Common and Distinctive Functions of the Hippo Effectors Taz and Yap in Skeletal Muscle Stem Cell Function

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ABSTRACT

Hippo pathway downstream effectors Yap and Taz play key roles in cell proliferation and regeneration, regulating gene expression especially via Tead transcription factors. To investigate their role in skeletal muscle stem cells, we analyzed Taz in vivo and ex vivo in comparison with Yap. Small interfering RNA knockdown or retroviral-mediated expression of wild-type human or constitutively active TAZ mutants in satellite cells showed that TAZ promoted proliferation, a function shared with YAP. However, at later stages of myogenesis, TAZ also enhanced myogenic differentiation of myoblasts, whereas YAP inhibits such differentiation. Functionally, while muscle growth was mildly affected in Taz (gene Wwtr1–/–) knockout (KO) mice, there were no overt effects on regeneration. Conversely, conditional KO of Yap in satellite cells of Pax3Cre-ERT2/; Yapfl/fl; Rosa26lac mice produced a marked regeneration deficit. To identify potential mechanisms, microarray analysis showed many common TAZ/YAP target genes, but TAZ also regulates some genes independently of YAP, including myogenic genes such as Pax7, Myf5, and Myod1 (ArrayExpress–E-MTAB-5395). Proteomic analysis revealed many novel binding partners of TAZ/YAP in myogenic cells, but TAZ also interacts with proteins distinct from YAP that are often involved in myogenesis and aspects of cytoskeleton organization (ProteomeXchange–PXD005751). Neither TAZ nor YAP bind members of the Wnt destruction complex but both regulated expression of Wnt and Wnt-cross talking genes with known roles in myogenesis. Finally, TAZ operates through Tead4 to enhance myogenic differentiation. In summary, Yap and Taz have overlapping functions in promoting myoblast proliferation but Taz then switches to enhance myogenic differentiation.

SIGNIFICANCE STATEMENT

Hippo pathway effectors Yap and Taz play key roles in cell proliferation and tissue growth. We analyzed Taz in comparison with Yap in muscle stem cells. Taz promoted proliferation, a function shared with Yap. However, Taz also enhanced differentiation into myotubes, unlike Yap. Muscle growth was affected in Taz (Wwtr1–/–) knockout (KO) mice, while KO of Yap produced a clear regeneration deficit. Taz regulates some genes independently of Yap, and Taz also interacts with proteins distinct from Yap, mainly involved in myogenesis/cytoskeleton. In particular, Taz operates through Tead4 to enhance differentiation. In summary, Yap and Taz have overlapping functions in promoting myoblast proliferation but later, Taz also enhances myogenic differentiation.

INTRODUCTION

Transcriptional cofactors Yap (Yap1) and Taz (Wwtr1) mainly regulate gene expression by binding Tead1–4 transcription factors. Together Yap, Taz, and Teads are the nexus of the Hippo signal transduction network that includes the Hippo kinase cascade, comprising kinases Mst1 (Stk4), Mst2 (Stk3), Lats1, and Lats2 [1–3]. Many other signaling modules also regulate Yap and Taz activity, such as mechanotransduction [4], glucose-signaling [5], GPCR [6], Wnt [7–9], Smad [10, 11], Notch [12], Pten-Akt-mTOR [13–16], and Lkb1-Ampk [17–20]. Yap and Taz are inhibited by phosphorylation of multiple HKRSS motifs by Lats1/2, which promotes localization to the cytosol, 14–3–3 binding, and degradation [3, 21]. Yap Ser127 and
Taz Ser89 are key phosphorylation sites, and mutations at Ser127 or Ser89 to alanine in Yap S127A and Taz S89A prevent phosphorylation at these residues and result in constitutive activity.

Signaling modules involving Yap/Taz control skeletal muscle myogenesis and adaptation to exercise. Tead transcription factors bind CATTCC/GGAAGT (MCAT or GTIIC motifs) often found near promoters of cardiac and skeletal muscle genes [3, 22], and by binding enhancers [23, 24]. However, Tead1 binds muscle genes repressed in rhabdomyosarcoma [25], consistent with observation that Yap and Taz can also repress gene expression [26].

Satellite cells are responsible for postnatal skeletal muscle growth, hypertrophy, and repair/regeneration [27], and we have shown that Yap promotes proliferation of myoblasts [28, 29] but inhibits myogenic differentiation. YAP1 S127A expression in activated, but not quiescent, satellite cells causes embryonal rhabdomyosarcoma-like tumours with short latency and 100% penetrance [25]. Expression of YAP1 S127A in muscle fibers causes myopathy [30], whereas other types of Yap delivery or Yap mutants can cause hypertrophy [31, 32], with outcome likely dependent on Yap levels.

Taz harbors the same functionally important WW and Tead-binding domains as Yap and often acts as a paralogue, but not always [2, 3]. This is demonstrated by Yap or Taz KO mice: while Yap KO causes early embryonic lethality [33], 50% of Taz (Wwtr1) KO mice are viable but develop glomerulocystic kidney disease [34]. Earlier reports suggest that Yap and Taz also have divergent functions in the skeletal muscle lineage: both Yap and Taz promote skeletal muscle fiber hypertrophy and regeneration [35, 36], but only Taz promotes fusion into multinucleated myotubes [29, 37, 38].

The distinct functions of Yap and TAZ are poorly understood, so we investigated their regulation, function, target genes, and binding partners in murine myoblasts. Taz and Yap have overlapping functions in promoting satellite cell proliferation. However, later in myogenesis, Taz enhances myogenic differentiation and function, a feature not shared by Yap. Taz (Wwtr1−/−) null mice had no overt muscle regeneration phenotype, unlike mice with conditional KO of Yap in satellite cells. To understand mechanism, we examined gene expression, and found, for example, that Yap and TAZ both alter expression of multiple Wnt pathway genes. In addition to common targets, TAZ also regulates a separate set of genes from Yap during myogenesis, including myogenic factors Pax7, Myf5, and MyoD. Using proteomics to identify binding partners of TAZ and Yap, we discovered both common and distinct interactors, with TAZ binding proteins that regulate myogenesis and aspects of cytoskeleton organization. Finally, we found that TAZ operates through Tead4 in myoblasts to enhance myogenic differentiation.
with 0.5% Triton-X100/PBS and blocked with 10% goat serum/PBS or 0.035% carrageenan/PBS followed by incubation with antibodies overnight at 4°C [41]. Antibodies: anti-Pax7 (DSHB); anti-myosin heavy chain (MyHC) (MF20, DSHB); anti-myogenin (F5D, DSHB); anti-MyoD (clone 5.8A, DakoCytoCytometry); anti-Taz (HPA007415, Sigma); anti-Yap1 (2F12, Abnova); anti-Tead4 (M01, Abnova).

Cryosections were fixed with 4% PFA/PBS followed by cooled methanol before antigen retrieval in heated citrate buffer [44] and blocking in 10% goat serum/PBS. Antibodies: anti-MyHC Type I (BA-D5, DSHB), anti-MyHC Type Ila (A4.74, DSHB), and anti-laminin (Sigma, L9393). Fluorochrome-conjugated secondary antibodies were from Thermofisher Scientific.

5-Ethyl-2'-deoxyuridine (EdU) (10 μM) was added for 2 hours before fixation and incorporation detected using Click-iT (Thermofisher Scientific) according to manufacturer’s instructions.

Western Blotting
Western blotting was performed using Run Blue precast native Page gels (Expeidon). Protein transfer was performed with the XCell II blot module (ThermoFisher Scientific). PVDF membranes were incubated with antibodies overnight/4°C and visualized using fluorochrome-conjugated secondary antibodies (Thermofisher Scientific) and digitally imaged.

Mass Spectrometry
C2C12 cells were grown in DMEM (D5761) with 10% FBS and 4 mM glutamine. Proliferating C2C12 cells were at 50% cell density. Confluent cultures were differentiated for 72 hours in DMEM, 2% horse serum, 4 mM glutamine.

For immunoprecipitation, 80,000 C2C12s were seeded per 10 cm dish. The following day, fresh medium was added 1 hour before addition of 1.5 diluted TAZ or YAP encoding retroviral supernatant. The next day, cells were replated and transduction confirmed with green fluorescent protein (GFP). Cells were washed on ice with PBS, and collected in lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1% Triton X-100) with 1 mM sodium orthovanadate, protease inhibitor cocktail (Sigma, p8340), and PMSF. Lysates were incubated for 1 hour on ice and centrifuged at 14,000 RPM at 4°C for 5 minutes and supernatant incubated at 4°C with anti-Flag M2 agarose beads (Sigma). Beads were washed three times with washing buffer (150 mM NaCl; 20 mM Tris-HCL pH 7.5). Sample preparation/mass spectrometry were as described [45], with proteomics data deposited to the ProteomeXchange Consortium via the PRIDE [46] partner repository: identifier PXD005751.

Microarray
Total RNA was isolated from TAZ S89A or YAP S127A transduced myoblasts after 24 or 48 hours using TRIzol (ThermoFisher Scientific) followed by purification and DNase digestion (RNeasy minikits, Qiagen). RNA quantification was performed on a Nanodrop spectrophotometer (ThermoFisher Scientific) and quality tested on an Agilent Tapestation (RIN 7.6–9.8).

Generation of sense strand cDNA, second strand synthesis, in vitro transcription cRNA synthesis, single stranded cDNA synthesis and RNA hydrolysis, fragmentation, and labeling were as manufacturer’s instructions (GeneChip WT Plus reagent kit, Affymetrix). Hybridization, washing, staining, and scanning of microarrays were carried out on Affymetrix Mouse Gene 2.0 ST microarrays using a GeneChip Fluidics station 450 and GCS3000 scanner (Affymetrix®).

Data preprocessing/quality control were performed using Affymetrix® GeneChip® Expression Console v1.2. Probe cell intensity data (CEL files) were processed using the RMA16 algorithm (Affymetrix), which fits a linear model at probe level by using background correction, quantile normalization of log2 transformed data, and summarization, for primary QC analysis. Performed in triplicate at Centre for Genome Enabled Biology and Medicine (University of Aberdeen).

Data were analyzed in Partek® Genomics Suite®, version 6.6, build 6.15.0730 Copyright; 2014 (Partek Inc.) using a Mouse Gene 2.0 ST annotation file from build mm10, MoGene-2.0-st-v1.na35.mm10.transcript. Affymetrix CEL files were imported to Partek® Genomics Suite®, data processed using RMA normalization with RMA background correction and quantile normalization of log2 transformed data and probeset summarization by median polish. Two-way analysis of variance (ANOVA) with time point (24 and 48 hours) and transcription factor (Control, TAZ S89A, YAP S127A) and time × transcription interaction to evaluate significantly differentially expressed genes. Fold change in TAZ S89A or YAP S127A compared with control vector as baseline at each time point calculated using geometric mean of samples in each group with significance calculated by Fishers Least significant difference. Fold change ≥1.3 and FDR of 10% were evaluated. Microarray data available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress), accession number E-MTAB-5395.

Histology
H&E and ATPase staining were described previously [30]. Muscle sections were stained with ATPase at pH 4.47 to detect type I, and pH 10.5 to detect type II, myofibers.

Image Acquisition and Analysis
Images were obtained at room temperature on a Zeiss microscope (Axiovert 200M) equipped with LD A-plan 20×/0.85 ph1, 10×/0.30ph1 and 40×/0.75ph1 objectives or on a Zeiss Exciter laser scanning microscope (LSM) with 40×/1.1 W Corr LD C-Apochromat objective. Images were quantified with image J software (NIH). Neonatal MyHC labeling was quantified with R software. Values are mean ± SEM with a Student’s t test (paired or unpaired as appropriate) or ANOVA with a post-hoc Tukey’s test for >2 groups.

Results

Taz Is Dynamically Regulated during Myogenic Progression
To assess Yap and Taz expression dynamics during muscle regeneration, we measured mRNA levels by RT-qPCR in vivo and myogenic progression in vitro. Murine TA muscle was injured by intramuscular injection of CTX and mRNA isolated from regenerating muscle after 1, 3, 5, 7, and 14 days post-injury (dpi). Taz mRNA increased markedly at 3 dpi compared with uninjured control, before dropping, while Yap was largely unchanged (Fig. 1A). Taz mRNA rose during differentiation of C2C12 myoblasts, while Yap mRNA remained relatively constant (Fig. 1B). Western blot analysis for total Yap and Taz protein in C2C12 reflected mRNA dynamics, with Taz levels rising...
Figure 1. Taz levels increase during skeletal muscle regeneration and myogenic differentiation. (A): Yap and Taz mRNA expression in regenerating tibialis anterior (TA) muscle collected at 1, 3, 5, 7, and 14 days post injury. Expression is fold change compared with control undamaged TA (n = 3). (B): Yap and Taz mRNA levels in C2C12 myoblasts at 24 and 48 hours in proliferation medium, and 24, 48, 72, and 96 hours in differentiation medium, expressed as fold change compared with 24 hours proliferation time-point (n = 3). (C): Representative Western blots from biological replicate 4 (n = 4) showing total Yap, Taz, and phosphorylated Yap and Taz levels in C2C12 myoblasts at 24 and 48 hours in proliferation medium, and 24, 48, 72, and 96 hours in differentiation medium, with relevant α-Tubulin or Gapdh loading controls. (D): Taz mRNA increased through differentiation in plated satellite cell-derived myoblasts at 12, 24, and 48 hours. Data are mean ± SEM from three experiments where an asterisk denotes significant difference (p < .05) from 0 hour using an unpaired Student’s t test. (E): C2C12 proliferating myoblasts coimmunolabeled for Yap and Taz. (F): Plated satellite cell-derived myoblasts coimmunolabeled with MyoD and Taz after 36 hours ex vivo. (G): Plated satellite cell-derived myotubes coimmunolabeled with myogenin and Taz after 48 hours ex vivo. (H): Confocal images of satellite cells coimmunolabeled with MyoD and Taz on isolated fibers cultured ex vivo for 72 hours; arrow points to Taz-containing nuclei. Scale bar = 100 μm (F, G). Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.
during differentiation, while Yap again remained relatively constant (Fig. 1C). Phosphorylated Yap and Taz levels both showed an increase during myogenic differentiation. Taz mRNA also increased during ex vivo differentiation of plated murine satellite cell-derived myoblasts (Fig. 1D).

Coimmunolabeling C2C12 myoblasts for Yap and Taz revealed Taz localized to the cytoplasm and nucleus, while Yap was nuclear (Fig. 1E), consistent with our previous observations [29]. Taz was similarly localized in satellite cells (Fig. 1F), but became cytoplasmic in mature multinucleated myotubes (Fig. 1G). Coimmunolabeling of satellite cells in their niche on a myofiber showed that Taz was expressed in satellite cells as shown by MyOD coexpression (Fig. 1H).

**Taz Promotes Myoblast Proliferation**

To compare Taz with Yap function, we either increased Taz activity via retroviral-mediated expression of human TAZ or constitutively active TAZ S89A, or after decreasing Taz and/or Yap levels using siRNA and performed proliferation and differentiation assays.

The retroviral backbone contains an IRES-eGFP that allows transduced cells to be identified by eGFP. Immunolabeling revealed that TAZ or TAZ S89A significantly increased the proportion of eGFP+ cells that had incorporated EdU, indicating that TAZ increases proliferation (Fig. 2A, 2B). siRNA knockdown of Taz, Yap, or both Taz/Yap, however, significantly reduced EdU incorporation (Fig. 2C, 2D). Thus, at this stage, TAZ promotes proliferation of myoblasts, as we reported previously for YAP [28, 29].

RT-qPCR showed that siRNA-mediated knockdown of Taz suppressed Myf5 and myogenin expression, but enhanced Pax7. Conversely, knockdown of Yap increased myogenin expression, but reduced Pax7 and Myf5. Simultaneous knockdown of both Taz and Yap rescued expression of myogenin (Fig. 2E).

**Taz and Yap Play Opposing Roles during Myogenic Differentiation**

We determined the effect of retroviral-mediated constitutive expression of TAZ or TAZ S89A on early myogenic differentiation in satellite cells cultured in their niches for 72 hours on an isolated myofiber, by coimmunolabeling for eGFP/Pax7 to mark undifferentiated/self-renewing cells (Fig. 3A) or eGFP/myogenin to mark myoblast entering differentiation (Fig. 3B). TAZ significantly decreased eGFP-containing self-renewing Pax7+ cells (Fig. 3A), while TAZ or TAZ S89A significantly increased eGFP-containing differentiating myogenin+ve myoblasts (Fig. 3B), compared with control. Thus, TAZ promotes entry into differentiation, at the expense of satellite cell self-renewal.

To investigate effects of TAZ or TAZ S89A throughout myogenic differentiation, we used expanded, plated satellite cell-derived myoblasts, which were first transduced and then switched to differentiation medium 24 hours later. Self-renewal and differentiation were again assessed by coimmunofluorescence for eGFP/Pax7 and eGFP/myogenin. Under differentiation stimulus, TAZ or TAZ S89A decreased the proportion of myocytes expressing Pax7, while enhancing the proportion expressing myogenin (Fig. 3C, 3D). At this early stage of differentiation, myotubes were also more prominent in myoblast cultures transduced with TAZ or TAZ S89A, than control retrovirus, suggesting a time-dependent switch of TAZ function from proproliferation to prodifferentiation (Fig. 3E).

At later stages of differentiation and fusion into multinucleated myotubes, constitutive expression of TAZ or TAZ S89A also increased incorporation of nuclei into myotubes (fusion index - Fig. 3F, 3G). siRNA-mediated Taz knockdown in expanded plated satellite cell-derived myoblasts did not significantly reduce (though p = .06) the fusion index to below control levels (Fig. 3H, 3I).

**Taz (Wwtr1–/–)-Null Myoblasts Differentiate Less Ex Vivo**

Skeletal muscles of Taz (Wwtr1–/–)-KO mice have not been well-characterized [40]. Consistent with the trend in siRNA data (Fig. 3H, 3I), satellite cells isolated and expanded from Taz (Wwtr1–/–)-null mice fused less into post-mitotic myotubes compared with WT cells (Fig. 3J, 3K).

Thus, Taz promotes proliferation of myoblasts, but at later stages of myogenesis, Taz switches to enhance differentiation, while, as we reported previously, Yap continues to promote proliferation while inhibiting differentiation [28, 29].

**Taz (Wwtr1–/–)-Null Mice Have Fewer Myofibers in Soleus**

In accordance with the role of the Hippo pathway in body/organ size regulation, 6-week-old Taz (Wwtr1–/–)-KO mice were lighter than WT mice (Fig. 4A) as were their TA, soleus, EDL, and gastrocnemius muscles (Fig. 4B-4E). However, the number of satellite cells and myonuclei per EDL myofiber was unchanged between Taz (Wwtr1–/–)-null and WT mice (Fig. 4F, 4G). Myofiber type composition in soleus was analyzed using ATPase activity at pH 4.47 to detect slow type I, or pH 10.5 to detect fast type Ila, myofibers. Soleus contained fewer muscle fibers in total, with both numbers of type I and type Ila myofibers significantly reduced compared with WT (Fig. 4H, 4I), but the proportion of slow type I myofibers was significantly increased, while the proportion of type Ila were significantly decreased, compared with control WT. Myofiber cross-sectional area was unchanged though (Fig. 4J).

Next, we investigated muscle regeneration in the Taz (Wwtr1–/–)-KO mice using intra-muscular injection of CTX into TA. Cryosections of 5 dpi regenerating TA were immunolabeled for neonatal MyHC to identify regenerating myofibers, but Taz (Wwtr1–/–)-KOs did not exhibit a reduction compared with controls (Fig. 4K-4L). At 10 dpi, the number of myofibers with centrally located nuclei in TA of Taz (Wwtr1–/–)-null mice was also unchanged (Fig. 4M, 4N).

**Inactivation of Yap in Satellite Cells Impairs Muscle Regeneration**

Administration of Tamoxifen to Paxx:Cre-ERT2/+;Yaplox/lox/Rosa26lacZ mice causes Pax7-driven Cre-ERT2-mediated deletion of Yap exons 1 and 2 in satellite cells, with simultaneous induction of lacZ expression. Paxx:Cre-ERT2/+;Yaplox/lox/Rosa26lacZ and age-matched control Paxx:Cre-ERT2/+;Yaplox/+;Rosa26lacZ mice were given three intraperitoneal injections of Tamoxifen, and then maintained on tamoxifen-containing food. TA muscles were injured by intramuscular injection of CTX, and regenerating muscles cryosectioned at 5 and 10 dpi (Fig. 5A).
Regenerating TA muscles at 5 dpi from Tamoxifen-treated Pax7Cre-ERT2/+:Yaplox/lox:Rosa26lacZ mice coimmunolabeled for neonatal MyHC and laminin had a 30% smaller area containing neonatal MyHC than control Pax7Cre-ERT2/+:Yaplox/lox:Rosa26lacZ (Fig. 5B, 5C). Immunolabeling for Pax7 revealed that there was a nonsignificant (p = .054) trend to reduced numbers of satellite cells in Tamoxifen-treated 5 dpi regenerating Pax7Cre-ERT2/+:Yaplox/lox:Rosa26lacZ muscle (Fig. 5D, 5F).

By 10 dpi, Pax7 immunolabeling revealed that satellite cell number in tamoxifen-treated Pax7Cre-ERT2/+:Yaplox/lox:Rosa26lacZ mice coimmunolabeled for neonatal MyHC and laminin had a 30% smaller area containing neonatal MyHC than control Pax7Cre-ERT2/+:Yaplox/lox:Rosa26lacZ (Fig. 5B, 5C). Immunolabeling for Pax7 revealed that there was a nonsignificant (p = .054) trend to reduced numbers of satellite cells in Tamoxifen-treated 5 dpi regenerating Pax7Cre-ERT2/+:Yaplox/lox:Rosa26lacZ muscle (Fig. 5D, 5F).

**Figure 2.** Taz controls satellite cell-derived myoblast proliferation. (A): Representative images of 5-ethyl-2'-deoxyuridine (EdU) incorporation in satellite cell-derived myoblasts 48 hours following transduction with control vector, or retroviral constructs encoding wild-type human TAZ or constitutively active TAZ S89A, where green fluorescent protein (GFP) marks transduced cells. (B): Quantification of GFP+ cells with EdU incorporation shows that TAZ or TAZ S89A significantly increases EdU incorporation, and so proliferation rate (n = 5 mice). (C): Representative images of EdU incorporation in satellite cell-derived myoblasts following Yap and/or Taz knockdown using siControl, siYap, siTaz, or siYap + Taz transfection. (D): Quantification of EdU incorporation shows that knockdown of Yap, Taz, or both Yap and Taz reduces the proliferation rate (n = 5 mice). (E): Relative gene expression measured by real-time quantitative polymerase chain reaction in satellite cell-derived myoblasts compared with siControl under proliferation conditions following siControl, siYap, siTaz, or siYap + Taz knockdown, normalized to Gapdh (n = 5 mice except for myosin heavy chain/MyoD, where n = 3). Data presented as mean ± SEM, where an asterisk denotes significant difference from control retrovirus/control small interfering RNA (p < .05) using Student’s t test. Scale bar = 100 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; EdU, 5-ethyl-2'-deoxyuridine; GFP, green fluorescent protein; RV, control vector.
Rosa26Lacz mice was as in control Pax7Cre-ERT2/+;Yapflo/+; Rosa26Lacz mice (Fig. 5E, 5F). The number of myofibers with centrally located myonuclei, a hallmark of regenerated muscle, was also unchanged between Pax7Cre-ERT2/+;Yapflo/+; Rosa26Lacz regenerating TA muscles and control (Fig. 5G, 5H). Thus, lack of Yap function in satellite cells slows, but does not prevent, muscle regeneration.

YAP and TAZ Regulate the Hippo Negative Feedback Loop, Myogenic Regulators, and Wnt Signaling

To compare the effects of TAZ and YAP on gene expression in the skeletal muscle lineage, we expressed TAZ S89A or YAP1 S127A in proliferating murine satellite cell-derived myoblasts for 24 and 48 hours and analyzed gene expression by microarray (Supporting Information Data 1), comparing our observations with those obtained by induction of YAP1 S127A in myoblasts derived from a transgenic mouse model [29] and from rhabdomyosarcomas driven by YAP1 S127A [25]. Combination of 24 and 48 hours time points revealed that TAZ S89A significantly changed expression of 860 genes, while YAP S127A altered expression of 294 genes, with an additional 316 genes regulated by both TAZ S89A and YAP S127A (Fig. 6A; Supporting Information Data 1). Satellite cells were already actively proliferating as cultured in high serum medium, and serum is a potent YAP activator [47], likely explaining why neither TAZ S89A nor YAP1 S127A significantly...
affected most mitotic genes, in contrast to earlier observations [25, 29].

Increased TAZ/YAP-Tead activity was indicated by both TAZ S89A and YAP S127A inducing the Hippo negative feedback loop genes Amotl2, Frmd6 (Willin), and Lats2, which was presumably to limit Taz/Yap activity. TAZ S89A and YAP S127A also regulate markers/regulators of the muscle lineage: Caveolin1 (Cav1) was upregulated, while Six1 and Six4 were downregulated. In addition, TAZ and YAP regulated multiple members of the mTOR/insulin-like growth factor (IGF) pathway, including Igfbp5, as well as Ndrg2 and Irs2, which regulate mTOR via Akt (Fig. 6B).

Wnts are TAZ/YAP regulators, and TAZ/YAP regulation of Wnt and Wnt-related proteins can inhibit Wnt signaling [48]. TAZ S89A and YAP S127A altered expression of key Wnt pathway regulators, reducing expression of Wnt-receptor proteins Fzd7 and Lgr5, while TAZ S89A alone suppressed expression of Fzd6, Lgr4, and Lgr6, suggesting that TAZ/YAP desensitize myoblasts to ligands that bind these receptors. Moreover, TAZ S89A increased expression of Wnt4, and altered expression of
other secreted Wnt-associated proteins and genes encoding the structurally related proteins Wisp1 and Wisp2 [49]. Additionally, both TAZ S89A and YAP S127A decreased Sox9 expression (Fig. 6B, 6C).

**TAZ Regulates a Unique Set of Genes**

Although the microarray was performed on proliferating myoblasts, genes regulated by TAZ only might explain the functional switch from proproliferation to prodifferentiation (Fig. 6C). Both Pax7 and MyoD were downregulated by TAZ S89A while Myf5 was upregulated, consistent with Taz siRNA knockdown (Fig. 2E). Upregulated cell junction proteins could contribute to enhanced fusion capability in TAZ-overexpressing myoblasts, since N-Cadherin is required for myoblast fusion [50]. TAZ overexpression also altered expression of olfactory receptors Olfr311 and Olfr1086 (Fig. 6C), a family of genes engaged in satellite cell self-renewal [51]. Mki67 (protein Ki67) involved in proliferation, but was downregulated by TAZ

**Figure 5.** Yap conditional knockout (KO) in satellite cells affects muscle regeneration. (A): Time course for analysis of Yap conditional KO and control mice after tamoxifen administration via intraperitoneal injection and food, and cardiotoxin (CTX)-induced injury. (B, C): Representative images and quantification of tibialis anterior (TA) cryosections 5 dpi after CTX injury of Pax7$^{CreER/1}$.Yapfl$^{8x8x}:Rosa26$^{	ext{Ind}}$ (Pax7Cre Yapfl/fl) and control Pax7$^{CreER/1}$.Yapfl$^{+/+}:Rosa26$^{	ext{Ind}}$ (Pax7Cre Yapfl/+) mice, communolabeled for neonatal myosin heavy chain (MyHC) (NeoMyHC)/laminin to identify regenerating myofibers ($n = 4$ mice per genotype). (D-F): Representative images and quantification of cryosections of TA muscles after 5 or 10 dpi, immunolabeled for Pax7 to label satellite cells (5 dpi: $n = 4$ per genotype; 10 dpi: $n = 4$ Pax7Cre Yapfl/fl and $n = 3$ Pax7Cre Yapfl/+). (G): Representative images of H&E staining and (H) quantification of centrally nucleated myofiber number per field of 10 dpi TA. Data are mean ± SEM, where an asterisk denotes significant difference ($p < .05$) between Pax7Cre Yapfl/+ and Pax7Cre Yapfl/fl using a Student’s t test. Scale bar = 100 μm. Abbreviations: CTX, cardiotoxin; DAPI, 4',6-diamidino-2-phenylindole; IP, intraperitoneal; MyHC, myosin heavy chain; TA, tibialis anterior.

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Figure 6. Genes regulated by TAZ S89A or YAP S127A and proteins bound by TAZ and/or YAP. (A): Venn diagram displaying the number of genes modulated by TAZ S89A and/or YAP S127A versus control retrovirus (up-reg: upregulated; down-reg: downregulated) at either 24 or 48 hours. (B): Expression fold change for genes that were regulated by both TAZ S89A and YAP S127A in transduced satellite cell-derived myoblasts versus control retrovirus at either 24 or 48 hours. (C): Expression fold change for genes that were regulated only by TAZ S89A at either 24 or 48 hours. (D): All TAZ and/or YAP interacting proteins identified by mass spectrometry in C2C12 myoblasts and myotubes. Those previously identified are in red [53] and novel proteins in black, with those of particular interest in blue. To minimize false positives, selected cut-off values were a ratio of ≥2 compared with empty vector and \( p < .05 \) Student’s \( t \) test between each data-set and control vector. Abbreviations: IGF, insulin-like growth factor; mTOR, •••.
in myoblasts (Fig. 6C), again highlighting the switch from promoting proliferation toward cell cycle withdrawal/differentiation.

**YAP and TAZ Bind Both Common and Unique Proteins**

Apart from Tead1-4, Yap and Taz also bind a plethora of upstream signaling proteins and downstream transcriptional regulators [52]. To identify binding partners in a non-biased way, we expressed flag-tagged WT human YAP or TAZ in proliferating murine C2C12 myoblasts and mature myotubes. Flag-immunoprecipitated YAP or TAZ complexes were identified using mass-spectrometry (Fig. 6D; Supporting Information Data 2).

Our TAZ and YAP binding proteins were first validated against previously characterized binding partners in human embryonic kidney 293T cells [53]. Overlap included angiomeotins, multiple 14-3-3 proteins, Tead1, Tead3, and Tead4 (red: Fig. 6D). However, we also identified many novel TAZ and/or YAP interacting proteins in myogenic cells (Fig. 6D). TAZ bound many more proteins in myoblasts than YAP, or that were common to both YAP and TAZ. In myotubes, however, YAP bound many more proteins than TAZ or both TAZ and YAP, with only YAP binding Wnt regulators Dvl2 and Dvl3. Of 26 TAZ and YAP binding proteins in mature myotubes (Fig. 6D), 10 are linked to actin cytoskeleton and the Chaperone assisted selective autophagy (CASA) complex, including the WW-domain containing Bag3. We also identified 20 proteins bound specifically by TAZ during differentiation, the majority associated with the actin cytoskeleton and myogenesis including Unc45a and Fhl3 [54, 55]. MyoD was not among YAP and/or TAZ binding partners, even at less stringent cut-offs.

**TAZ Operates through Tead4 to Control Myogenic Differentiation**

We next assayed Tead expression during myogenesis. TA muscle was injured using CTX and mRNA isolated from regenerating muscle after 1, 3, 5, 7, and 14 dpi. RT-qPCR showed that Tead2 and Tead4 exhibited similar expression profiles, peaking around day 3, before falling to levels seen in undamaged muscle (Fig. 7A). Tead1-3 mRNA was at a constant level in proliferating and differentiating C2 myoblasts, but Tead4 expression increased robustly at the onset of myogenic differentiation, being maintained at elevated levels through fusion (Fig. 7B). Western blot revealed that Tead1 and Tead4 protein levels increased upon entry into C2C12 myoblast differentiation and were maintained (Fig. 7C).

As Tead4 is critical for myoblast fusion due to inducing myogenin by binding MCAT elements in its promoter [56], we further examined its function. Communualabeling of Tead4 and Taz showed both proteins present in the nucleus of differentiating myoblasts, at a time when Tead4 levels rise markedly (Fig. 7D). Thus, the TAZ-Tead4 interaction identified in proliferating myoblasts (but not in mature myotubes) (Fig. 6D) could be maintained during induction and the early phases of myogenic differentiation, not assayed by proteomics.

We used retroviral-mediated expression to enhance TAZ activity and/or siRNA to reduce Tead4 levels in satellite cell-derived myoblasts during myogenic differentiation, and assayed fusion index after communualabeling for eGFP/MyHC. TAZ S89A with control siRNA augmented myoblast fusion (Fig. 7E, 7F), but TAZ S89A with concomitant Tead4 knockdown reversed this enhanced fusion index (Fig. 7E, 7F). Knockdown of Tead4 alone resulted in shorter and thinner myotubes, reflected in a reduced fusion index, but expression of TAZ S89A did allow recovery of the fusion index back to control levels (Fig. 7E, 7F).

To investigate the role of TAZ/Tead4 in entry into myogenic differentiation versus fusion, we also transduced satellite cell-derived myoblasts with TAZ S89A and/or transfected with Tead4 siRNA and differentiated cells as myocytes. After 24 hours in differentiation medium, communualabeling revealed that TAZ S89A increased the proportion of eGFP-expressing cells containing myogenin, compared with controls. Knockdown of Tead4 alone significantly reduced myogenin expression (Fig. 7G, 7H). Simultaneous expression of TAZ S89A and Tead4 siRNA knockdown though, brought the proportion of GFP-expressing myoblasts containing myogenin back to control levels (Fig. 7G, 7H).

**Discussion**

Yap, Taz, and Tead1-4 constitute a dynamic system in satellite cells and the muscle lineage, with mRNA and protein levels of some changing extensively during muscle regeneration and myogenic differentiation in vitro. Three are of note: Yap [29] and Taz only become detectable during satellite cell activation, suggesting that Yap and Taz only operate during myogenesis; mRNA/protein levels of some Teads also change, suggesting that Teads are not just static targets of Yap/Taz; Taz and Tead4 increase dramatically during myogenic differentiation, while Yap expression remains fairly constant. During regeneration, Taz and Tead4 levels peak around 3–5 dpi, as myogenic differentiation is underway [57–59]. Our observations are consistent with Taz and Tead4 as pro-differentiation factors [28, 29, 37, 56].

Manipulation of Yap and Taz activity/levels via retroviral-mediated expression of WT or TAZ S89A, or knockdown of Taz, revealed that TAZ promotes proliferation. Yap [28, 29] and Taz synergistically promote proliferation in many cell types [60], with a recent ChIP-Seq study in breast cancer cells showing that Yap and TAZ frequently operate through the same enhancer elements [24]. Later, in myogenesis, however, TAZ switches to promote differentiation, in accordance with an earlier report in immortalized murine myoblasts [37, 38], unlike Yap, which inhibits differentiation [28, 29]. TAZ actually augments the myogenic differentiation program under differentiation stimuli, as shown by the precocious expression of myogenin in many myocytes. Thus, the greater fusion index with TAZ overexpression is not merely due to having more myoblasts available, due to the proproliferation effects of TAZ. Consistent with its later pro-differentiation role, constitutive TAZ expression also reduced self-renewal of satellite cells, while Taz (Wwtr1<sup>−/−</sup>)-null satellite cells exhibited reduced fusion ex vivo.

Taz (Wwtr1<sup>−/−</sup>)-null mice have functional and growth defects in kidneys and other organs, consistent with Taz as a regulator of organ growth and transcriptional regulator of genes involved in kidneys, heart, and bone growth [21, 61, 62]. Taz (Wwtr1<sup>−/−</sup>)-null mice were generally lighter, with TA, soleus, EDL, and gastrocnemius muscle mass all reduced, but with unchanged numbers of satellite cells and myonuclei per

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Figure 7. TAZ operates through Tead4 to control myogenic differentiation. (A): Tead2–4 mRNA expression in regenerating tibialis anterior (TA) muscle collected at 1, 3, 5, 7, and 14 days post injury with cardiotoxin. Expression is presented as fold change compared with control undamaged TA (n = 3). (B): Tead1–4 mRNA levels in C2C12 myoblasts at 24 and 48 hours in proliferation medium, and 24, 48, 72, and 96 hours in differentiation medium, expressed as fold change compared with 24 hours proliferation time-point (n = 3). (C): Representative Western blots of C2C12 myoblast lysate collected at 24 and 48 hours in proliferation medium and 24, 48, 72, and 96 hours in differentiation medium and immunoblotted for Yap, Taz, Tead1 (biological replicate 3), phosphorylated Yap and Taz (biological replicate 2) and Tead4 (biological replicate 1) (n = 4), with relevant α-Tubulin or Gapdh loading controls. (D): Representative images of plated satellite cell-derived myoblasts coimmunolabeled with Tead4 and Taz cultured under differentiation conditions for 24 hours. (E): Representative images of coimmunolabeling of myotubes formed from satellite cell-derived myoblasts following TAZ S89A or control vector (RV) transduction and/or small interfering RNA (siRNA) knockdown of Tead4 or siRNA control. Green fluorescent protein (GFP) labels transduced cells and myosin heavy chain (MyHC) identifies myotubes. (F): Comparison of the proportion of the nuclei in GFP+ve/MyHC+ve myotubes under each condition shows that TAZ requires Tead4 to enhance differentiation (n = 3 mice). (G): Representative images of coimmunolabeling of myocytes formed from satellite cell-derived myoblasts following TAZ S89A or RV transduction and/or siRNA knockdown of Tead4 or siRNA control, and 24 hours in differentiation medium. GFP labels transduced cells and Myogenin identifies myocytes. (H): Comparison of the proportion of GFP+ve/Myogenin+ve myocytes under each condition shows that TAZ-induced myogenic differentiation operates through Tead4 (n = 3 mice). Data are mean ± SEM from three mice, where an asterisk denotes significant difference (p < .05) from RV/siRNA Control, or as indicated by bars, using a paired Student’s t test. Scale bar = 100 μm. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; MyHC, myosin heavy chain; RV, control vector; siRNA, small interfering RNA.
myofiber. However, absence of Taz reduced the total number of myofibers in soleus, with both fewer slow type I and fast Type IIa muscle fibers present, although there was a greater proportion of slow type I myofibers.

Lack of an overt muscle regeneration phenotype in Taz (Wttr1−/−)-null mice could be due to compensation by Yap in promoting satellite cell proliferation, as Yap or Taz knockout did not affect expression of the other gene [63]. In comparison, genetic deletion of Yap specifically in satellite cells caused delayed regeneration, so Taz is unable to compensate for Yap in early regeneration. This regeneration defect in tamoxifen-treated Pax7CreERT2/+, Yapflx/flx;Rosa26lox/lox mice was transient although, maybe due to Taz promoting myogenic differentiation later in regeneration. Therefore, skeletal muscle joins other systems where Yap is required for regeneration, for example, where defects in crypt regeneration are observed when Yap is inactivated in epithilium of the small intestine, and in the skin [64–66].

We also examined gene expression in myoblasts in response to TAZ S89A or YAP S127A. As expected, YAP and TAZ increased expression of Hippo marker gene Cyr61 and Hippo negative-feedback loop genes Lats2, Frmd6 (Willin), and Amotl2: a typical response to increased Yap and Yap activity [29]. Intriguingly, TAZ also downregulates Bag3, a WW domain-containing member of the CASA mechanosensitive Z-disc linked complex [67] that binds to Yap and Taz, and Yes1, the earliest reported binding partner of Yap [68]. Bag3 and Yes1 might represent additional members of the Hippo negative feedback loop in myoblasts. Several TAZ- and YAP-regulated genes have been linked to myogenesis. For example, TAZ and YAP reduced expression of Six1 and Six4, two factors controlling migration of muscle precursor cells [69] with a role in muscle regeneration [70]. TAZ and YAP also regulated many IGF/mTOR-related genes (Ndr2g, Ifgfbp5, Lsr2, and lgf2bp1), confirming crosstalk between Hippo and mTOR signaling [2, 3].

TAZ also regulates a unique cohort of genes, including Pax7, whose expression was reduced by TAZ S89A, consistent with the switch toward differentiation. TAZ also modulates multiple oflactory receptor genes, whose levels vary between mdx and control mice [51], and a family of genes that can affect muscle regeneration [71].

TAZ and YAP bind proteins in murine myoblasts/myotubes that have been identified as binding partners in human 293T cells [53]. These generic YAP and TAZ binding proteins include the angiomotins (Amot and Amotl1), Tead1 and Tead4 and several 14-3-3 proteins (Ywhae, Ywhaq, Ywhaz). Additionally, we identified many novel TAZ and/or YAP binding partners in the skeletal muscle lineage, including in myotubes, Bag3 [72] and syncolin (Sync), an interleukin mediated protein linked to the dystrophin-associated protein complex [73]. TAZ-specific binding partners that could potentially explain pro-differentiation effects include Fhl3, which binds MyoD to inhibit differentiation [55] and Unc45a, which increases muscle cell proliferation and fusion, and when inhibited, muscle cell fusion [54]. Runx1 bound to TAZ in myoblasts and downregulation of Runx1 leads to cell cycle exit and differentiation [54]. Taz has been reported to bind MyoD using immunoprecipitation assays [37], but while we observed MyoD and Taz in the same nuclei, we found no significant direct interaction using mass spectrometry.

Our combined gene expression and binding partner analyses in myoblasts reveals that YAP or TAZ does not bind members of the Wnt destruction complex [8]. YAP bound the diseveled proteins Dvl2 and Dvl3 in myotubes, relevant as Dvl2 can affect satellite cell polarity and migration downstream of Wnt7a/Fzd7 [74]. TAZ S89A downregulates Fzd6 and Fzd7, and Lgr4, Lgr5, and Lgr6, important Wnt receptors in developmental and regenerative myogenesis [75, 76], suggesting that TAZ desensitize cells to ligands that bind these receptors. For example, quiescent satellite cells express high levels of Fzd7 but not Taz or Yap, but during activation, the increased abundance/activity of Yap/Taz could be involved in the down-regulation of Fzd7.

Additionally, TAZ S89A increases expression of Wnt4, decreases Sox9 expression and alters expression of secreted Wnt-associated proteins and genes encoding the structurally related proteins Wisp1, Wisp2, Igfbp4, Cyr61, and Bmp4 [49]. Many of these genes have been implicated in regulation of developmental myogenesis, satellite cell function and muscle fibers. For example, over-expression of Wnt4 increased Pax7 and MyoD1 expression in chick embryos [77]. Bmp4 promotes satellite cell proliferation but inhibits differentiation [78].

Interestingly, Tead4 bound TAZ and YAP in proliferating myoblasts but not in mature multinucleated myotubes. Tead4 increases dramatically as myoblasts enter myogenic differentiation and levels are then maintained. Thus the TAZ-Tead4 interaction identified in proliferating myoblasts by proteomics is likely maintained during induction and the early phases of myogenic differentiation, not assayed by proteomics. When Tead4 was knocked down, myogenic differentiation was suppressed, which recovered to controls levels when TAZ S89A was also expressed. However, TAZ S89A could no longer enhance entry into the differentiation programme and augment fusion when Taz4 levels were reduced. Thus, the role of TAZ in inducing and promoting myogenic differentiation is linked to interaction with Tead4 [37, 56].

**Conclusion**

In conclusion, in the skeletal myogenic lineage, we demonstrate similar roles of TAZ and YAP in promoting myoblast proliferation, but during the later stages of myogenesis, Taz switches toward influencing satellite cell fate by promoting myogenic differentiation.

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**Author Contributions**

A.M.T.: conception and design; N.V.: data analysis and interpretation; H.W. and P.S.Z.: conception and design, data analysis and interpretation, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
The authors indicate no potential conflicts of interest.

REFERENCES


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