The Transcriptional Coactivator TAZ Is a Potent Mediator of Alveolar Rhabdomyosarcoma Tumorigenesis

Michael D. Deel, Katherine K. Slemons, Ashley R. Hinson, Katia C. Genadry, Breanne A. Burgess, Lisa E.S. Crose, Nina Kuprasertkul, Kristianne M. Oristian, Rex C. Bentley, and Corinne M. Linardic

Purpose: Alveolar rhabdomyosarcoma (aRMS) is a childhood soft tissue sarcoma driven by the signature PAX3-FOXO1 (P3F) fusion gene. Five-year survival for aRMS is <50%, with no improvement in over 4 decades. Although the transcriptional coactivator TAZ is oncogenic in carcinomas, the role of TAZ in sarcomas is poorly understood. The aim of this study was to investigate the role of TAZ in P3F-aRMS tumorigenesis.

Experimental Design: After determining from publicly available datasets that TAZ is upregulated in human aRMS transcriptionome, we evaluated whether TAZ is also upregulated in our myoblast-based model of P3F-initiated tumorigenesis, and performed IHC staining of 63 human aRMS samples from tissue microarrays. Using constitutive and inducible RNAi, we examined the impact of TAZ loss of function on aRMS oncogenic phenotypes in vitro and tumorigenesis in vivo. Finally, we performed pharmacologic studies in aRMS cell lines using porphyrin compounds, which interfere with TAZ-TEAD transcriptional activity.

Results: TAZ is upregulated in our P3F-initiated aRMS model, and aRMS cells and tumors have high nuclear TAZ expression. In vitro, TAZ suppression inhibits aRMS cell proliferation, induces apoptosis, supports myogenic differentiation, and reduces aRMS cell stemness. TAZ-deficient aRMS cells are enriched in G2-M phase of the cell cycle. In vivo, TAZ suppression attenuates aRMS xenograft tumor growth. Preclinical studies show decreased aRMS xenograft tumor growth with porphyrin compounds alone and in combination with vincristine.

Conclusions: TAZ is oncogenic in aRMS sarcomagenesis. While P3F is currently not therapeutically tractable, targeting TAZ could be a promising novel approach in aRMS. Clin Cancer Res; 24(11); 2616–30. ©2018 AACR.
Translational Relevance

Alveolar rhabdomyosarcoma (aRMS) is a soft tissue sarcoma associated with the skeletal muscle lineage, affecting mostly children and adolescents. aRMS tumors are characterized by the PAX3-FOXO1 fusion gene, a chimeric transcription factor thought to illegitimately reactivate embryonic myogenesis programs. While the majority of aRMS tumors initially respond to multimodal therapy, most will become resistant, underscoring the 5-year survival rate of <50% for all-comers and <10% when metastatic. Understanding the mechanisms responsible for aRMS cell self-renewal and chemoresistance is critical. In adult carcinomas, the TAZ transcriptional coactivator (encoded by the WWTR1 gene and a paralog of the Hippo pathway effector YAP) has been found to support tumor cell proliferation, survival, and stemness. Far less is known about the role of TAZ in sarcomas, including aRMS. Here, we show that TAZ is highly expressed in human aRMS tumors and supports aRMS tumorigenic phenotypes in vitro and in vivo. Suppression of TAZ through genetic or pharmacologic approaches diminishes aRMS tumor growth and prolongs survival in xenograft studies, and potentiates the activity of the antitubulin agent vincristine, a standard agent in aRMS therapy. These studies identify TAZ inhibition as a potential adjunct therapy to target fusion-positive aRMS stemness and chemoresistance.

When unphosphorylated, YAP and TAZ localize to the nucleus and coactivate proproliferation transcription factors (12, 13), most notably the TEAD family (14, 15). Functionally, YAP/TAZ are essential for cellular proliferation, amplification of tissue-specific progenitor cells during tissue regeneration, and ultimately control of organ size (11, 16). In many contexts, YAP and TAZ have overlapping roles. However, they share only 50% homology and have divergent functions in development. For example, YAP knockout mice are embryonic lethal, while TAZ knockout mice are viable but frequently develop polycystic kidney disease (24, 25). However, an understanding of the roles of YAP and TAZ in mesenchymal cancers, including RMS, is just beginning. In eRMS, higher YAP/TAZ expression at the IHC level correlates with reduced patient survival (8, 26, 27), and a subset of tumors have copy-number gains in the YAPI and/or WWTR1 loci (26, 27). YAP contributes to eRMS tumorigenesis by supporting proliferation and stemness, and opposing myogenic differentiation (8, 26, 28), potentially at the early steps of tumorigenesis based on a human myoblast model of eRMS (28). Similarly, TAZ contributes to eRMS by supporting proliferation, colony formation, and increasing the expression of select cancer-related genes (27). Expression of TAZS89A (a constitutively active TAZ mutant) transforms C2C12 myoblasts (27), again suggesting that YAP/TAZ exert oncogenic effects early during tumorigenesis.

Less is known about the roles of YAP/TAZ in aRMS. We had previously shown that YAP is highly abundant in P3F-aRMS cells, supporting proliferation and evasion of senescence (8). Given this, we expected to find in our previously established myoblast-based model of P3F-initiated tumorigenesis that YAP1 would be upregulated. Instead, WWTR1 was increased at the mRNA level in this model, suggesting that TAZ has a specific role in aRMS tumorigenesis. A potential functional role for TAZ in aRMS is further suggested by studies showing that TAZ is essential to the transcriptional activity of wild-type PAX3 (29, 30) and that the binding of TAZ to PAX3 occurs via domains that are retained in the P3F fusion (5). The aim of this study was to elucidate the oncogenic activity of TAZ in P3F-aRMS sarcomagenesis.

Materials and Methods

Generation of cell lines and constructs

Human RMS cell lines Rh28 (31) and Rh30 (32) were gifts from Tim Triche (Children’s Hospital of Los Angeles, Los Angeles, CA) in 2005; Rh3 (33), Rh41 (34), and CW9019 (35) were gifts from Brett Hall (Columbus Children’s Hospital, Columbus, OH) in 2006. All cell lines tested negative for mycoplasma (using Lonza MycoAlert PLUS test at the Duke University Cell Culture Facility) and were also authenticated by short tandem repeat analysis (Promega Powerplex 18D at Duke University DNA analysis facility) in 2014; Rh28 and Rh30 were reauthenticated in 2016. Human skeletal muscle myoblasts (HSMMs) and 293T cells were obtained from Lonza and the ATCC through the Duke University Cell Culture Facility, respectively, and cultured as described previously (36). Knockdown and overexpression constructs described below were stably expressed using established lentiviral and selection methods resulting in polyclonal cell lines (37). TAZ shRNA oligos (23, 38) or nontargeting (NT) scrambled controls (Supplementary Table S1) were annealed and ligated into pLKO.1-puro or Tet-pLKO-puro (Addgene plasmids #8435 and #21915, respectively). Lenti-EF-ires-blast and pLenti-EF-FH-TAZ S89A-ires-blast (Addgene #52084) vectors were gifts from Yutaka Hata (Tokyo Medical and Dental University, Tokyo, Japan). pQCXIH-Flag-YAP-S127A (Addgene 33092) was a gift from Kun-Liang Guan (University of California San Diego, CA; ref. 39).

Growth curves, MITT, BrdUrd, and cell-cycle analysis assays

Standard growth curves using manual and automated hemocytometer counting, as well as MTT and bromodeoxyuridine (BrdUrd) assays, were performed as described (40). Cell-cycle analyses were performed and analyzed by the Duke University Flow Cytometry core as described previously (41).
Rhabdosphere and limiting dilution assays

To establish and propagate aRMS cell spheres, we modified a protocol developed for eRMS cells (42). Briefly, aRMS cells were grown in ultra-low attachment plates or flasks (Corning) in Neurobasal media (Gibco) supplemented with 1× B27 (2× B27 for Rh28 cells; Invitrogen), 80 ng/mL bFGF (Corning), 40 ng/mL EGF (Sigma), and 50 μg/mL insulin. Limiting dilution assays were based on sphere formation, and assessed 48 wells per condition. Wells were scored positive (≥1 sphere/well) or negative (0 spheres/well) for sphere formation after seven days in culture. Sphere-forming frequency and statistics were calculated using ELDA software (43).

Luciferase reporter assays

Rh28 (1×10^5 cells/well) or Rh30 (5×10^4 cells/well) cells stably expressing TAZ shRNA, TAZs89A, or NT vector were transiently transfected with TEAD luciferase reporter (8× GTHC plasmid) or empty vector (pGL3-P-E) and 5 ng of Renilla reporter (pHRCTR1) for 48 hours in triplett in 24-well plates. Treatment (see Drug Treatment section below) with verteporfin (VP) or protoporphyrin IX (PPIX) was performed for 24 hours prior to reading. Luciferase activity was assayed using the Dual Luciferase Reporter Assay (Promega) as per manufacturer’s protocol in a luminometer (Turner Biosystems Modulus). Data are presented as Firefly/Renilla luciferase activity.

Cell-fractionation, qRT-PCR, and immunoblotting

For cell fractionation, 1×10^6 cells were plated in a 10-cm dish; after 48 hours in culture they were trypsinized, washed, and fractionated into subcellular components using a detergent-based kit (Cell Signaling Technology, #9038). Quantitative real-time PCR (qRT-PCR) was performed as described previously (8) using primer sets listed in Supplementary Table S1. Immunoblotting was performed as described previously (40) using anti-TAZ, anti-PAK, anti-cleaved PAK (Asp214), anti-phospho-histone (Cell Signaling Technology, #4883, #9542, #9541, #9701), anti-β-tubulin, anti-actin (Sigma #T8328, #A5441), anti-histone H3 (Abcam ab1791), and anti-MF20 (DSHB Hybridoma Product MF20) antibodies.

Mouse xenograft studies

Xenograft studies utilized 1×10^7 Rh28 cells resuspended in Matrigel (BD Biosciences) and implanted subcutaneously into the flanks of immunodeficient SCID/beige mice as done previously (8, 44). For the TAZ shRNA study, Rh28 cells stably expressing doxycycline-inducible TAZ shRNAs were used. The drinking water was supplemented with 1 mg/mL doxycycline (Sigma-Aldrich) in 5% w/v sucrose or 5% w/v sucrose (control). Mice were monitored twice weekly, and upon observing palpable tumors, randomly assigned to the doxycycline or sucrose group. We had previously demonstrated no difference in tumor growth dynamics in Rh28 cell xenografts with a NT vector control in mice treated with doxycycline versus sucrose (8, 44). Tumors were measured after 48 hours in culture they were trypsinized, washed, and after 48 hours in culture they were trypsinized, washed, and

drug treatments

For in vivo experiments, two studies were evaluated: (i) DMSO vs. VP and (ii) DMSO vs. VP vs. VCR. Drugs were administered in PBS to a concentration of 10% DMSO, 10 mg/mL VP, or 1 mg/mL VCR and administered by intraperitoneal injection. Upon palpable tumors, mice were randomly assigned to treatment groups of equal number and were dosed with DMSO, 100 mg/kg VP, and/or 1 mg/kg VCR. DMSO and VP were administered every other day for eight total treatments. VCR was administered weekly for four total treatments. Tumors were harvested at day 28 following tumor formation. A 28-day duration of therapy was predetermined in the study design as prior xenograft studies using 1×10^7 Rh28 cells had reached maximum tumor burden by this point (8, 44).

Microarray

Microarray analysis of transcriptional changes in HSMMs stably expressing P3F was described previously (8, 36). The associated
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Institutional Review Board of Duke University (Durham, NC). Study approval IACUC-approved protocols. Human TMAs obtained from the COG had been generated from deidentified patient tissue collected with informed consent and were approved for use by the Institutional Review Board of Duke University (Durham, NC).

Results

WWTR1/TAZ is upregulated in PAX3-FOXO1 (P3F)-expressing primary human skeletal muscle myoblasts, aRMS cell lines, and aRMS tumors

To gain insight into genes regulated by P3F, our laboratory developed a cell-based model of aRMS based on P3F-initiated transformation of HSMMs. In brief, forced expression of P3F in combination with p16INK4A loss in HSMMs enabled senescence bypass (37); additional expression of hTERT and MycN yielded aRMS tumors (36). Microarray analysis of P3F-initiated transformation of HSMMs revealed upregulated expression of RASSF4, which is a transcriptional target of P3F and promotes aRMS tumorigenesis by restraining the Hippo tumor suppressor kinase MST1 (8). As the transcriptional coactivator YAP is a critical downstream effector of oncogenic Hippo signaling, we anticipated finding that YAP1 would also be upregulated. However, analysis of the microarray dataset showed that YAP1 expression was unchanged, while WWTR1 (encoding TAZ) was upregulated (Fig. 1A). These findings were validated by qRT-PCR using cDNA generated from the original cells represented in the microarray (Fig. 1B and C). While the increase in expression was only about twofold, other genes known to be targets of P3F, such as CYR61 and C. Decreased expression of TAZ target genes TWAR and CTGF, and CYR61 verified functional knockdown (Fig. 2D and E). We next analyzed the phenotypic response to TAZ inhibition, and in cell counting assays found that, compared with the NT vector, the population growth of both cell lines declined over time (Fig. 2F and G).

TAZ suppression decreases cell proliferation, induces apoptosis, and supports myogenic differentiation in P3F-aRMS

To determine whether this occurred in human aRMS tumors, we queried the NIH Pediatric Oncology Branch Oncogenomics database (47) and found that WWTR1 mRNA levels are higher in human aRMS tumors than in skeletal muscle (Fig. 1C). Using the same human RMS TMA block that we had previously used to analyze YAP (8), we examined TAZ protein expression by IHC. Compared with skeletal muscle, where TAZ is membrane-bound or cytoplasmic, in aRMS tumors TAZ was predominantly nuclear (Fig. 1H). This held true for P3F, P7F, and PF-neg tumors. Interestingly, TAZ abundance is not specific to P3F aRMS and was also observed in P7F aRMS, PF-neg aRMS, and eRMS cases (Fig. 1I, Supplementary Fig. S3A–S3C), suggesting that TAZ is important for RMS tumorigenesis across the mutational landscape. Because TAZ was upregulated in our P3F-initiated model, and because TAZ is a critical coactivator for wild-type PAX3 in other cell types (29, 30), we focused our studies on P3F-positive aRMS.

As TAZ and YAP have overlapping functions, and YAP is also abundant in aRMS (8), we hypothesized that individual tumors might preferentially express one or the other, as seen in malignant peripheral nerve sheath tumors (48). To determine whether TAZ and YAP abundance inversely correlate, we reanalyzed the TMA previously immunostained for YAP (8) and observed a similar degree of YAP expression among the P3F, P7F, and PF-neg aRMS groups (Fig. 1J and K, Supplementary Fig. S3D). In the nine P3F-aRMS tumors where YAP was scored as 1 (<25% nuclear staining), two had a TAZ score of 2, and seven had a TAZ score of 3. However, using a Pearson correlation coefficient, TAZ and YAP levels do not significantly correlate in P3F-aRMS samples, in the other subgroups, or overall in aRMS (Supplementary Fig. S3E and S3F). This suggests that most aRMS tumors coexpress TAZ and YAP, which was also observed in synovial sarcoma (48).

TAZ suppression inhibits P3F-aRMS cell growth

After determining TAZ abundance in human aRMS cell lines and tumor tissue, we sought to determine the phenotypic consequence of TAZ loss of function in Rh28 and Rh30 (both P3F-positive) human aRMS cell lines. However, because prior reports had noted mostly cytoplasmic TAZ in aRMS IHC studies (27), to be sure that there was a significant nuclear (active) TAZ pool in human aRMS cells, we took a complementary biochemical approach and fractionated aRMS cells into cytoplasmic, nuclear, and membrane-bound compartments (Fig. 2A). As expected, TAZ was predominantly cytoplasmic or membrane-bound in undifferentiated C2C12 myoblasts. On the other hand, TAZ was predominantly nuclear in both aRMS cell lines, corroborating our TMA IHC studies. We thus tested the effect of TAZ loss of function using RNAi, generating five independently targeting, lentiviral-delivered, constitutive shRNAs to TAZ. Of these, two (sh2 and sh5) were selected for further study as they consistently demonstrated TAZ knockdown. Rh28 and Rh30 cells were studied in tandem, and in response to TAZ-directed shRNAs both showed increased TAZ expression was also only upregulated about twofold (Fig. 1D), suggesting that the increase in TAZ could be important.

After confirming that TAZ was upregulated in the HSMM-based model, we examined WWTR1 mRNA and TAZ protein expression in a panel of P3F or P7F fusion-positive human aRMS cell lines. In contrast to quiescent mature skeletal muscle, both WWTR1 and TAZ expression were significantly higher in aRMS (Fig. 1E and F). To determine whether this occurred in human aRMS tumors, we queried the NIH Pediatric Oncology Branch Oncogenomics database (47) and found that WWTR1 mRNA levels are higher in human aRMS tumors than in skeletal muscle (Fig. 1G). Using the same human RMS TMA block that we had previously used to analyze YAP (8), we examined TAZ protein expression by IHC. Compared with skeletal muscle, where TAZ is membrane-bound or cytoplasmic, in aRMS tumors TAZ was predominantly nuclear (Fig. 1H). This held true for P3F, P7F, and PF-neg tumors. Interestingly, TAZ abundance is not specific to P3F aRMS and was also observed in P7F aRMS, PF-neg aRMS, and eRMS cases (Fig. 1I, Supplementary Fig. S3A–S3C), suggesting that TAZ is important for RMS tumorigenesis across the mutational landscape. Because TAZ was upregulated in our P3F-initiated model, and because TAZ is a critical coactivator for wild-type PAX3 in other cell types (29, 30), we focused our studies on P3F-positive aRMS.

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Figure 1.
TAZ is upregulated in PAX3-FOXO1–expressing primary human skeletal muscle myoblasts, aRMS cell lines, and tumors. A, Expression profile of human skeletal muscle myoblast (HSMM) vector control cells (Vpre) compared with PAX3-FOXO1 (P3F)–expressing HSMM cells presenescence bypass (PFpre) or postsenescence bypass (PFpost). This image is modified with permission from the Journal of Clinical Investigation. Portions of these data and validation of internal controls (WNT5A, DUSP4, MYOD, FGFR4, CXCR4) were previously reported (8, 44). qRT-PCR verifies increased WWTR1 (TAZ) expression (B), unchanged YAP expression (C), and validation of IL4R as a low-expressing internal control gene induced by P3F (refs. 45, 46; D). AAs measured by qRT-PCR (E) and immunoblot (F), P3F and P7F human aRMS cell lines express high levels of TAZ compared with human skeletal muscle. G, From microarray data in the Oncogenomics database (47), TAZ expression is higher in fusion-positive and fusion-negative primary human aRMS tumors than in human skeletal muscle. Representative images of RMS TMA cores immunostained for TAZ (H) and YAP protein (I). Scale bars, 100 μm. Quantification of TAZ (J) and YAP staining (K) in RMS shows increased expression of both proteins. For TAZ staining, muscle, N = 11; P3F, N = 34; P7F, N = 13; PF-neg, N = 13. For YAP staining, muscle, N = 11; P3F, N = 36; P7F, N = 10; PF-neg, N = 15. While the gene name for TAZ is WWTR1, for simplicity the label TAZ is used throughout the remainder of the figures.

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Figure 2.
Genetic suppression of TAZ inhibits aRMS cell growth, decreases cell proliferation, and increases apoptosis. A, Cell fractionation reveals enrichment of nuclear (active) TAZ in Rh28 and Rh30 aRMS cells, compared with primarily cytoplasmic/membrane expression in C2C12 murine myoblasts. β-tubulin and histone H3 are used as markers of cytoplasmic and nuclear expression. B and C, Lentiviral-mediated suppression of TAZ in Rh28 and Rh30 cells shows consistent knockdown as measured by qRT-PCR and immunoblot. TAZ knockdown also leads to suppression of TAZ target genes CTGF and CYR61 as measured by qRT-PCR (D and E), decreased cell growth as measured by cell counting in culture (F and G), and decreased proliferation as measured by BrdUrd incorporation (H and J). Conversely, aRMS cells expressing constitutively active TAZ (TAZS89A) show increased proliferation (I and K). TAZ suppression also led to increased apoptosis, as measured by immunoblots of both full-length and cleaved PARP (L and M). Actin used as loading control. C, cytoplasmic; N, nuclear; M, membrane; NT, nontargeting scrambled control vector. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
TAZ suppression promotes myogenic differentiation, and enriches the G2–M population. A and B, Rh28 and Rh30 cells stably expressing TAZ shRNAs and cultured in differentiation-inducing conditions display morphologic elongation as well as increased staining for MF20 (myosin heavy chain) expression, increased MF20 expression as measured by immunoblot (C and D), and increased myogenic marker expression as measured by qRT-PCR (E and F). Scale bars, 100 µm. G and H, Rh28 and Rh30 cells stably expressing TAZ shRNAs show an increase in the G2–M phase of the cell cycle as measured by flow cytometry of DNA content. Bars represent the average and SE of each group. N = 3 (Rh28s), N = 2 (Rh30s). I, As measured by immunoblot for M-phase-specific phosphorylation of histone H3, TAZ suppression in Rh30 cells leads to an accumulation of cells in M phase. Actin used as loading control. NT, nontargeting scrambled control vector.
immunoblot for MF20 (sarcomeric myosin), and qRT-PCR for expression of MYOD1, MYOG, and MYF6. TAZ suppression also increased myogenic differentiation (Fig. 3A–E). Of note, increased myogenic differentiation was observed only in aRMS cells cultured in fusion media; aRMS cells cultured in normal growth media did not undergo morphologic elongation and had no or minimal increase in myogenic markers (Supplementary Fig. S4A and S4B). These findings were in contrast to earlier reports in C2C12 myoblasts and skeletal muscle, which showed that TAZ has a prodifferentiation effect (20, 27). Given these contrasting findings, we also knocked down TAZ in C2C12 myoblasts and found that, as others had observed (20, 49, 50), differentiation of C2C12s is TAZ dependent (Supplementary Fig. S4C–S4E). To begin to understand the differential response to TAZ suppression in transformed (aRMS) versus nontransformed (C2C12) cells, we hypothesized that the cellular response to TAZ expression might depend upon whether a cell is “poised” for differentiation, and that in a heterogeneous population we might see both phenotypes. To investigate this, we turned to a complementary gain-of-function approach and ectopically expressed a constitutively active TAZ mutant (TAZ89A, Supplementary Fig. S4F) in Rh28 and Rh30 cells. While a small proportion of the cells initially underwent a morphologic elongation reminiscent of myotubes, the majority had higher proliferation, as shown by increased BrdUrd incorporation (Fig. 2I and K), suggesting a heterogeneous response to TAZ expression that is cell-context dependent. In summary, TAZ suppression decreases cell proliferation, induces apoptosis, and supports myogenic differentiation in P3F-aRMS cells.

TAZ-deficient aRMS cells are enriched in the G2–M phase of the cell cycle

To determine additional mechanisms by which TAZ suppression blocks aRMS cell growth, we performed cell-cycle analysis of aRMS cells stably expressing TAZ shRNAs. Compared with the NT control vector, TAZ-deficient cells were enriched in the G2–M phase of the cell cycle (Fig. 3G and H), with an increase from 19% to 32%–34% in Rh28 cells and an increase from 16% to 21%–22% in Rh30 cells. To determine whether cells were arrested in G2 or M, we blotted for phospho-histone H3 (p-HH3), which is specifically phosphorylated during mitosis. We did not see an increase in p-HH3 expression with TAZ suppression in Rh28 cells (Supplementary Fig. S4G) but did see a modest increase in p-HH3 in Rh30 cells (Fig. 3I). These data suggest that TAZ depletion results in an accumulation of cells in G2–M; however, a primary reduction of cells in G1–S is not ruled out and needs further study.

P3F-aRMS cancer cell stemness is TAZ dependent

As TAZ confers stem-like properties and chemoresistance in cancer cells of epithelial origin (21, 23, 24), we hypothesized that TAZ might also be important for stemness in RMS. In addition, although fusion-positive aRMS is typically sensitive to initial chemotherapy, acquired resistance and/or recurrence are common, occurring in up to 50% of cases (3). This implies that within a population of aRMS cells, some may be initially quiescent but have the ability to self-renew. To determine whether TAZ mediates P3F-aRMS stemness, we developed a three-dimensional (3D) sphere culture system that is a well-established method for studying cancer stem cell biology (23, 24), including in eRMS cells (42). We adapted an existing eRMS sphere culture protocol to permit growth of aRMS and showed that aRMS cells are also capable of forming and being propagated as spheres (Fig. 4A and C). Passage of these aRMS spheres resulted in a 3- to 12-fold increase in WWTR1 expression as measured by qRT-PCR (Fig. 4B and D), suggesting that TAZ may be important for stem cell enrichment.

We then examined whether the expression of stem cell markers SOX2, OCT4, and NANOG were TAZ dependent in aRMS cells. As demonstrated via qRT-PCR, TAZ suppression led to decreased expression of these markers (Fig. 4E–H). To determine the functional role of TAZ in aRMS cancer cell stemness, we performed limiting dilution assays (LDA) using aRMS rhabdospheres (Fig. 4I and J). We stably expressed the TAZ shRNAs or NT control in Rh28 and Rh30 cells, then plated them at varying densities. While the estimated sphere-forming frequency of the NT control for Rh28 and Rh30 cells was 1/24 and 1/21, respectively, it was reduced to <1/44 in both TAZ shRNA groups, in both cell lines. These data suggest that TAZ is functionally required for P3F-aRMS stemness and cancer cell self-renewal.

TAZ suppression attenuates tumor growth in vivo in Rh28 aRMS xenografts

After determining that suppression of TAZ decreases proliferation, induces apoptosis, supports myogenic differentiation, and decreases stemness in P3F-aRMS cells in vitro, we used Rh28 cell xenografts to examine the role of TAZ in aRMS sarcomagenesis in vivo. As constitutive suppression of TAZ in Rh28 cells inhibited cell viability, we used a conditional doxycycline-inducible shRNA system to suppress TAZ expression after tumor formation. After validating the constructs in vitro (Supplementary Fig. S5 and S6), we generated SQ xenografts and found that inducible TAZ knockdown decreased tumor growth and prolonged survival (Fig. 5A and B). By day 21, IACUC-defined maximum tumor burden was reached in all of the mice in the control groups, compared with only one tumor in the doxycycline groups, which had a mean day 21 tumor volume of 801 mm3. Using qRT-PCR (Fig. 5C, left) and IHC (Fig. 5D, second column), we validated that TAZ suppression, including decreased nuclear TAZ expression, (Fig. 5E, left) was maintained throughout the duration of the study at the mRNA and protein levels. We then evaluated potential mechanisms through which TAZ inhibition caused in vivo growth delay. We performed IHC analysis of Ki67 and TUNEL (Fig. 5D, third and fourth columns) to evaluate proliferation and apoptosis, respectively. Similar to our findings in vitro, TAZ suppression in vivo led to decreased proliferation, as well as a slight increase in apoptosis (Fig. 5F and G).

While we observed tumor growth inhibition with TAZ suppression, by day 21 the mice receiving doxycycline began to exhibit similar growth as the control groups, suggesting the P3F-aRMS cells either developed resistance to TAZ suppression or mechanisms to compensate for TAZ inhibition. Anticipating that YAP upregulation could be a compensatory mechanism, we examined YAP1 expression via qRT-PCR (Supplementary Fig. S7A), as well as TAZ/YAP-TEAD targets CTGF and CYR61 (Fig. 5C). While YAP1, CTGF, and CYR61 mRNA expression was slightly higher in the two doxycycline-treated tumors that had grown most rapidly, YAP1 expression did not change and targets CTGF and CYR61 were overall suppressed, indicating these were not mechanisms of overcoming TAZ inhibition. This result is similar to the in vitro observations made with TAZ suppression in C2C12 myoblasts and RD eRMS cells, where no compensatory increase in YAP expression was seen (27), and suggests that the
P3F-aRMS xenografts developed alternate means for overcoming TAZ suppression.

Pharmacologic inhibition of TAZ-TEAD activity diminishes aRMS cell and tumor growth

As TAZ is a transcriptional coactivator, it does not directly bind DNA but rather exerts tumorigenic activities through binding to and activating a host of oncogenic transcription factors, including TEADs (14). TEAD activity was shown to be TAZ dependent in skeletal muscle and RD eRMS cells (27). To verify this was the case for P3F-aRMS, we transiently transfected constitutively active TAZS89A along with an 8xGTIIC TEAD reporter in Rh28 and Rh30 cells and observed a 7- to 11-fold increase in TEAD activity (Fig. 6A; Supplementary Fig. S7B).

Porphyrin compounds were identified in a drug screen to disrupt the association between YAP and TEADs (51). Although not used clinically as an anticancer therapy, verteporfin (VP) is FDA approved for the treatment of macular degeneration. Both VP and protoporphyrin IX (PPIX), another porphyrin derivative, are commonly used in preclinical studies of the Hippo pathway (28, 51, 52). We tested these compounds in the TEAD reporter assay in P3F-aRMS cells, and both drugs decreased TEAD activity (Fig. 6B and C; Supplementary Fig. S7C–S7F). In addition, VP treatment partially abrogated constitutive TAZS89A activity (Fig. 6A, Supplementary Fig. S7B), and enhanced the effects of TAZ shRNA suppression (Fig. 6B). These studies suggest TEADs are activated by TAZ in P3F-aRMS, but targeting both TAZ and YAP may be necessary to completely abolish TEAD activity.

Figure 4.
TAZ supports and is necessary for aRMS cancer cell stemness. Rh28 (left) and Rh30 (right) aRMS cells were grown as 3D rhabdospheres. A and C, Representative images of spheres. Scale bars, 100 μm. B and D, Compared with cells grown as an adherent monolayer, serial sphere passage increases TAZ expression, as measured by qRT-PCR. TAZ suppression (E, G) also leads to decreased expression of stem cell genes SOX2, OCT4, and NANOG, as measured by qRT-PCR. mRNA expression was normalized to GAPDH (F and H). I and J, Limiting dilution assays in cells stably expressing TAZ shRNAs show TAZ is necessary for stem cell renewal in aRMS cells. The average sphere-forming frequency is shown, with the expected range in parentheses. During LDA optimization, neither Rh28 nor Rh30 cells were able to form spheres when plated at 1 cell/well, and compared to Rh30 cells, Rh28 cells were less able to form spheres at lower cell densities. P, passage number.

P3F-aRMS xenografts developed alternate means for overcoming TAZ suppression.

Pharmacologic inhibition of TAZ-TEAD activity diminishes aRMS cell and tumor growth

As TAZ is a transcriptional coactivator, it does not directly bind DNA but rather exerts tumorigenic activities through binding to and activating a host of oncogenic transcription factors, including TEADs (14). TEAD activity was shown to be TAZ dependent in skeletal muscle and RD eRMS cells (27). To verify this was the case for P3F-aRMS, we transiently transfected constitutively active TAZS89A along with an 8xGTIIC TEAD reporter in Rh28 and Rh30 cells and observed a 7- to 11-fold increase in TEAD activity (Fig. 6A; Supplementary Fig. S7B).

Porphyrin compounds were identified in a drug screen to disrupt the association between YAP and TEADs (51). Although not used clinically as an anticancer therapy, verteporfin (VP) is FDA approved for the treatment of macular degeneration. Both VP and protoporphyrin IX (PPIX), another porphyrin derivative, are commonly used in preclinical studies of the Hippo pathway (28, 51, 52). We tested these compounds in the TEAD reporter assay in P3F-aRMS cells, and both drugs decreased TEAD activity (Fig. 6B and C; Supplementary Fig. S7C–S7F). In addition, VP treatment partially abrogated constitutive TAZS89A activity (Fig. 6A, Supplementary Fig. S7B), and enhanced the effects of TAZ shRNA suppression (Fig. 6B). These studies suggest TEADs are activated by TAZ in P3F-aRMS, but targeting both TAZ and YAP may be necessary to completely abolish TEAD activity.
Figure 5.
Suppression of TAZ inhibits aRMS tumor growth and prolongs survival in murine xenografts. TAZ suppression in Rh28 xenografts (A) delays tumor progression, as measured by time to maximum tumor burden, and (B) prolongs survival as measured by Kaplan–Meier survival plot. Median survival and 95% confidence interval for the groups were: Suc sh2 (17.5/C6 0.98 days), Dox sh2 (33/C6 3.49 days), Suc sh5 (17.5/C6 2.47 days), and Dox sh5 (30/C6 3.39 days). C, qRT-PCR validation of TAZ suppression and downregulation of TAZ target genes (CTGF, CYR61). D, Representative IHC images of H&E, TAZ, Ki67, and TUNEL staining for each of the groups. EHC quantitation showing the TAZ shRNA groups have (E) decreased nuclear TAZ staining, (F) decreased proliferation (Ki67), and (G) increased apoptosis (TUNEL). Scale bars: 100 μm. N = 4 in each arm except Dox TAZ_sh5 group, where one mouse did not develop a tumor, so N = 3.
Pharmacologic inhibition of TAZ-TEAD activity diminishes aRMS cell and tumor growth. TEAD luciferase activity (8XGTIIC–Luc reporter) is increased in Rh28 cells expressing constitutively activate TAZ mutant TAZ89A, but partially reversible with treatment of 10 μmol/L VP (A), decreased in Rh28 cells treated with 10 μmol/L VP, as well as in Rh28 cells stably expressing TAZ shRNAs; and decreased in Rh28 cells treated with 1 or 10 μmol/L PPIX. D and E, Dose–response curve in Rh28 cells treated with VP or PPIX. F, Rh28 cells treated with 10 μmol/L PPIX have decreased cell growth, as measured by manual cell counting. G, Rh28 xenografts treated with 100 mg/kg VP have decreased tumor growth as compared with vehicle control (DMSO). H, TAZ89A decreases Rh28 cell sensitivity to VCR. I, Dose-dependent cooperativity with either 0.3 or 1 μmol/L VP and VCR in Rh28 cells. J, The combination of VP and VCR in vivo is more effective than either agent alone in inhibiting tumor growth. N = 5 mice in each group. VP, verteporfin; PPIX, protoporphyrin IX.
After validating target inhibition using porphyrin compounds in aRMS cells, we evaluated Rh28 cell viability following treatment with PPIX and VP. We determined that 10 μmol/L of either drug inhibits cell growth (Fig. 6D and E), which is consistent with the doses used in other cancer studies (28, 51, 52). We then tested these drugs on P3F-aRMS cell and tumor viability. PPIX treatment dramatically reduced Rh28 cell growth in vitro as measured by cell counting over 5 days (Fig. 6F). Delayed tumor growth was also seen with VP treatment of Rh28 SQ xenografts in vivo (Fig. 6G). As tumors became palpable, mice were randomly assigned to treatment with VP or DMSO vehicle via intraperitoneal injection every other day for eight doses. Similar to what was seen with VP treatment in eRMS (28), responses were variable. While a near-complete response was seen in two of the mice, one tumor grew to a tumor mass over 5 days (Fig. 6H; Supplementary Fig. S7J), suggesting that TAZ suppression decreased aRMS cell growth and proliferation, increased apoptosis, and prolonged survival. Our finding that TAZ opposes myogenic differentiation was not unexpected—indeed a signature phenotype of RMS is its loss of the ability to differentiate (59). However, as in normal skeletal myogenesis, TAZ associates with MyoD and promotes differentiation, we speculate that TAZ behaves differently depending upon whether the cellular milieu (epigenetic state) is permissive for differentiation signals. This has been noted with other proteins such as mTOR, which antagonizes myogenic differentiation during conditions favoring proliferation but facilitate myogenic differentiation if activated during conditions favoring differentiation (60).

Because TAZ suppression leads to a G2–M arrest in P3F-aRMS cells, and TAZ mediates resistance to antitubulin drugs in other malignancies (23, 52–55), we assessed whether VP might cooperate with VCR to increase its therapeutic effect. As measured via MTT assay, an additive effect is seen in Rh28 cells treated with the combination of VCR and either 0.3 or 1 μmol/L VP (Fig. 6I). To test whether TAZ inhibition could potentiate the effects of VCR in vivo, we evaluated the combination of VP and VCR in Rh28 SQ xenografts. VP was again administered every other day for eight doses and VCR was injected weekly for four doses. While efficacy was seen in monotherapy of both drugs, the combination of VP plus VCR was most effective (Fig. 6I), suggesting that TAZ-TEAD inhibition augments the activity of anti-tubulin drugs in fusion-positive aRMS.

Discussion

Although more than 20 years have passed since the PAX3-FOXO1 (P3F) fusion was discovered as the principal mutation responsible for otherwise karyotypically simple aRMS tumors, survival rates for patients with P3F-aRMS remain dismal. VAC; ref. 3). In an MTT assay, we treated Rh28 and Rh30 cells expressing either constitutively active TAZS89A or control with VP treatment in eRMS (28), responses were variable. While a near-complete response was seen in two of the mice, one tumor grew to a tumor mass over 5 days (Fig. 6H; Supplementary Fig. S7J), suggesting that TAZ suppression decreased aRMS cell growth and proliferation, increased apoptosis, and prolonged survival. Our finding that TAZ opposes myogenic differentiation was not unexpected—indeed a signature phenotype of RMS is its loss of the ability to differentiate (59). However, as in normal skeletal myogenesis, TAZ associates with MyoD and promotes differentiation, we speculate that TAZ behaves differently depending upon whether the cellular milieu (epigenetic state) is permissive for differentiation signals. This has been noted with other proteins such as mTOR, which antagonizes myogenic differentiation during conditions favoring proliferation but facilitate myogenic differentiation if activated during conditions favoring differentiation (60).

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actively investigating ways to target TAZ and/or the TAZ provides proof-of-principle that interfering with TAZ–TEAD and YAP–TEAD interactions is a promising approach. We predict that dual inhibition of TAZ and YAP may be more efficacious than monotherapy, as there is feedback between these paralogs, and inhibition of one may induce the upregulation of the other. For example, combining TAZ suppression with 5-fluorouracil paradoxically increased hepatocellular carcinoma cell growth through upregulation of YAP protein (64). In the current work, the xenograft tumors eventually grew despite TAZ RNAi or VP treatment, reinforcing the need to target TAZ in the context of rational combination therapies. On the other hand, VP combined with VCR was effective at blocking tumor growth.

While the P3F transcription factor is currently not therapeutically tractable, inhibiting TAZ may be a means of attenuating its activity. In melanocytes, TAZ is essential to the transcriptional activity of wild-type PAX3 (29, 30), and the binding of TAZ to PAX3 occurs through domains that are retained in the P3F fusion (5). TEAD1 and AP-1, progrowth transcription factors that depend on TAZ/YAP activation (10), are among the top enriched motifs in P3F-binding sites (46), suggesting that these proteins may work together to control P3F-mediated transcription, a future research direction in our laboratory.

Finally, our study of TAZ biology in P3F-aRMS may shed light on the role of TAZ in sarcomas in gene expression analysis of TAZ. In a large number of sarcomas showed that TAZ may be associated with more undifferentiated and/or higher-grade tumors (48). For example, 87% (13/15) of high-grade dedifferentiated and pleomorphic liposarcomas stained positive for nuclear TAZ, while only 6% (1/17) of low-grade myxoid and well-differentiated liposarcomas were positive. When analyzing all sarcomas, nuclear YAP and TAZ were expressed in 50% and 66% of 159 samples, respectively, and higher YAP or TAZ expression independently correlated with higher tumor grade and worse survival (48).

In conclusion, understanding TAZ biology may provide insight into molecular mechanisms of sarcomagenesis and treatment resistance (48). Here, we show that TAZ is abundant in human aRMS tumor samples, and that TAZ suppression decreases proliferation, promotes differentiation, and inhibits cancer cell stemness. TAZ-deficient aRMS cells are also enriched in G0–M, suggesting that TAZ may be important for G0–M cell-cycle progression. Constitutive activation of TAZ diminishes the efficacy of VCR, and combining VP with VCR is more effective than either agent alone in blocking aRMS xenograft tumor growth. Inhibiting TAZ is a promising adjunctive therapy for targeting the aRMS CSC population and reducing chemoresistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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The Transcriptional Coactivator TAZ Is a Potent Mediator of Alveolar Rhabdomyosarcoma Tumorigenesis

Michael D. Deel, Katherine K. Slemmons, Ashley R. Hinson, et al.

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