of TAZ in oncogenesis of breast cancer cells. Among the many proteins known to interact with TAZ, we have focused on the interaction of TAZ with TEADs for several reasons. First, our large scale immunoprecipitation of FLAG-tagged TAZ expressed in transiently transfected 293 cells uncovered TEAD1, TEAD3, and TEAD4 among the proteins coimmunoprecipitated with FLAG-TAZ (the proteomic analysis of TAZ-interacting proteins as studied by large scale immunoprecipitation will be described elsewhere). Second, the recent characterization of TEADs and Scalloped as functional partners for YAP and Yorkie, respectively, suggests that YAP-homologous TAZ may also functionally engage TEADs (8, 9, 17, 18, 40–42). Third, interaction of TAZ with TEAD1 has been shown previously, although the structural basis and functional consequence of this interaction were not defined (32, 33).

We first verified the interaction of TAZ with TEADs by pull-down experiment. Because the C-terminal domains of TEADs were earlier shown to mediate interaction with YAP (43), we expressed the C-terminal fragments of TEAD1–4 as recombinant proteins fused with GST. Cell lysates derived from Hs578T cells, which express both YAP and TAZ (24), were incubated with immobilized GST (as a negative control) and GST-TEAD1, -TEAD2, -TEAD3, and -TEAD4, respectively. After extensive washing, the proteins retained by the beads were resolved by SDS-PAGE followed by immunoblotting using antibodies against TAZ, which also cross-react with YAP (24), so that we can examine retention of TAZ in comparison with YAP. As shown in Fig. 1A, like YAP (the upper band), TAZ (the lower band) was efficiently retained by immobilized GST-TEADs but not by GST. The interaction was next confirmed by coimmunoprecipitation of FLAG-TAZ and HA-TEADs coexpressed in transiently transfected 293 cells (Fig. 1C). The cell lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-HA to detect coimmunoprecipitated HA-TEADs. HA-TEAD1, 2, 3, and 4 were efficiently coimmunoprecipitated with FLAG-TAZ (lanes 6–9). As a negative control, HA-TEAD4 was not recovered by anti-FLAG antibody when FLAG-TAZ was not coexpressed (lane 10). These results, taken together, suggest that TEAD1–4 can all interact efficiently with TAZ.

FIGURE 1. TAZ interacts with TEADs. A, retention of TAZ and YAP by immobilized C-terminal regions of TEAD1–4. Cell extracts derived from Hs578T cells were incubated with immobilized GST-TEAD1 (amino acids 96–411), GST-TEAD2 (amino acids 121–447), GST-TEAD3 (amino acids 115–435), GST-TEAD4 (amino acids 119–434), or GST. Proteins retained by the beads, together with the starting material (input), were resolved by SDS-PAGE followed by immunoblotting with antibodies that react with both TAZ and YAP. B, GST-TEADs and GST resolved by SDS-PAGE and stained with Coomassie Blue. C, coimmunoprecipitation of TAZ and TEADs. 293 cells were transfected to express FLAG-TAZ and HA-TEAD1–4. The cell lysates were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were resolved by SDS-PAGE followed by immunoblotting with anti-HA-horseradish peroxidase to detect coimmunoprecipitated HA-TEADs.
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A

vector  Flag-TAZ  Flag-TAZ-S89A

B

vector  Flag-TAZ  Flag-TAZ-S89A

75 kD  50 kD

C

colony number (%)

vector  Flag-TAZ  Flag-TAZ-S89A

D

S89A+vector  S89A+TEADs-KD-1  S89A+TEADs-KD-2

E

relative amount

TEAD1  TEAD2  TEAD3  TEAD4

vector  KD-1  KD-2  vector  KD-1  KD-2  vector  KD-1  KD-2

F

colony number (%)

S9A+control  S9A+TEADs-KD-1  S9A+TEADs-KD-2
Uncoupling TAZ from Regulation by the Hippo Pathway Enhances Its Transforming Ability—A previous study has shown that TAZ is phosphorylated by the Hippo pathway primarily at Ser89, and the phosphorylated TAZ is sequestered in the cytoplasm by interaction with 14-3-3 proteins (20). Phosphorylation of Ser89 of TAZ has also been shown by an earlier study to be responsible for interaction with 14-3-3 proteins (19). If this Hippo pathway-mediated phosphorylation serves to regulate TAZ negatively, mutation of Ser89 should render TAZ refractory to this inhibition. Because our earlier study has shown that TAZ promotes migration and invasion and is important for transformed phenotype of breast cancer cells (24), we examined whether expression of TAZ is sufficient to confer tumorigenic property to immortalized MCF10A cells as assessed by anchorage-independent growth in soft agar. As shown in Fig. 2 (A–C), MCF10A cells transduced with pBABE-puro vector did not form colonies in soft agar, whereas cells expressing FLAG-TAZ formed a significant number of colonies in soft agar, suggesting that TAZ, like YAP (11, 44, 45), is sufficient to confer oncogenic phenotype to MCF10A cells. Using this assay, it was shown that cells expressing TAZ-S89A, refractory to Hippo phosphorylation and having lost the ability to interact with 14-3-3 proteins (20), formed more colonies in the soft agar. These results suggest that TAZ has transforming ability that is negatively regulated by the Hippo pathway and that ablation of this regulation increases its transforming ability.

Knockdown of TEAD Expression Suppresses S89A-driven Transformation—Because the Hippo refractory S89A has enhanced ability to transform MCF10A cells, we have used it to investigate the underlying molecular mechanism. Because interaction of TAZ with TEADs was confirmed in our independent analysis, we first examined whether S89A-mediated transformation of MCF10A cells is dependent on TEADs. To provide evidence for a role of endogenous TEADs in S89A-mediated transformation, we have suppressed the expression of endogenous TEAD1, 3, and 4 using a retroviral vector to express shRNAs that are known to knockdown endogenous TEAD1, 3, and 4 (18). As shown in Fig. 2 (D and E), the shRNAs (KD-1 and KD-2) noticeably reduced the soft agar colony numbers of S89A-expressing cells. As shown in Fig. 2E, KD-1 has
more efficient ability than KD-2 to suppress endogenous expression of TEAD1, TEAD3, and especially TEAD4. Importantly, KD-1 reduced number of colonies grown in the soft agar to a greater extent as compared with the less efficient KD-2. The transcript for TEAD2 was increased in cells expressing KD-1 or KD-2, likely because of cellular feedback mechanism to

FIGURE 4. Mutants of S89A defective in interaction with TEAD4 are no longer able to drive anchorage-independent growth. A, MCF10A cells expressing S89A-M4 and S89A-M9, defective in binding to TEAD4, are not able to induce anchorage-independent growth on soft agar, whereas TEAD interaction competent S89A-M1, S89A-M2, and S89A-M3 (to a less extent), like S89A, are transforming. B, quantification of colony numbers for all the S89A mutants in comparison with vector-, TAZ-, and S89A-transduced cells. The average colony number from three independent experiments was presented with standard deviations. The average number derived from S89A-expressing cells was arbitrarily set at 100%. C, whole cell lysates derived form cells stably expressing TAZ, S89A, and its mutants were analyzed by immunoblotting using anti-FLAG and anti-actin antibodies. D, fusion of TEAD4 to the C terminus of S89A-M9 restored the ability to drive anchorage-independent growth. Soft agar assays were done on cells expressing S89A, TEAD4, S89A-M9, and S89A-M9-TEAD4. E, quantification of colony numbers for various proteins in comparison with vector and S89A cells. The average colony number from two independent experiments was presented with standard deviations. The average number derived from S89A-expressing cells was arbitrarily set at 100%. F, lysates derived from cells expressing the indicated proteins were analyzed by immunoblotting with anti-FLAG and anti-TEAD4 antibodies.
compensate the reduced expression of other TEADs. The correlation of efficiencies of knockdown with those of inhibiting colony formation in soft agar driven by S89A supports the notion that endogenous TEAD proteins (especially TEAD4) are necessary for S89A to mediate oncogenic transformation of MCF10A cells.

Identification of Residues in the N-terminal Region of TAZ Important for Interaction with TEADs—Because TAZ interacts with TEADs and its transforming ability depends on endogenous TEADs, one likely mode of action is that interaction with TEADs is important for its transforming ability. To examine this possibility directly, we have first identified several residues of TAZ that are important for interaction with TEADs. The N-terminal region of human YAP encompassing residues 32–102 has earlier been shown to represent the minimal region of YAP that is sufficient for interaction with TEADs (43). We have aligned the amino acid sequence of the N-terminal 102-residue region of human YAP with corresponding region of YAP and TAZ from several species and have identified several stretches of amino acids that are evolutionally conserved as well as conserved between TAZ and YAP (Fig. 3A). Because the interaction with TEADs is shared between YAP and TAZ and also evolutionally conserved, some of these conserved residues are likely involved in interaction with TEADs. We have thus created nine different mutants of S89A covering most of these conserved residues, with each of them having two consecutive residues being replaced with two Ala residues, except for mutant 2, which has a single residue (Asp23) being replaced by Ala (Fig. 3A). These FLAG-tagged mutants, together with TAZ and S89A, were each coexpressed stably with HA-TEAD4 in MCF10A cells. Whole cell lysates were immunoprecipitated with anti-FLAG antibody, and the amounts of coimmunoprecipitated HA-TEAD4 were assessed by immunoblotting analysis. As shown in Fig. 3B, mutants 1, 2, 5, and 6 coimmunoprecipitated TEAD4 (Fig. 3B, upper panel, lanes 1, 2, 5, and 6) efficiently (upper panel, lanes 11 and 12). These mutants are therefore not affected in their ability to interact with TEAD4. However, mutants 4, 7, 8, and 9 have much reduced ability to interact with TEAD4 because the amounts of coimmunoprecipitated TEAD4 were dramatically reduced (Fig. 3B, upper panel, lanes 4 and 7–9). Mutant 3 displayed slightly reduced ability to interact with TEAD4 (upper panel, lane 3). The expression of S89A is consistently less than TAZ in MCF10A cells. Hence the immunoprecipitated S89A is expected to be less compared with TAZ, and this is not due to the loading errors (Fig. 4C). These results suggest that the region of TAZ encompassing residues 43–53 (covered by mutants 7–9) is mostly involved in interaction with TEADs with participation of other residues preceding this region (such as LE32 covered by mutant 4).

S89A Mutants Defective in TEAD Interaction Lose Transforming Ability—To investigate the functional importance of TAZ interaction with TEADs, the consequence of ablating interaction with TEADs on oncogenic property was investigated by examining the ability of these different mutants (in comparison with S89A and TAZ) to confer anchorage-independent growth of MCF10A cells in soft agar. Interestingly, mutants 1, 2, 5, and 6, whose interaction with TEADs were not significantly affected by the mutations, exhibited transforming abilities that are comparable with S89A, whereas mutants 4, 7, 8, and 9, whose interactions with TEADs were dramatically
**TEADs Mediates Nuclear Retention of TAZ in Oncogenesis**

![Diagram](image_url)

**Figure A:**
- Hippo and 14-3-3-mediated cytoplasmic sequestration

**Figure B:**
- TEAD interaction-mediated nuclear retention

**Figure C:**
- Hippo pathway

**Figure D:**
- Cytoplasmic sequestration

**Figure E:**
- TAZ

**Figure F:**
- Nuclear retention & Transcriptional program

**Figure G:**
- TAZ

**Hippo- and 14-3-3-mediated cytoplasmic sequestration**

**Cytoplasm**

**Nucleus**

**TEAD interaction-mediated nuclear retention**
abrogated by the mutations, have essentially lost the transforming ability (Fig. 4, A and B). Mutant 3, which displayed slight loss of interaction with TEADs, has marginally reduced ability to transform MCF10A cells. Representative results for some of these mutants in comparison with S89A and TAZ are shown in Fig. 4A, whereas the quantitative results obtained from three independent experiments for all mutants were shown in Fig. 4B. Fig. 4C shows the levels of expression of S89A, TAZ, and all the mutants. The strict correlation of the loss of TEAD interaction with the loss of ability to transform MCF10A cells suggests strongly that interaction with TEADs is essential as well as is the underlying basis for S89A to execute the transforming program in MCF10A cells.

**Fusion of TEAD4 to the C Terminus of S89A-M4 and S89A-M9 Restores Transforming Activity**—To confirm that the loss of transforming activity of S89A-M4, 7, 8, and 9 is indeed due to the loss of their interaction with TEADs, we generated S89A-M4-TEAD4 and S89A-M9-TEAD4 fusion proteins by directly joining the TEAD4 to the C terminus of the respective S89A mutants. As shown in Fig. 4D, fusion protein S89A-M9-TEAD4 transformed MCF10A cells more efficiently than S89A. Interestingly, TEAD4 itself also exhibited some transforming ability, although it is not as efficient as S89A. The quantitative results from two independent experiments for the transforming abilities were shown in Fig. 4E, and the expression levels of these proteins are shown in Fig. 4F. The very potent transforming ability exhibited by S89A-M4-TEAD4 and S89A-M9-TEAD4 as compared with modest transforming ability of TEAD4 and nontransforming ability of S89A-M4 and S89A-M9 strongly suggests that a defect in transforming ability of S89A-M4 and S89A-M9 is indeed caused by their lost interaction with TEADs rather than by misfolding or conformational changes. Taken together, these results suggest that interaction of TAZ and S89A with endogenous TEADs is the underlying mechanism for TAZ and S89A to transform MCF10A cells.

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**TAZ Mutants Defective in TEAD Interaction Are Defective in Nuclear Accumulation**—To understand the mechanistic aspect of the TEAD interaction-dependent transforming ability of S89A, we have examined the subcellular localization of these mutants in comparison with S89A and TAZ. As shown in Fig. 5A, TAZ was present in both cytoplasm and nucleus, whereas Hippo refractory S89A was more enriched in the nucleus, which is consistent with its lost interaction with 14-3-3 proteins (19). S89A mutants 1, 2, 3, 5, and 6, which retain the ability to interact with TEADs and to transform MCF10A cells, displayed similar enrichment in the nucleus. Representative results for mutants S89A-M1 and S89A-M5 are shown in Fig. 5A. Interestingly, mutants 4, 7, 8, and 9, which have lost the ability to interact with TEADs and to transform MCF10A cells, failed to accumulate in the nucleus, because they are primarily detected in the cytoplasm. Representative results for mutants S89A-M4 and S89A-M9 are shown in Fig. 5A. These results suggest that the loss of interaction with TEADs led to a defect for S89A to accumulate in the nucleus. Because all of these mutants are created in the context of S89A that is refractory to Hippo inhibition and is not able to interact with 14-3-3 proteins, their cytoplasmic distribution is not due to sequestration by 14-3-3 proteins but a failure to accumulate in the nucleus. These results suggest a possible mechanism in that interaction with TEADs is important for S89A to accumulate in the nucleus and to execute the transforming program.

**Fusion of TEAD4 to the C Terminus of S89A-M9 Restores Nuclear Accumulation of S89A-M9**—Because fusion of TEAD4 to the C terminus of S89A-M9 restored the transforming activity of S89A-M9, we investigated whether this correlates with restored nuclear accumulation. As shown in Fig. 5B, S89A-M9-TEAD4 was predominantly localized in the nucleus, supporting the notion that TEADs-mediated nuclear accumulation of S89A correlates with its transforming ability.

**Live Cell Imaging of GFP-TAZ Supports TEAD-mediated Nuclear Retention of TAZ**—To gain additional insight into the mechanism responsible for TEAD interaction-mediated nuclear accumulation of TAZ, we have performed live cell imaging to monitor the dynamic distribution of GFP-TAZ. As with endogenous and FLAG-TAZ, GFP-TAZ is distributed both in the cytoplasm and the nucleus. The rate of nuclear import and export was assessed by fluorescence recovery after photobleaching (FRAP). By bleaching the nuclear pool of GFP-TAZ followed by monitoring the migration of cytosolic pool into the nucleus, we observed a rapid nuclear import of GFP-TAZ with a half-time of ~0.65 min (Fig. 6A). On the other hand, after bleaching the cytoplasmic pool followed by monitoring the migration of nuclear pool to the cytoplasm, it was noted that export of nuclear pool occurred at a slower rate with a half-time of ~1.6 min (Fig. 6B). FRAP analysis of GFP-TAZ-M9 (equivalent to the M9 mutation in S89A context) indicted that nuclear import of GFP-TAZ-M9 exhibited similar kinetics with a half-time of ~0.62 min (Fig. 6, C and E), whereas nuclear export of GFP-TAZ-M9 was significantly enhanced with a half-time of ~0.8 min (Fig. 6, D and F). These results suggest that interaction with TEAD prevents nuclear export of TAZ, and therefore TEAD interaction serves to mediate nuclear retention of TAZ.

**DISCUSSION**

Previous studies suggest that TAZ promotes cell migration, invasion, proliferation, epithelial-mesenchymal transition, and

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**FIGURE 6.** GFP-TAZ-M9 mutant shows more rapid rates of nuclear export compared with wild type GFP-TAZ, indicating that nuclear retention is compromised. **A**, normalized fluorescence recovery kinetics of GFP-TAZ following repeated photobleaching of the nucleus as highlighted in yellow. **B**, normalized fluorescence recovery kinetics of GFP-TAZ following repeated photobleaching of the cytoplasm as marked in yellow. **C**, normalized fluorescence recovery kinetics of GFP-TAZ-M9 following repeated photobleaching of the nucleus as highlighted in yellow. **D**, normalized fluorescence recovery kinetics of GFP-TAZ-M9 following repeated photobleaching of the cytoplasm as indicated in yellow. Gray scale images show points i–v as indicated in each FRAP curve, respectively. **E**, scatter plot of half-life of GFP-TAZ and GFP-TAZ-M9 nuclear import. GFP-TAZ p, means ± S.E. = 0.67 ± 0.06 min; GFP-TAZ-M9 p, means ± S.E. = 0.65 ± 0.08 min. **F**, scatter plot of half-life of GFP-TAZ and GFP-TAZ-M9 nuclear export. GFP-TAZ p, means ± S.E. = 1.52 ± 0.07 min; GFP-TAZ-M9 p, means ± S.E. = 0.57 ± 0.07 min, p < 0.0001. Each point is the average half-life of 2 FRAP curves from the same cell, each experiment was performed in at least five different cells, and similar results were obtained. G, a model to depict two major mechanisms regulating the distribution and function activity of TAZ. Hippo pathway and 14-3-3 proteins mediate cytoplasmic sequestration, whereas TEAD interaction drives nuclear retention of TAZ.
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tumorigenesis (24). Because YAP is sufficient to transform MCF10A cells (11), we first examined whether TAZ is also oncogenic as assessed by anchorage-independent growth in soft agar. Introducing FLAG-TAZ into MCF10A cells conferred the cells the ability to grow in soft agar. Because residue Ser89 of TAZ is the major site targeted by the Hippo pathway to create a biding site for 14-3-3 proteins for cytoplasmic sequestration (20), we have created the Hippo refractory S89A mutant of TAZ and shown that S89A mutant exhibited enhanced transforming ability, consistent with the notion that Hippo pathway negatively regulates TAZ action. In our hands, the transforming ability of S89A is comparable with activated forms (such as G12V and G13D) of K-Ras. These results suggest that TAZ behaved like an oncogene in its ability to transform MCF10A cells, and its transforming ability is significantly enhanced by uncoupling its inhibition by the Hippo pathway (for example, by S89A mutation described here). The Hippo core machinery is activated upon cell-cell contact in vitro and reaching right organ size in vivo by upstream activators such as FAT4 (48), NF2-encoded Merlin (49, 50), and likely mammalian homologues (DCH51 and/or DCH52) of fly Dachsous (51, 52). The reported mutation and/or down-regulation of Hippo upstream activators such as FAT4 and Merlin may lead to a similar uncoupling of TAZ (and like YAP) from the Hippo core machinery. Similarly, mutations and/or down-regulation of Hippo core machinery may uncouple TAZ and YAP from the respective negative regulation. The reported mutation of WW45 in human cancer cells is supportive to this notion (1, 2). Whether TAZ and/or YAP are also uncoupled from the Hippo regulation by mutations of themselves remains to be examined. Because the Hippo upstream activators and Hippo core machinery consist of many distinct proteins, inactivating mutations and/or down-regulation of these proteins might represent a possible mechanism to uncouple TAZ and YAP from the Hippo regulation during cancer development.

Because TAZ has been shown to regulate diverse cellular and developmental processes via its interaction with many different proteins, many of which are transcriptional factors (27), TAZ may engage any of these known interacting proteins or new proteins to mediate its transforming ability. Inspired by our independent observation that TEAD1, TEAD3, and TEAD4 were coimmunoprecipitated by FLAG-TAZ expressed in 293 cells and the recent demonstration that YAP and Yorkie engage TEADs and Scalloped, respectively, as the transcriptional factors in mediating the cellular function (7–9, 17, 18, 53), we have investigated the role of endogenous TEADs and TAZ-TEAD interaction in S89A-mediated transformation of MCF10A cells. Our results suggest that endogenous TEADs, and especially TEAD4, are important for TAZ to transform MCF10A cells. Identification of several residues important for TAZ to interact with TEADs enabled us to demonstrate that interaction with TEADs is important for TAZ to transform MCF10A cells. Therefore, interaction of TAZ with TEADs dictates its transforming ability. Although our results clearly suggest that TEAD interaction-mediated nuclear retention of TAZ is crucial for the oncogenic transformation of TAZ, it remains to be defined whether this is a passive consequence of engagement of TAZ with chromosome-bound TEADs or represents an independent mechanism. A future experiment aiming to address whether nuclear localization of TEADs (and thus TAZ) is dependent on their DNA binding ability will help to resolve this issue.

The mechanistic consequence of TAZ-TEAD4 interaction was revealed by examining the subcellular localization of these mutants. Our results suggest that TEAD interaction is important for TAZ to be accumulated in the nucleus. Live cell imaging of GFP-TAZ and FRAP experiments supports the notion that interaction with TEAD leads to nuclear retention and thus slowed export of TAZ. These results suggest that TAZ distribution (and thus transcriptional activity) is regulated by two major regulatory mechanisms (Fig. 6G). The first is the well-defined cytoplasmic sequestration by interaction with 14-3-3 proteins upon its phosphorylation by the Hippo pathway, whereas the other, revealed here, is nuclear retention mediated by its interaction with TEADs. Whether TEAD-mediated nuclear retention of TAZ is also important for TAZ to act as a transcriptional coactivator for other transcriptional factors is unknown. It remains to be determined whether the rescued nuclear localization and transforming ability of S89A-M9-TEAD4 depend on transcriptional activity of TEAD4. Although it remains to be experimentally tested, our inclination is that the transcriptional activity of TEAD4 is important for S89A-M9-TEAD4 to be transforming, because S89A-M9 portion is expected to provide the transcriptional coactivating property to the fused TEAD4. If nuclear targeting of TEAD4 is not dependent on its DNA binding property, then introducing mutations into TEAD4 portion that abolish DNA binding (and thus transcriptional activity) but support nuclear targeting will help to resolve this issue.

Interestingly, it was observed that TEAD4 alone is able to transform MCF10A cells with efficiency comparable with that of TAZ, and this transforming ability is synergistically enhanced when it is fused to S89A-M4 or S89A-M9. This raises the possibility that TEAD4 itself may act like an oncogene during cancer development. Supporting this conclusion is the observation that TEAD4 gene is amplified, and its transcript level is increased in several cancers, including basal-like breast cancer (25, 26, 54), testicular germ cell tumors (55, 56), and serous Fallopian tube carcinoma (57). In addition, the 12p13 chromosome region where the TEAD4 gene is located has been reported to be amplified in additional cancers such as glioblastoma multiforme and desmoplastic medulloblastoma (58, 59), nasopharyngeal carcinoma (60), and head and neck squamous cell carcinoma cell lines (38). Whether TEAD4 gene is indeed amplified and/or overexpressed in these other cancers remains to be experimentally examined. It is envisioned that overexpressed TEAD4 in these cancers may engage endogenous TAZ and/or YAP as the transcriptional coactivators to mediate its oncogenic property. Consistent with this possibility is our preliminary observation that shRNA-mediated knockdown of endogenous TAZ suppressed TEAD4-mediated transformation of MCF10A cells (data not shown).

In view of the importance of TAZ-TEAD4 (and YAP-TEAD) interaction in transformation and the fact that TAZ and YAP are often uncoupled from negative Hippo regulation because of mutations or down-regulation of upstream activators and
Hippo core components, in the context that TEAD4 is frequently amplified and overexpressed in various cancer, TAZ, YAP, and TEAD4 as well as TAZ-TEAD4 and YAP-TEAD4 interactions may offer realistic targets to screen for drug candidates for targeted therapy of various cancer because of overactivation of TAZ/YAP-TEAD4. For example, the lack of targeted therapy for basal-like or triple negative breast cancer and the frequent amplification and overexpression of TEAD4, as well as increased transcript of TAZ in this type of breast cancer (24), indicate that TAZ, TEAD4, and TAZ-TEAD4 interaction will be useful drug targets for the invasive breast cancer. Future studies along these lines will provide additional insight into the role of TAZ, YAP, and TEAD4 in various cancers and the feasibility for them as therapeutic targets.

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