TGF-β1 regulates the expression and transcriptional activity of TAZ protein via a Smad3-independent, myocardin-related transcription factor-mediated mechanism

Maria Zena Miranda‡§, Janne Folke Bialik†§, Pam Speight†, Qinghong Dan†, Tony Yeung†, Katalin Szászi‡§, Stine F. Pedersen†§, and András Kapus†§¶

From the †Keenan Research Centre for Biomedical Science of the St. Michael's Hospital, Departments of ‡Surgery and §Biochemistry, University of Toronto, Toronto, Ontario M5B 1T8N, Canada and the ¶Department of Cell and Developmental Biology, University of Copenhagen, Copenhagen DK-2100, Denmark

Edited by Eric R. Fearon

Hippo pathway transcriptional coactivators TAZ and YAP and the TGF-β1 (TGFβ) effector Smad3 regulate a common set of genes, can physically interact, and exhibit multilevel cross-talk regulating cell fate-determining and fibrogenic pathways. However, a key aspect of this cross-talk, TGFβ-mediated regulation of TAZ or YAP expression, remains uncharacterized. Here, we show that TGFβ induces robust TAZ but not YAP protein expression in both mesenchymal and epithelial cells. TAZ levels, and to a lesser extent YAP levels, also increased during experimental kidney fibrosis. Pharmacological or genetic inhibition of Smad3 did not prevent the TGFβ-induced TAZ up-regulation, indicating that this canonical pathway is dispensable. In contrast, inhibition of p38 MAPK, its downstream effector MK2 (e.g. by the clinically approved antifibrotic pirfenidone), or Akt suppressed the TGFβ-induced TAZ expression. Moreover, TGFβ elevated TAZ mRNA in a p38-dependent manner. Myocardin-related transcription factor (MRTF) was a central mediator of this effect, as MRTF silencing/inhibition abolished the TGFβ-induced TAZ expression. MRTF overexpression drove the TAZ promoter in a CC(A/T-rich)₆GG (CArG) box-dependent manner and induced TAZ protein expression. TGFβ did not act by promoting nuclear MRTF translocation; instead, it triggered p38- and MK2-mediated, Nox4-promoted MRTF phosphorylation and activation. Functionally, higher TAZ levels increased TAZ/TEAD-dependent transcription and primed cells for enhanced TAZ activity upon a second stimulus (i.e. sphingosine 1-phosphate) that induced nuclear TAZ translocation. In conclusion, our results uncover an important aspect of the cross-talk between TGFβ and Hippo signaling, showing that TGFβ induces TAZ via a Smad3-independent, p38- and MRTF-mediated and yet MRTF translocation-independent mechanism.

This work was supported by Canadian Institutes of Health Research (CIHR) Grants MOP-106625 and MOP-130463 (to A. K.), the Kidney Foundation of Canada (to A. K. and K. S.), and Natural Sciences and Engineering Research Council of Canada (NSERC) Grants RGPIN227-908-13 (to A. K.) and RGPIN 327407 (to K. S.). The authors declare that they have no conflicts of interest with the contents of this article.

To whom correspondence should be addressed: Keenan Research Centre for Biomedical Science, Rm. 621, 209 Victoria St., Toronto, Ontario M5B 1T8, Canada. Tel.: 416-847-1751; E-mail: kapusa@smh.ca.

Yes-associated protein (YAP)² and its paralog, transcriptional coactivator with a PDZ-binding domain (TAZ), are central effectors of the Hippo pathway and play essential roles in the control of organ size, proliferation (contact inhibition), stemness, differentiation, cellular plasticity (e.g. epithelial-mesenchymal transition (EMT)), regeneration, and the mechanosensitive regulation of gene expression (1–4). Congruent with these cell fate-determining functions, YAP and TAZ have emerged as key mediators of major disease entities, particularly cancer (5, 6) and, as recent studies by us (7–10) and others (11–14) reveal, organ fibrosis. YAP and TAZ are primarily regulated at the level of their nucleocyttoplasmic traffic. Under resting conditions (e.g. in contact-inhibited cells) the constitutive activity of Hippo kinases, Mst1/2, and their downstream targets, Lats1/2, keep YAP and TAZ in a phosphorylated state thereby ensuring their cytosolic retention via binding to sequestering proteins (e.g. 14–3-3) (15). Upon Hippo kinase inhibition, YAP and TAZ get dephosphorylated and translocate to the nucleus, where they bind to cognate transcription factors (TFs), predominantly to members of the TEAD family, and drive a large set of genes involved in the above-mentioned functions (16, 17). Another major input regulating YAP/TAZ nuclear accumulation is the state of the cytoskeleton; actin polymerization accompanied by myosin phosphorylation (e.g. as a result of Rho activation) leads to nuclear YAP/TAZ translocation through partially Hippo-independent, incompletely understood mechanisms (18–20). Through this cytoskeletal pathway mechanical cues, such as cell contractility, stretch, or extracellular matrix stiffness, impact YAP/TAZ distribution, thereby initiating mechanoresponsive gene transcription (4, 21).

²The abbreviations used are: YAP, Yes-associated protein; 3DA, promoter containing a triplicate of CArG sequence; AKTi, Akt1/2 kinase inhibitor; CArG, CC(A/T-rich)₆GG; CCG, CCG-1423; CHX, cycloheximide; DORA, doramipimod; Luc, luciferase; MRTF, myocardin-related transcription factor; MK2, MAPK-activated protein kinase 2; NR, non-related; PF, PF-3644022; ROK, Rho-associated protein kinase; S1P, sphingosine 1-phosphate; SBE, Smad-binding element; SMAu, smooth muscle actin; SRF, serum-response factor; TAZ, transcriptional coactivator with a PDZ-binding domain; TEAD, TEA-domain transcription factor; TF, transcription factor; UUO, unilateral ureteral obstruction; VAS, VAS2870; NRKF, normal rat kidney fibroblast; PFA, paraformaldehyde; ANOVA, analysis of variance; EMT, epithelial-mesenchymal transition; mTOR, mechanistic target of rapamycin; qPCR, quantitative PCR.
Although nucleocytoplasmic shuttling of YAP and TAZ is a central aspect of their regulation, mounting evidence indicates that the expression of YAP and/or TAZ also exhibits significant changes under various conditions. In fact, increased YAP or TAZ levels are not only characteristic of a wide range of cancers, but they are often negative prognostic factors, likely due to their contribution to proliferation and metastasis (5, 6). Recently, diabetic nephropathy, a fibrogenic state, has also been associated with increased YAP expression (22). Despite the potentially crucial importance of changes in net YAP and/or TAZ levels, and the demonstration of the involvement of some TFs in this process (see under “Discussion”), the regulation of YAP and TAZ expression (transcription), the relevant stimuli, and the underlying mechanisms remain largely unexplored.

YAP/TAZ signaling exhibits extensive cross-talk with other pathways; a chief example is transforming growth factor β1 (TGFβ) signaling (23–25). This pleiotropic cytokine, which upon binding to its receptors triggers both “canonical,” i.e. Smad2/3-dependent, and non-canonical signaling, is the main inducer of EMT and fibrogenesis (26–28). Because TGFβ also regulates cancer cell proliferation (29, 30), its cardinal effects show a strong functional overlap with those of YAP/TAZ. The molecular underpinning of the cross-talk between TGFβ and YAP/TAZ signaling is at least 2-fold. First, YAP and TAZ were shown to bind Smad2 and -3, and nuclear YAP/TAZ were proposed to act as retention factors for Smads (31, 32). Second, a multitude of genes harbors both Smad-binding elements (SBEs) and TEAD-binding elements in their promoters (33). Binding of the Smad3–TAZ or YAP–TEAD complexes to one or both of these cis-elements has been shown to exert synergistic (or in certain cases antagonistic) transcriptional effects in a promoter-dependent fashion (33, 34). Considering fibrogenic gene expression, we have recently shown that TAZ confers Smad3 sensitivity to the promoter of smooth muscle actin (SMA), the hallmark of the myofibroblast (9). Interestingly, an impact of TGFβ on TAZ expression has also been noted in a few studies, including our own (7, 31, 35). However, despite the potential key importance of TGFβ-induced changes in TAZ expression in the TGFβ/TAZ cross-talk, the underlying signaling mechanisms (canonical versus non-canonical), the relevant transcription factors, their mode of regulation, and the functional significance of this phenomenon have not been elucidated. Therefore, we set out to characterize if and, if so, how TGFβ impacts TAZ (or YAP) expression, and whether this manifests in altered TAZ activity.

To address this issue, we built on our recent study wherein we discovered that myocardin-related transcription factor (MRTF) regulates TAZ at multiple levels (9). MRTF is a transcriptional coactivator of serum-response factor (SRF), which stimulates gene expression via CC(A/T)_{n+1} GG elements, the so-called CArG box (36). MRTF is primarily regulated by its nuclear uptake; binding of monomeric (G) actin to MRTF prevents its nuclear accumulation (37). Upon actin polymerization, G-actin dissociates from MRTF allowing its nuclear entry. We previously identified a CArG box in the TAZ promoter and showed that MRTF silencing reduces basal TAZ expression (9). We also demonstrated that MRTF can bind TAZ. This direct interaction inhibits both MRTF and TAZ activity on the SMA promoter (but is synergistic on others), and it is disrupted by TGFβ, which redistributes TAZ from MRTF to Smad3 (9). Cognizant of this scenario, we asked if MRTF might also be mediating the effect of TGFβ on TAZ expression and, if so, whether this occurs via Smad3-dependent or non-canonical pathways. We also asked whether MRTF acts in a “classic” translocation-dependent fashion or via alternative mechanisms. Here, we show that TGFβ induces robust TAZ expression by activating MRTF in a p38 MAPK (p38)- and redox-dependent, translocation-independent manner and that this phenomenon primes cells for exaggerated TAZ activation in response to stimuli inducing TAZ translocation.

**Results**

**TAZ protein expression increases in various cell types upon TGFβ treatment and in an experimental model of fibrosis**

To test whether TGFβ impacts TAZ protein expression, we exposed C3H/10T1/2 cells (a pericyte-like fibroblast line) to this cytokine for varying times. This line was chosen because both fibroblasts and pericytes have been implicated as major drivers of fibrogenesis (38, 39). TGFβ caused a significant increase in TAZ protein, which was detectable as soon as 3 h and rose to ~6-fold above the resting level at 6 h. TAZ levels then slowly declined but remained several fold higher than the basal level even after 48 h of stimulation (Fig. 1A). Remarkably, this response was specific for TAZ, as YAP showed no change or only marginal change throughout the entire time course (Fig. 1A). The effect of TGFβ on TAZ expression was prevented by SB-431542, indicating that TGFβ receptor 1 kinase is required for this effect (Fig. 1B). TGFβ induced a robust increase in the TAZ protein level in normal rat kidney fibroblasts (NRKFs) as well (Fig. 1C). To test whether a similar response was present also in epithelial cells, LLC-PK1 cells were exposed to TGFβ either under normal conditions or under low calcium condition, wherein intercellular contacts are disassembled. This dual stimulation regimen (two-hit scheme) was used as our earlier studies have indicated that cell contact injury (uncoupling) synergizes with TGFβ to cause epithelial-myofibroblast transition (hallmarked by SMA expression) (40–42). Although TGFβ alone is insufficient to induce mesenchymal transformation in intact epithelial cells (40–42), it was capable of enhancing TAZ protein expression, and this effect was further potentiated by low calcium (Fig. 1D). Next, we asked whether TAZ levels would also change under pathological conditions in which TGFβ is known to play a key role. To this end, we probed whole-kidney lysates obtained from mice, which were either sham-operated or underwent unilateral ureteral obstruction (UOO). This procedure provokes a robust renal fibrosis which, as our recent study shows, is mitigated by YAP/TAZ inhibition (8). UOO provoked a substantial increase in TAZ protein expression, which was seen as early as 3 days after UOO and increased further by day 7 (Fig. 1E). Albeit less pronounced than TAZ expression, YAP expression also increased in most samples. Taken together, these results indicate that TGFβ induces robust TAZ protein expression in fibroblast-type and epithelial cells and in an in vivo model of fibrosis.
TGFβ induces p38-dependent, MRTF-mediated TAZ expression

TGFβ induces p38-dependent, MRTF-mediated TAZ expression

TGFβ activates both Smad2/3-dependent (canonical) and a variety of Smad2/3-independent (non-canonical) signaling pathways (28). To assess the role of the canonical signaling in TGFβ-induced TAZ expression, we applied two approaches. First, we treated C3H/10T1/2 cells with the Smad3 inhibitor SIS3. This failed to prevent the effect of TGFβ for 6 h. Changes in TAZ and YAP expression were detected as in A. C and D, NRKF (C) and LLC-PK1 kidney tubular cells (D) were incubated without or with TGFβ for 48 h and then processed for Western blotting for the indicated proteins. E, whole-kidney lysates were prepared from mice 1, 3, and 7 days after sham operation or UUO and probed for the indicated proteins. Representative blots are shown from four similar experiments. Densitometric quantitation of TAZ expression normalized to GAPDH is shown below the blot. Errors bars represent standard deviation.

TGFβ-induced TAZ expression required non-canonical (Smad3-independent) pathways

TGFβ in the absence or presence of the inhibitor (Fig. 2C). SIS3 completely abolished the TGFβ-induced rise in luciferase activity, verifying effective prevention of Smad3-dependent transcription. Accordingly, SIS3 also prevented the TGFβ-induced PAI-1 up-regulation (Fig. 2A). To substantiate these pharmacological observations, cells were transfected with control (non-related, NR) or Smad3-specific siRNAs for 24 h prior to stimulation (Fig. 2B). Although the Smad3 siRNA reduced Smad3 expression to 21 ± 4% (n = 3, data not shown) and inhibited SBE-luciferase activity to a similar extent (Fig. 2D), it failed to reduce the ensuing
TAZ expression (Fig. 2B). In agreement with these data, siRNA-mediated suppression of Smad3 did not significantly inhibit the TGFβ-induced TAZ expression either (Fig. 2E).

Smad2 is also activated by TGFβ, and in a small subset of effects Smad2 and Smad3 play selective roles (43, 44). Therefore, we also tested the impact of Smad2 by using Smad2 siRNA. Interestingly, Smad2 silencing led to a dramatic decrease in the basal TGFβ-independent expression of YAP and also reduced basal TAZ expression (Fig. 2F). TGFβ had no effect on YAP levels in the Smad2 silenced cells (similar to the controls) but was still able to increase TAZ expression. Nevertheless, at least partly due to the lower basal level, the attained TAZ expression was reduced (Fig. 2F). The fact that Smad2 (as opposed to Smad3) silencing affected YAP levels, and that this effect was independent of TGFβ, pointed to a fundamentally different role and mechanism of Smad2 and -3, wherein the former is indispensable for basal YAP expression and may play a permissive role in maintaining TAZ levels and regulation as well (see also “Discussion”).

To investigate the involvement of major non-canonical pathways, we first used their respective pharmacological inhibitors.
**TGFβ induces p38-dependent, MRTF-mediated TAZ expression**

Thus, we determined the TGFβ-induced change in TAZ expression in the absence (vehicle controls, DMSO) or presence of the selective p38 MAPK inhibitor, doramapimod (DORA), the Akt inhibitor AKT1-1/2 (AKTi), the MEK/ERK1/2 inhibitor U0126, and the Rho kinase inhibitor Y-27632. The corresponding Western blottings and their quantifications show that DORA (Fig. 3A) and AKTi (Fig. 3B) significantly suppressed the TGFβ-induced TAZ response, whereas U0126 (Fig. 3C) and Y-27632 (Fig. 3D) did not exert significant effects. Efficiency of the latter two drugs (on ERK phosphorylation and the actin skeleton) was verified in separate experiments (data not shown). These results suggest that p38 and Akt pathways significantly contribute to the mediation of the TGFβ-induced Smad3-independent induction of TAZ expression.

**Inhibition of TAZ degradation cannot account for the TGFβ-induced rise in TAZ expression**

Several kinase pathways have been reported to induce the proteolysis of TAZ through phosphorylation of a phosphodegron site (45, 46). Because some of these (e.g. GSK-3β) are inhibited by TGFβ (47), it was conceivable that TGFβ elevates TAZ levels by suppressing TAZ degradation. To assess this possibility, we first tested the impact of proteasome inhibition on TAZ expression in the relevant time period (Fig. 4A). The proteasome inhibitor MG132 caused a strong increase in steady-state TAZ protein expression within 3–6 h, an effect whose time dependence and magnitude were comparable with that of TGFβ (Fig. 4B) and therefore compatible with a potential role of TGFβ-inhibited TAZ proteolysis. TGFβ and MG132 added together appeared to exert a slightly stronger effect than MG132 alone (Fig. 4B). Thus, we tested the half-life of TAZ upon blocking protein synthesis by cycloheximide (CHX) in the absence or presence of TGFβ (Fig. 4C). CHX induced a rapid reduction in TAZ levels without causing significant changes in YAP or actin levels, verifying that TAZ is a fast turnover protein (45) with a half-life of ~2 h in C3H/10T1/2 cells. However, the drop in TAZ levels upon CHX treatment was similar in the absence and presence of TGFβ (Fig. 4C). We considered that CHX might have reduced the expression of a protein necessary to inhibit TAZ proteolysis. To address this possibility, we expressed HA-tagged TAZ in C3H/10T1/2 cells for various times in the absence and presence of TGFβ (Fig. 4D). This approach has the following two advantages: it allowed us to test the potential TAZ-stabilizing effect of TGFβ in the absence of CHX, and it eliminated any transcriptional effects via the TAZ promoter, because HA-TAZ expression was driven by an artificial CMV-based promoter. Under these conditions, we did not observe a significant increase in TAZ protein expression in TGFβ-treated cells at any of the investigated time points. Although these experiments do not fully exclude some contribution of altered degradation, they strongly suggest that other (e.g. transcriptional) mechanisms play a central role.

**MRTF plays a key role in TGFβ-induced TAZ mRNA and protein expression**

We next tested TAZ mRNA expression and found that TGFβ induced a significant rise in the TAZ message in C3H/10T1/2 mesenchymal cells (Fig. 5A). Interestingly, the increase in TAZ mRNA was prevented by DORA but not by AKTi, suggesting that p38 but not Akt promotes TAZ expression by mediating TGFβ-induced TAZ transcription (Fig. 5B). Because Akt is an important regulator of protein translation (e.g. via the mTOR/S6 kinase pathway (48)), we surmised that Akt might contribute to TAZ expression through this mechanism. Given that TAZ is a very fast turnover protein, suppression of translation is expected to impact TAZ levels to a greater extent (i.e. more rapidly) than more stable proteins. Indeed, AKTi significantly reduced TGFβ-induced S6 protein phosphorylation (Fig. 5C), whereas the mTOR inhibitor rapamycin suppressed the TGFβ-induced TAZ expression (Fig. 5D). These findings are consistent with the notion that inhibition of TAZ translation, at least in part, accounts for the effect of AKTi.

We next focused on the mechanism underlying TGFβ-induced changes in transcription. Because our recent studies implicated the transcriptional coactivator MRTF in basal TAZ expression, and the TAZ promoter harbors a CArG box, we asked whether MRTF might be a mediator of TGFβ-induced
TAZ transcription. To this end we treated the cells with CCG-1423, an inhibitor of MRTF/SRF-dependent transcription (49). The drug nearly abolished the stimulatory effect of TGFβ/H9252 on TAZ mRNA expression (Fig. 5B). Similar observations were made in LLC-PK1 tubular cells, in which TGFβ/H9252 induced a significant rise in TAZ mRNA, which was nearly completely prevented by CCG-1423 (Fig. 5E).

To substantiate these findings, we next determined the TGFβ-induced TAZ protein expression upon pharmacological or genetic inhibition of MRTF. CCG-1423 dramatically reduced the TGFβ-provoked rise in TAZ protein level both in C3H/10T1/2 and LLC-PK1 cells (Fig. 5B). Similar observations were made in LLC-PK1 tubular cells, in which TGFβ induced a significant rise in TAZ mRNA, which was nearly completely prevented by CCG-1423 (Fig. 5E).

To substantiate these findings, we next determined the TGFβ-induced TAZ protein expression upon pharmacological or genetic inhibition of MRTF. CCG-1423 dramatically reduced the TGFβ-provoked rise in TAZ protein level both in C3H/10T1/2 and LLC-PK1 cells (Fig. 5B). Similar observations were made in LLC-PK1 tubular cells, in which TGFβ induced a significant rise in TAZ mRNA, which was nearly completely prevented by CCG-1423 (Fig. 5E).

**TGFβ induces p38-dependent, MRTF-mediated TAZ expression**

**Figure 4. Altered TAZ degradation cannot account for the TGFβ-induced increase in TAZ expression.** A, to assess the role of the proteasome in regulating steady-state TAZ levels, C3H/10T1/2 cells were treated with the proteasome inhibitor MG132 (40 μM) for the indicated times, and changes in TAZ expression were determined by Western blotting. B and C, to assess whether TGFβ prevents the constitutive degradation of TAZ, cells were treated with MG132 (B) or the protein synthesis inhibitor CHX (25 μg/ml) (C) in the absence or presence of TGFβ. B, cells were treated with vehicle (DMSO) or MG132 (40 μM) and, where indicated, exposed to TGFβ for 6 h. C, cell lysates were collected at the allotted times and change in TAZ protein was quantified relative to the 0-h conditions. The graph shows the respective degradation profiles for both conditions (n = 3). D, cells were transfected with a HA-TAZ construct (0.5 μg/ml) and 24 h later treated with or without TGFβ for the indicated times. HA-TAZ expression was monitored using an anti-HA antibody.

TAZ transcription. To this end we treated the cells with CCG-1423, an inhibitor of MRTF/SRF-dependent transcription (49). The drug nearly abolished the stimulatory effect of TGFβ on TAZ mRNA expression (Fig. 5B). Similar observations were made in LLC-PK1 tubular cells, in which TGFβ induced a significant rise in TAZ mRNA, which was nearly completely prevented by CCG-1423 (Fig. 5E).

To substantiate these findings, we next determined the TGFβ-induced TAZ protein expression upon pharmacological or genetic inhibition of MRTF. CCG-1423 dramatically reduced the TGFβ-provoked rise in TAZ protein level both in C3H/10T1/2 and LLC-PK1 cells (Fig. 5B). Similar observations were made in LLC-PK1 tubular cells, in which TGFβ induced a significant rise in TAZ mRNA, which was nearly completely prevented by CCG-1423 (Fig. 5E).

**TGFβ drives TAZ expression by inducing post-translational modification rather than inducing a net increase in nuclear translocation of MRTF**

Activation of MRTF-dependent transcription is, in most cases, due to the nuclear translocation of MRTF. However, previous reports have indicated that TGFβ is a weak/marginal inducer of MRTF translocation in C3H/10T1/2 cells (50) and our previous studies led to a similar conclusion in LLC-PK1 cells as well (41, 42). Nonetheless, we tested the impact of TGFβ on the localization of both endogenous and GFP-tagged MRTF C3H/10T1/2 cells. Under resting conditions, endogenous
MRTF resided both in the cytosol and the nucleus (Fig. 7A). TGFβ caused no change or only marginal changes in nuclear localization both after 0.5 and 3 h of treatment (i.e., when the TAZ transcription was already markedly increased), and only a slight increase after 6 h, as verified by immunofluorescence microscopy and the quantitation of the cytoplasmic to nuclear ratio (Fig. 7A). This finding was not due to a general unresponsiveness of MRTF localization in C3H/10T1/2 cells, as serum provoked rapid and sizable MRTF accumulation in the nucleus. Because non-specific (background) antibody binding might mask small but observable changes in MRTF distribution, we monitored MRTF localization in an antibody-independent manner as well. We transfected cells with GFP-MRTF and determined the percentages of cells showing cytosolic or nuclear GFP-MRTF distribution. As shown by Fig. 7B, 90% of resting cells exhibited cytosolic or even GFP-MRTF localization, and this distribution was unaltered upon TGFβ treatment. In contrast, serum caused a 5-fold increase in the percentage of cells showing nuclear GFP-MRTF accumulation. Finally, these results were confirmed by determining MRTF protein levels in cytoplasmic and nuclear preparations (Fig. 7C). Some MRTF resided in the nucleus in control cells, and there was no measurable increase in the nuclear MRTF level upon TGFβ treatment (Fig. 7C). However, both the cytosolic and the nuclear MRTF fraction exhibited an upward shift in the apparent molecular mass of MRTF (51). MRTF has recently been shown to contain 24 serine/threonine phosphorylation sites, the collective mutation of which (to alanine) inhibited MRTF activity, even when MRTF was rendered constitutively nuclear (see under "Discussion") (52). We therefore tested whether such post-translational modifications, even without a net increase in the nuclear MRTF content, could account for increased MRTF activity, leading to increased TAZ expression. To address this issue, first we checked whether the transcriptional activity of MRTF was indeed increased by TGFβ (despite the lack of translocation) under our conditions. Cells were transfected with 3DA-Luc, a reporter construct in which three CArG boxes precede the coding region of luciferase. TGFβ readily activated 3DA-Luc, and importantly, both the basal activity and the TGFβ-induced acti-
vation were completely abolished by siRNA-mediated down-regulation of MRTF (Fig. 7D). These findings indicated that TGFβ activates MRTF-dependent transcription without inducing robust MRTF translocation. Finally, to check that MRTF/SRF signaling can indeed regulate the endogenous TAZ promoter, we performed ChIP experiments (Fig. 7E). Indirect immunoprecipitation using anti-MRTF antibody resulted in a 3.5-fold increase in the associated TAZ promoter region over the isotype control, and this was further increased by TGFβ (despite the fact that the MRTF antibody is suboptimal for ChIP). Direct immunoprecipitation with anti-SRF indicated strong basal binding, which was substantially elevated by TGFβ. These experiments verified that MRTF/SRF interacts with the endogenous TAZ promoter.
Next, we investigated whether the kinase inhibitors that mitigated TAZ expression impacted the post-translational modification of MRTF. The TGFβ-induced shift was reduced by 50% by the p38 inhibitor DORA (Fig. 8A), whereas AKTi did not significantly alter the shift (Fig. 8B). To substantiate the role of p38, we used siRNA as well. Combined targeting of p38 isoforms significantly reduced both the TGFβ-induced TAZ expression and the molecular weight shift in MRTF (Fig. 8, C and D). Accordingly, DORA but not AKTi significantly suppressed the TGFβ-dependent activation of 3DA-Luc (Fig. 8E, panels i and ii). Of note, Smad3 silencing not only failed to prevent but in fact potentiated the basal activity and TGFβ-induced activation of 3DA-Luc (Fig. 8E, panel iii). The latter data are in agreement with our earlier report showing that Smad3 is an inhibitor of MRTF (42). Together, these findings indicate that both TAZ mRNA and reporter 3DA-Luc activity
are regulated by TGFβ in an MRTF-dependent, Smad3-independent manner.

A recent elegant study has shown that MRTF can be directly phosphorylated both by p38 and its major downstream substrate MK2 (53). We therefore asked whether MK2 might be involved in TGFβ-induced TAZ expression. To assess this, cells were pretreated with PF 3644022 (PF), a potent MK2 inhibitor (54). PF significantly reduced the TGFβ-induced TAZ expression (Fig. 8F) and also mitigated the TGFβ-induced shift in the apparent molecular weight of MRTF (Fig. 8G). Finally, we tested the impact of pirfenidone, the first clinically approved drug against pulmonary fibrosis (55). Although its mechanism of action remains incompletely defined, pirfenidone was proposed to interfere with the p38/MK2 axis (56). Pirfenidone markedly suppressed both the TGFβ-induced TAZ expression (Fig. 8H) and the concomitant MRTF phosphorylation (Fig. 8I). Taken together, these experiments suggest that TGFβ induces MRTF phosphorylation partially via p38 and possibly MK2, which is in turn critical for TGFβ-induced MRTF translocation-independent activation of MRTF.

MRTF is sufficient to drive the TAZ promoter and increase TAZ protein expression

Although the data so far suggested that MRTF is indispensable for TAZ expression, we sought to establish whether TGFβ can indeed activate the TAZ promoter and whether MRTF and/or SRF is sufficient to induce TAZ protein expression. For this purpose, we cloned a 1200-bp segment of the human TAZ promoter and inserted it into pGL3 luciferase vector (TAZ-Luc). Cells were then transfected with TAZ-Luc and exposed to vehicle or TGFβ. As shown in Fig. 9A, TGFβ caused a 2.5-fold increase in TAZ-Luc activity. Moreover, DORA prevented the TGFβ-triggered activation of the TAZ promoter (Fig. 9A). We then overexpressed either MRTF or SRF along with the TAZ-Luc reporter and found that MRTF but not SRF drove the TAZ promoter (Fig. 9B). In accordance with this finding, overexpression of MRTF but not SRF was capable of inducing TAZ protein expression; cells transfected with HA-MRTF but not HA-SRF showed enhanced staining for endogenous TAZ protein, compared with their non-transfected neighbors (Fig. 9C). Of note, endogenous SRF is abundantly expressed and largely nuclear, likely accounting for the fact that further SRF expression, without concomitant activation, failed to evoke TAZ expression. Finally, to test whether the observed transcriptional effect of MRTF was indeed mediated directly via the CArG box within the TAZ promoter, we mutated and thereby inactivated this sequence (TAZmut-Luc). In contrast to TAZ-Luc (Fig. 9A), TAZmut-Luc was not activated by TGFβ (in fact it was significantly inhibited by the cytokine) (Fig. 9D). MRTF overexpression also failed to stimulate this promoter construct (Fig. 9E). Taken together, MRTF is sufficient to drive the TAZ promoter activation and protein expression in a CArG-box-dependent manner.

NADPH oxidase Nox4 is a mediator of TGFβ-induced, MRTF-mediated expression and transcriptional activity of TAZ

TGFβ is a prime inducer of Nox4 (57–59), and this enzyme has recently been implicated as a potential regulator of MRTF phosphorylation via a redox-dependent mechanism (51). Furthermore, Nox4 has been shown to activate p38 (59, 60). Therefore, we asked whether Nox4 might be involved in the regulation of TGFβ-induced TAZ expression and TAZ promoter activation. To test this, we preincubated the cells with VAS2870 (VAS), a potent inhibitor of Nox4. Remarkably, VAS reduced the TGFβ-triggered TAZ expression by 60% (Fig 10A) and, concomitantly, mitigated the TGFβ-induced shift in the molecular mass of MRTF by 50% (Fig 10B). To assess whether the inhibition of TAZ protein expression by VAS could be attributed to an effect on the TAZ promoter, we transfected the cells with TAZ-Luc and stimulated them with TGFβ in the absence or presence of VAS. The inhibitor caused a 50% reduction in the TGFβ-evoked TAZ-Luc activity, reflecting a strong suppression of the relative (fold) change compared with the TGFβ effect measured in the vehicle-treated controls (Fig 10C). Together, these data imply that Nox4 plays an important role in the TGFβ-induced TAZ promoter activation and protein expression, predominantly as an upstream activator of MRTF.

Functional significance of increased TAZ expression: Priming and synergy with TAZ-translocating inputs

Although our experiments so far document that TGFβ induces TAZ expression, and allows insight into the underlying mechanisms, they do not inform us about the potential functional significance of this phenomenon. Given that TAZ, as a transcription factor, acts in the nucleus, we surmised that increased overall TAZ expression might influence TAZ-dependent transcription by two ways. First, having more TAZ could proportionally increase the nuclear presence and thus the transcriptional activity of TAZ, even without a change in the nuclear/cytoplasmic ratio. Second, a larger TAZ pool might prime the cells for enhanced TAZ-mediated transcription once a second stimulus, promoting TAZ nuclear translocation, challenges the cell. One difficulty in conclusively addressing this problem using natural TAZ target genes is that a large number of these harbor both TEAD-binding elements and CArG boxes (61), as well as Smad-binding elements (33) in their promoter. Moreover, studies from us and others have shown that, in addition to the individual effect of these factors, they may negatively or positively influence each other’s action at the promoter, depending on the distance of their respective binding sites (9, 62). To overcome these complexities, we used a TEAD reporter construct (TEAD-Luc), which contains exclusively TEAD-binding elements. As shown in Fig 11A, TGFβ stimulated TEAD-Luc. Importantly, siRNA-mediated down-regulation of TAZ strongly (80%) inhibited the activation of TEAD-Luc. These findings imply that TGFβ, without any additional stimulus, is capable of activating TEAD-mediated transcription in a TAZ-dependent manner. We next assessed whether TGFβ-induced TAZ up-regulation can prime the cell for a TAZ-mobilizing stimulus. Sphingosine 1-phosphate (S1P) has been shown to promote nuclear localization of TAZ predominantly by inhibiting Hippo kinases (63). Indeed, S1P promoted the nuclear translocation of TAZ (Fig 11B) without affecting net TAZ expression (Fig 11C). In contrast, TGFβ increased TAZ protein expression (Fig 11C), which occurred roughly propor-
**Discussion**

Cross-talk between TGFβ-induced and YAP/TAZ-mediated signaling has been shown to play a key role in cell plasticity and the pathogenesis of cancer and organ fibrosis (8, 23, 31, 33). Our studies uncover a new aspect of this important interplay; we show that TGFβ is a strong and fast inducer of TAZ (but not YAP) transcriptional activity provoked by a TAZ-translocating stimulus, and conversely, the TAZ-mediated transcriptional effect of TGFβ is strongly potentiated by stimuli causing concomitant TAZ translocation.

**Figure 9. TGFβ or overexpression of MRTF but not SRF can induce TAZ protein expression and TAZ promoter activation.**

A. C3H/10T1/2 cells were cotransfected with a ~1.2 kb segment of the TAZ promoter coupled to firefly luciferase (TAZ-Luc) along with Renilla luciferase for 24 h. Subsequently cells were pretreated with vehicle (DMSO) or DORA (10 µM) for 30 min, then stimulated with TGFβ for 24 h. Normalized TAZ promoter activity was determined by the dual-luciferase assay. B, to assess whether MRTF or SRF was sufficient to drive the TAZ promoter, C3H/10T1/2 cells were cotransfected with TAZ-Luc and Renilla along with either FLAG-MRTF or HA-SRF. Twenty four h later TAZ-Luc activity was measured. C, verification of expression of FLAG-MRTF and HA-SRF and assessment of their impact on TAZ expression was achieved by double immunofluorescence staining for the corresponding epitope tag and endogenous TAZ. Note that HA-SRF is strongly expressed and exhibits robust nuclear localization, yet it does not cause a detectable increase in TAZ expression, as opposed to FLAG-MRTF. Scale bar, 20 µm. D, C3H/10T1/2 cells were transfected with a mutant version (TAZmut-Luc) of TAZ-Luc, in which the CArG box has been inactivated, and with Renilla. Twenty four h later cells were left untreated or treated with TGFβ for an additional 24 h, and luciferase activities were determined (n = 3). E, conditions were as in D, except cells were transfected with FLAG-MRTF-B along with the TAZmut-Luc/Renilla system for 24 h, and luciferase activities were measured without further treatment (n = 3). Cntrl, control. n.s., non-significant.
mediator of TGFβ regulation reduced basal TAZ expression in tubular cells (9). The TAZ promoter contains a CArG box and (... TGFβ activation of MRTF, which in turn stimulates the TAZ promoter. In a non-conventional (net translocation-independent) manner and increases TAZ protein expression.

Figure 10. Nox4 contributes to TGFβ-induced TAZ expression and the post-translational modification of MRTF. A and B, cells were pretreated with vehicle (DMSO) or VAS2870 (10 μM, VAS), a potent inhibitor of Nox4 for 30 min and, where indicated, exposed to TGFβ for 6 h. TAZ expression (A) and the shift in the molecular mass of MRTF (B) were then quantified (n = 3). C, CH1/10T1/2 cells were transfected with TAZ-Luc and Renilla luciferase for 24 h, followed by pretreatment with DMSO or VAS2870 and, where indicated, with TGFβ for 24 h (n = 3).

YAP expression in both fibroblasts and epithelial cells, and we provide new insight into the underlying mechanisms. Specifically, we demonstrate that TGFβ stimulates TAZ expression by a Smad3-independent, p38-mediated and redox-sensitive pathway, leading to non-conventional (net translation-independent) activation of MRTF, which in turn stimulates the TAZ promoter. TGFβ-induced TAZ expression increases TAZ-dependent transcription and, importantly, strongly sensitizes the cells for a subsequent TAZ-activating stimulus (TGFβ-induced priming).

Enhanced TAZ expression upon TGFβ exposure was observed in a few previous studies including our own (7, 31, 35), but the mechanism, the critical TFs, and the significance of this process remained undefined. The rationale to test the potential role of MRTF originated from our observations that (a) the TAZ promoter contains a CArG box and (b) that MRTF down-regulation reduced basal TAZ expression in tubular cells (9). The conclusions that MRTF is both an indispensable and a sufficient mediator of TGFβ-induced TAZ expression are based on our current findings that (a) pharmacological inhibition (CCG-1423) or genetic down-regulation (siRNA) of MRTF abolishes the TGFβ-triggered TAZ expression and (b) that overexpression of MRTF (but not SRF) drives the TAZ promoter in a CArG box-dependent manner and increases TAZ protein expression.

Considering the signaling that links TGFβ to TAZ expression (and thus MRTF), our finding that neither Smad3 down-

regulation nor its pharmacological inhibition interfered with this process excluded the involvement of this canonical pathway. In fact, the absence of Smad3 tended to increase the TGFβ-triggered rise in TAZ protein expression, which may well be explained by our earlier finding that Smad3 can bind to MRTF and inhibit its transcriptional activity on certain promoters (e.g. that of SMA) (9, 42). Silencing Smad2 induced a dramatic drop in basal YAP expression and reduced TAZ levels as well. This effect was observed in the absence of TGFβ, suggesting a constitutive role for Smad2 in YAP and likely TAZ expression. This effect is distinct from the TAZ-specific and TGFβ-induced regulation. Although TAZ remains responsive to TGFβ stimulation in Smad2-silenced cells, the attained levels are lower. Thus, at this point we cannot exclude that Smad2 might play a role in the TGFβ-regulated TAZ response as well. It is noteworthy in this regard that Smad2 was reported to interact with MRTF-B, and the complex may synergize on the SMA promoter in smooth muscle cells (64). Further studies are warranted to test whether a similar mechanism might be relevant for TAZ as well. If so, it would point to an intriguing, distinct, and opposite regulation by Smad2, since our earlier studies have shown that Smad3 inhibits MRTF signaling. Thus, it remains to be tested whether one arm of the canonical pathway (Smad2) can selectively synergize with non-canonical pathways regulating TAZ expression.

Of the non-canonical TGFβ pathways, activation of the MEK/ERK signaling does not seem to play a role in this process either. This finding may be surprising in light of a recent study wherein insulin-like growth factor was found to increase TAZ expression in a MEK/ERK-dependent manner during osteogenic differentiation of mesenchymal stem cells (65). However, the insensitivity to ERK inhibition in our setting is in full agreement with the central role of MRTF as the key mediator of the TGFβ effect, inasmuch as ERK-mediated phosphorylation (at Ser-454) was shown to inhibit MRTF activity by increasing actin binding and promoting MRTF efflux from the nucleus (66, 67). As a recent detailed analysis shows, the picture is more complex because MRTF can be phosphorylated at least on 24 Ser/Thr sites, many of which are targeted by ERK, and a subset of which plays either negative or positive roles, affecting nuclear influx, efflux, or transcriptional activity (52). Although such modifications may fine-tune MRTF responses to various stimuli, our data reveal that ERK-dependent phosphorylation cannot account for the TGFβ-induced elevated TAZ expression. Similarly, ROK also fails to convey this effect, although it can promote MRTF phosphorylation (37), and its inhibition can mitigate Rho-mediated MRTF translocation in response to certain stimuli (41, 68). In contrast, the lack of Rho/ROK involvement is consistent with the facts that (a) in many cells TGFβ is a weak and late-onset activator of Rho (42) and (b) it induces none or only marginal MRTF translocation (42, 50). Indeed, we observed only a late onset (≥6h) and mild nuclear accumulation of MRTF upon TGFβ exposure, an effect likely due to TGFβ-induced expression of Rho-GEFs (69). Because TAZ expression preceded these changes, their causal contribution (at the early stage investigated) can be excluded. However, this does not mean that the effect of TGFβ on TAZ was independent of the F-actin status. Actin depolymerization by latruncu-
lin B prevented the effect of TGFβ on TAZ (data not shown). Because actin depolymerization drives MRTF out of the nucleus by inhibiting influx and activating efflux (37, 70), this finding points to the need for the constitutive nuclear presence of some MRTF, which then can be post-translationally modified. It is noteworthy in this regard that during the late stages of our studies, a paper by Liu et al. (71) was published, which implicated MRTF as a mediator of heregulin (HRG1)-induced TAZ expression in breast cancer cells. However, HRG1, as opposed to TGFβ, caused robust nuclear translocation of MRTF. Together these observations imply that, depending on the stimulus, MRTF may drive TAZ expression by two distinct mechanisms, namely in a nuclear translocation-dependent and -independent manner. Perhaps such differential regulation might confer target selectivity (e.g. turning on only a subset of MRTF-dependent genes, whose promoter might show selective affinity for such post-translationally modified MRTF). Clearly, these modes do not have to be mutually exclusive. Indeed, we found that serum induced robust MRTF translocation and also induced TAZ expression, albeit its potency was less than that of TGFβ, which failed to induce translocation. Such dual regulation (which is supported by the fact that the transcriptional activity of nuclear resident MRTF mutants is still modified by mutations in the phospho-target sites (52)) offers distinct cytoskeleton remodeling-dependent (e.g. mechanosensitive) and -independent (e.g. TGFβ) control of TAZ expression, integrated via MRTF.

What could this translocation-independent, TGFβ-provoked signaling be, and how could it impact MRTF activity? Our findings suggest a key role for p38, a well-known non-

**Figure 11. TGFβ-induced TAZ expression increases TAZ-dependent transcriptional activity and primes cells for augmented TAZ activity in response to a stimulus inducing TAZ translocation.** A, to demonstrate TAZ-dependent transcriptional activity, luciferase assays were performed using a reporter construct harboring tandem TEAD-binding elements in front of firefly luciferase (TEAD-Luc). Cells were transfected with the TEAD-Luc/Renilla system along with siNR or TAZ-specific siRNA (siTAZ, 25 nM) for 24 h and then left untreated or exposed to TGFβ for an additional 24 h, followed by the determination of TEAD activity (*n* = 3). TAZ knockdown was confirmed by Western blotting performed in parallel. B, cells were incubated in the absence or presence of TGFβ for 5 h and subsequently challenged, where indicated, with S1P (1 μM) for 1 h. Cells were then stained for endogenous TAZ, and nuclei were visualized by DAPI. Scale bar, 20 μm. C and D, to demonstrate the priming effect of TGFβ on TAZ activity, cells were pretreated with TGFβ for 6 h and then further stimulated with S1P (1 μM) to induce translocation of TAZ to the nucleus. The effect on TAZ protein expression (C) and TEAD-Luc activity (D) was then measured (*n* = 3). Cntrl, control. n.s., non-significant.
TGFβ induces p38-dependent, MRTF-mediated TAZ expression

canonical transducer in the TGFβ pathway (72, 73). Inhibition or down-regulation of p38 strongly reduced the TGFβ-induced TAZ mRNA and protein expression and the concomitant shift in the apparent molecular weight of MRTF (phosphorylation). Importantly, a very recent and elegant study has shown that p38 can promote MRTF phosphorylation by two mechanisms: it can directly phosphorylate MRTF at unidentified site(s), and it activates MK2, which phosphorlates MRTF at Ser-312 and Ser-333 (53). However, the authors found no functional consequence (e.g. altered nuclear transport, SRF binding, and transcriptional effect) of the MK2-mediated MRTF phosphorylation. We found that pharmacological inhibition of MK2 reduced TAZ expression and MRTF phosphorylation. Although the exact mechanism warrants further studies, these results suggest that p38-mediated MRTF phosphorylation alone or in combination with MK2-mediated phosphorylation promotes TAZ transcription. Of note, TGFβ-induced TAZ and SMA expression show remarkable similarities in that both are MRTF-dependent, partly p38, and MK2-mediated processes (59, 74, 75). In fact, inhibition of p38 mitigates the MRTF-dependent activation of SMA expression by other stimuli (contact uncoupling, osmotic stress) (68, 76) as well.

As opposed to MRTF, the overexpression of its partner SRF is insufficient to increase TAZ expression. Nonetheless, in agreement with previous reports (77), we found that TGFβ also increases SRF expression in a p38-, Akt-, and MRTF-dependent manner (data not shown). This effect might also facilitate TAZ expression when combined with MRTF activation. It is also conceivable that p38 and/or MK2 may modify SRF, thereby increasing the activity of the MRTF–SRF complex. Furthermore, Akt likely acts via a post-transcriptional mechanism because its inhibition did not or only marginally affected TAZ mRNA. Our data suggest that Akt might support TAZ expression via the Akt/mTOR/S6 kinase pathway, which increases protein translation. Interestingly, SRF expression can be regulated by this pathway (77).

Besides their direct actions, MRTF and p38 may also promote TAZ expression via indirect mechanisms, some of which involve strong positive feedback loops. For example, we have recently described that the Nox4 promoter harbors a CArG box, and MRTF potentiates Nox4 expression (7). In parallel, Lee et al. (51) reported that Nox4 promotes MRTF phosphorylation in a redox-sensitive manner. We confirmed this finding and found that inhibition of Nox4 reduced TAZ expression and TAZ-mediated transcription. Because Nox4-derived H₂O₂ can activate p38 (59), this could be a mechanism whereby Nox4 promotes MRTF phosphorylation. Together, these findings not only suggest that TAZ expression is a redox-regulated process, but they also imply that the interplay among TGFβ, p38, MRTF, and Nox4 contains at least two self-augmenting circuits. Interestingly, TAZ itself may participate in these feed-forward processes. We recently found that YAP and TAZ are also required (at least in epithelial cells) for the TGFβ-induced Nox4 expression (7). Finally, our ongoing studies suggest that mechanical stimuli induce TGFβ production via MRTF- and TAZ-dependent processes, and a recent paper reported that enhanced YAP signaling results in the overexpression of the TGFβ and the TGFβ receptor (78). In summary, the TGFβ/p38/MRTF pathway and its interplay with YAP/TAZ signaling contains an abundance of self-perpetuating interactions. Such positive feedbacks may underlie the focal and rapidly progressive characteristic of fibrogenic processes.

So far, the majority of studies addressing the TF control of Hippo transcriptional coactivators concentrated on YAP. Given that the effect of MRTF is specific to TAZ, it remains to be tested whether previously identified YAP-regulating factors (e.g. cAMP-response element-binding protein (79) and GATA-binding protein (80)) modulate or not TAZ expression as well. HIF-1 (81) and NFκB (82) were shown to transcriptionally regulate TAZ. Future studies should clarify whether these TFs act in parallel or (at least in part) in series with SRF/MRTF.

Finally, we briefly consider the physiological and pathobiological relevance of our findings. We have shown that TGFβ-promoted TAZ expression results in enhanced TAZ transcriptional activity and strongly sensitizes cells to the effect of other TAZ activators. Physiologically increased TAZ expression was observed in the context of TGFβ-induced osteogenic differentiation of mesenchymal stem cells (35) and during embryonic stem cell renewal or differentiation (31). TAZ is a key mediator of osteogenesis and stem cell differentiation, and its overexpression is likely an essential contributor to these processes. Interestingly, MRTF was also reported to be a key regulator of osteogenesis and mesenchymal cell fate (34, 83, 84), effects that may well be related to its TAZ-inducing capacity. Pathologically, TAZ overexpression is characteristic in several tumors. Moreover, this study shows that TAZ is strongly up-regulated in UUO, a robust fibrosis model, and a recent work demonstrates YAP overexpression in diabetic nephropathy (22). Thus, the picture is emerging that TAZ (and YAP) overexpression is a major feature of cancer and fibrosis. It is noteworthy in this regard that pirfenidone, a clinically used antifibrotic drug potently inhibited TGFβ-induced TAZ expression. Heightened TAZ expression may have two important functional consequences, particularly in the context of increased/altered TGFβ signaling. First, the higher TAZ levels, especially when combined with TAZ-translocating mechanical stimuli (such as a stiff extracellular matrix) will result in vastly enhanced TAZ-mediated transcriptional responses. These will augment the production of extracellular matrix components and fibrogenic mediators, creating a feed-forward pathomechanism. Second, prolonged TGFβ exposure, both in cancer and fibrosis, often results in a drop of Smad3 expression (85, 86). This phenomenon contributes to the switch between the tumor suppressor to the tumor promoter role of TGFβ in cancer and may facilitate MRTF-mediated fibrogenic processes in the late phases of fibrosis (42, 87). Because Smad3 facilitates TAZ nuclear localization and transcription, the loss of Smad3 could result in diminished TAZ signaling. However, Smad3-independent, MRTF-mediated overexpression of TAZ might compensate for the loss of Smad3, thereby maintaining the fibrogenic process. Taken together, we propose that the TGFβ-induced, MRTF-mediated TAZ up-regulation described herein is an important pathomechanism in organ fibrosis. Accordingly, strategies targeting MRTF and TAZ may be promising therapeutic approaches in this disease entity.
TGFβ induces p38-dependent, MRTF-mediated TAZ expression

Experimental procedures

Reagents

TGF-β1 was purchased from R&D Systems (Minneapolis, MN), and sphingosine 1-phosphate (SIP) was from Cayman Chemical Co. (Ann Arbor, MI). The following inhibitors were purchased from the indicated sources. ROK inhibitor Y-27632, Akt1/2 kinase inhibitor AKTI-1/2, protein synthesis inhibitor CHX, proteasome inhibitor MG132, and MRTF/SRF inhibitor CCG-1423 were from Sigma. The p38 MAPK inhibitor doral-mapiim (BIRB-76) was from Cayman Chemical Co. (Ann Arbor, MI); MEK1/2 inhibitor U0126 was from Alexis Biochemicals (San Diego, CA); Smad3 inhibitor SIS3 and NADPH oxidase inhibitor VAS2870 were from Calbiochem/EDM Millipore (Billerica, MA); mTORC1 inhibitor rapamycin was from Abcam (Cambridge, UK); Food and Drug Administration-approved anti-fibrotic drug pirfenidone and MAPK-activated protein kinase-2 (MK2) inhibitor PF-3644022 were from Tocris (Bristol, UK); and cortactin (mouse monoclonal, 1:3000, catalog no. 9212S), p38 (rabbit polyclonal, 1:300, catalog no. 2211S), p97 (rabbit monoclonal, 1:100, catalog no. 19715S), SRF (rabbit monoclonal, catalog no. 5147S), S6 protein (rabbit polyclonal, 1:2000, catalog no. 25812S), and tubulin (rabbit polyclonal, 1:10,000, catalog no. 2128S) were from Cell Signaling Technology (Beverly, MA); TAZ (mouse monoclonal, 1:200 for immunofluorescence, m2-615 560235) was from Pharmingen; MRTF-A (rabbit polyclonal, 1:3000 for Western blotting or 1:200 for immunofluorescence, H-1401: sc-32909) and GAPDH (mouse monoclonal, 1:10,000, 0411; sc-47724) were from Santa Cruz Biotechnology (Santa Cruz, CA); α-actin (mouse monoclonal, 1:10,000, catalog no. 31345), and tubulin (rabbit monoclonal, 1:10,000, catalog no. 2128S) were from Cell Signaling Technology (Beverly, MA); TAZ (mouse monoclonal, 1:200 for immunofluorescence, m2-615 560235) was from Pharmingen; MRTF-A (rabbit polyclonal, 1:3000 for Western blotting or 1:200 for immunofluorescence, H-1401: sc-32909) and GAPDH (mouse monoclonal, 1:10,000, 0411; sc-47724) were from Santa Cruz Biotechnology (Santa Cruz, CA); β-actin (mouse monoclonal, 1:10,000, A1978) was from Sigma; HA (mouse monoclonal, 1:3000, 16B12 B207195) was from BioLegend (San Diego); PAL-1 (rabbit polyclonal, 1:3000, ab66705) was from Abcam (Cambridge, UK); and cortactin (mouse monoclonal, 1:5000, 05-180) was from Upstate Cell Signaling Solutions (Lake Placid, NY).

Cell culture

C3H/10T1/2 cells (mouse embryonic mesenchymal cells, clone 8, American Type Culture Collection) and LLC-PK1 (Cl 4 cells) (porcine kidney tubular epithelial cells, a gift from R. C. Harris, Vanderbilt University School of Medicine, Nashville, TN) were maintained in low-glucose (1 g/liter) and sodium pyruvate-containing DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Invitrogen). NRKFs (American Type Culture Collection) were cultured in high-glucose (4.5 g/liter) and sodium pyruvate-containing DMEM (Invitrogen) with similar supplements. Where indicated, cells were treated with 5 ng/ml TGFβ, 1 μM SIP, or 10% FBS. For transfections, antibiotic-free medium was used. If not specified otherwise, C3H/10T1/2 cells were used in the experiments.

Plasmids and transfection

The luciferase reporter constructs, SBE4-Luc, the MRTF/SRF-sensitive promoter (3DA) containing three CArG repeats, the TEAD reporter (a gift from L. Attisano (University of Toronto)) were used as in our previous studies (9, 42, 68). A segment of the human TAZ promoter (−1060/+74) was cloned into a pGL3-luciferase vector using primers (sense, 5′-GTAGGTACCGGGAAGGCCTCTCTCT- ACTTT-3′, and antisense, 5′-AGATCTCGAGGCGCTAAGGCCTAGTGG(//)CTTG-3′). The thymidine kinase minimal promoter-driven Renilla reporter (pRL-TK) was purchased from Promega (Madison, WI). The following expression constructs were used: pcDNA3 was originally purchased from Invitrogen (Burlington, Ontario, Canada); HA-SRF (88) and FLAG, hemagglutinin (HA) or GFP-tagged MRTF constructs (9, 42) were generated as described previously. HA-TAZ (plasmid 32839) provided by Dr. Kunliang Guan was obtained from Addgene (Cambridge, MA). C3H/10T1/2 and LLC-PK1 cells were transfected at 30–40 or 70–80% confluence, respectively, using transfection reagents JetPRIME (Polyplus Transfection SA, New York) at 2 μl/μg DNA or X-tremeGene 9 (Sigma) at 2.5 μl/μg DNA according to the manufacturers’ instructions. The following plasmid concentrations were used: HA-TAZ (0.5 μg/ml), GFP-MRTF (1 μg/ml), FLAG-MRTF (1 μg/ml), and HA-MRTF (3 μg/ml) or HA-SRF (0.2–0.5 μg/ml) to yield similar protein expression from the various constructs.

Luciferase reporter assays

These were carried out as described previously (9). Briefly, cells were cotransfected at 40% confluence with luciferase reporter construct (0.15–0.25 μg/ml) and the pRL-TK (0.025 μg/ml) for internal control. Cells were treated as indicated and 24 h later lysed on ice with 1× Passive Lysis Buffer (Promega). The luciferase assay was performed with the Dual-Luciferase kit (Promega) as per the manufacturer’s instructions.

RNA interference

siRNA was designed to target the following sequences in (no. 1) C3H/10T1/2 cells: siSmad3, 5′-GGCCAUCACCGACGACA- CAtt-3′; siSmad2, 5′-GAAUUGCCAGCACGAGUAU-3′; siMRTF-A and -B (A/B), (A) 5′-CCAAGGAGCUGAAGCG- CAAAUU-3′; (B) 5′-CGAAACACCGUAAGCUAAUU-3′; sip38α, 5′-GAGCCGACCAUGUAAGUAU-3′; sip38β, 5′-CCGAGGAGCAUAAUU-3′; sip38γ, 5′-CCUGUCC- UGCCUCUUCAUU-3′; siTaz, 5′-CCUAAUCAUCAGA- GAAAUU-3′. siRNA was designed to target the following sequences in (no. 2) LLCPK cells: siSmad3, 5′-GAGUUCUACACCCAAUUCUCC-3′; siMRTF-A and -B (A/B), (A) 5′-CCAAGGAGCUGAAGCGCA CAAAUU-3′; (B) 5′-CGAAACACCGUAAGCUAAUU-3′; sip38α, 5′-GAGCCGACCAUGUAAGUAU-3′; sip38β, 5′-CCGAGGAGCAUAAUU-3′; sip38γ, 5′-CCUGUCCUC- UGCCUCUCAAU-3′; siTaz, 5′-CCUAAUCAUCAGAAGAAAUU-3′. siRNA synthesis was completed by Applied Biosystems (Burlington, Ontario, Canada) or by Thermo Fisher Scientific (Waltham, MA). NR control siRNA was purchased from Applied Biosystems (Silencer Negative Control siRNA number 2). siRNA transfections were completed using either JetPRIME or LipofectamineRNAiMAX (Invitrogen) as per manufacturer’s instructions.
Real-time quantitative PCR (qPCR)

Following the indicated treatments, mRNA was extracted using the RNeasy mini kit (Qiagen Venlo, Netherlands) according to the manufacturer’s instructions. mRNA was reverse-transcribed using iScriptTM reverse transcription (Bio-Rad). qPCR was performed in triplicate on each cDNA sample using specific primers targeting: mouse TAZ (sense, 5'-GAC GAG AGT GAT ACA CCT GAA-3', and antisense, 5'-GAA GGC AGT CCA GGA AAT CA-3'); mouse GAPDH (sense, 5'-TGT CAA GCT CAT TCC CTG GTA T-3', and antisense, 5'-GTG GTC CAG GGT TTC TTA CTC-3'); mouse RPL13a (sense, 5'-GGT CTC AAG GTT GTT CCG CTG A-3', and antisense, 5'-AGA TCT GCT TCT TCT TCC GAT A-3'); pig TAZ (sense, 5'-GAT GAG ATG GAC ACA GGA GAA A-3', and antisense, 5'-CCC GGA AGA CAG TCA AGA AA-3'); and pig GAPDH (sense, 5'-GGA AAG TGG ACA TGG TCG CCA TCA-3', and antisense, 5'-AGC TCC TCA TCC TCA GCC TTG ACT-3'). qPCR was performed by a ViiATM 7 real-time PCR system or a Bio-Rad iCycler thermal cycler/Q5 multicolor real-time PCR detection system (95 °C 3 min; 48 °C 30 s, 55 °C, 1 min) using 2× IQ™ SYBR® Green Supermix (Bio-Rad) or Quantifast SYBR Green (Qiagen) and GAPDH or RPL13a as reference genes, as described (7). Data were analyzed using Viia 7 software or the iQ5 software. Melt curves confirmed the presence of one amplicon. Quantification of mRNA was assessed using the Pfaffl Method.

Chromatin immunoprecipitation (ChIP)

ChIP was performed essentially as in our previous studies (9). Briefly, C3H/10T1/2 cells were grown in 10-cm dishes until reaching confluence and then treated in the presence or absence of TGFβ (5 ng/ml) or AKT (10 μM), TGFβ (5 ng/ml), or AKT+ TGFβ for 6 h. Cells were fixed with 4% PFA for 30 min and permeabilized with 0.3% Triton X-100 and blocked with 5% goat serum. Cells were incubated with the primary antibodies (S6 and pS6) at 1:300 dilution for 2 days at 4 °C, washed, and exposed to secondary antibodies (goat anti-mouse catalog no. A28175 and goat anti-rabbit catalog no. A27039 from Molecular Probes) (1:3000) overnight at 4 °C. The secondary antibodies were removed; the cells were stained with Hoechst 33342 (1 μM for 15 min) and washed three times with PBS. The 96-well plate was loaded onto INCELL6000 (GE Healthcare) high-content fluorescence microscope and imaged using the appropriate channels. Twelve random fields were imaged within each well. TIFF images were then loaded into the Acapella automated image analysis software (PerkinElmer Life Sciences) for nuclei identification and quantification of fluorescence intensities. Routinely 1000–3000 cells were quantified per condition. Data were imported into FlowJo (version 10.2) cytometry analysis software to determine the mean fluorescence intensities for the different channels. The pS6 kinase signal was normalized to the corresponding total S6 kinase signal. None of the treatments caused significant changes in the total S6 protein level.

Detection of molecular weight shift

To monitor changes in the apparent molecular weight of MRTF, proteins from cell lysates were separated using 6% mini gel. Samples were run until the ~75-kDa marker reached the edge of the gel. MetaMorph Premiere software (Molecular Devices) line tool was used to determine the distance between the lowest ends of the MRTF bands obtained from control and treated samples. The shift (S) between vehicle- and TGFβ-treated samples was taken as unity (100%). Changes detected

TGFβ induces p38-dependent, MRTF-mediated TAZ expression
upon inhibitor treatments (I) were compared with this control and expressed as I/S ratio.

**Immunofluorescence microscopy**

Cells were plated on glass coverslips. Subconfluent cells were treated and fixed after indicated times with 4% PFA (Calmenco and Marivac, Lakefield, Quebec, Canada) and incubated in 100 mM glycine and 0.1% Triton X-100 in PBS. Samples were then blocked with 3% BSA/PBS, incubated with primary antibody overnight, washed with PBS, and incubated with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Lonza, Basel, Switzerland) and fluorescent-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h in the dark. Coverslips were mounted on slides with DAKO mounting fluid (Agilent, Burlington, Ontario, Canada). Images were taken using a WaveFX spinning-disk confocal microscope system (Quorum Technologies, Guelph, Canada) equipped with an ORCA-Flash4.0 digital camera or an Olympus IX81 microscope (Olympus, Tokyo, Japan) coupled to an Evolution QE monochrome camera (Media Cybernetics, Silver Spring, MD). Images were processed and analyzed using MetaMorph Premiere software (Molecular Devices). Cell midsections were taken to analyze the nuclear/cyttoplasmic distribution of the indicated proteins. Signal intensity in the nucleus was divided by intensity in the cytoplasm for all cells. The nuclear/cytosolic ratio was determined under each condition and compared with the untreated control.

**Unilateral ureteral obstruction (UUO)**

The UUO model was performed as described previously (7). Briefly, 6–8-week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) underwent either left-sided UUO or sham surgery. Mice were anesthetized, and a flank incision was made on the left side to identify the left kidney and ureter. Subsequently, two 4-0 silk suture knots were tied on the left ureter. One, 3 or 7 days post-surgery, the mice were sacrificed, and the left kidneys were harvested. Half of the kidney was snap-frozen in liquid nitrogen, and the samples were stored at −80 °C until processed. The Animal Care Committee of the St. Michael’s Hospital approved this study.

**Whole-kidney lysates**

Kidney samples were processed as described previously (7). Briefly, kidney samples from sham and UUO mice were cut and added to ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA) supplemented with 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and Complete Mini Protease Inhibitor (Roche Applied Science, Mississauga, Ontario, Canada). Samples were homogenized with a rotator-stator homogenizer and then centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatant was collected and used to determine protein concentration, and samples were analyzed using SDS-PAGE and Western blotting as above.

**Statistical analysis**

Data are presented as representative blots or images from at least three similar experiments or as means ± S.E. of the mean (S.E.) for the number of experiments indicated. Statistical significance was determined by two-tailed Student’s t test or ANOVA (Tukey or Dunn post hoc testing for parametric and nonparametric ANOVA, as appropriate) using Excel (Microsoft) or Prism software. p < 0.05 was accepted as significant. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

**Author contributions**—M. Z. M. and J. F. B. performed all the experiments on fibroblasts and epithelial cells, respectively, analyzed the data, prepared the corresponding figures, and contributed to the writing of the manuscript. P. S. generated constructs by mutagenesis, performed the ChIP assay, and provided experimental advice; Q. D. performed the in vivo experiments and data analysis; T. Y. helped with experiments for the revision; K. S. and S. F. P. contributed to the design of studies, to the critical interpretation of the data, and to the final assembly of the manuscript; and A. K. conceived the study and wrote the majority of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

**References**

1. Piccolo, S., Dupont, S., and Cordenonsi, M. (2014) The biology of YAP/TAZ: hippo signaling and beyond. *Physiol. Rev.* 94, 1287–1312

2. Hansen, C. G., Moroishi, T., and Guan, K. L. (2015) YAP and TAZ: a nexus for Hippo signaling and beyond. *Trends Cell Biol.* 25, 499–513

3. Low, B. C., Pan, C. Q., Shivashankar, G. V., Bershadsky, A., Sudol, M., and Sheetz, M. (2014) YAP/TAZ as mechanosensors and mechanotransducers in regulating organ size and tumor growth. *FEBS Lett.* 588, 2663–2670

4. Dupont, S. (2016) Role of YAP/TAZ in cell-matrix adhesion-mediated signalling and mechanotransduction. *Exp. Cell Res.* 343, 42–53

5. Zanconato, F., Cordenonsi, M., and Piccolo, S. (2016) YAP/TAZ at the roots of cancer. *Cancer Cell* 29, 783–803

6. Zhang, K., Qi, H. X., Hu, Z. M., Chang, Y. N., Shi, Z. M., Han, X. H., Han, Y. W., Zhang, R. X., Zhang, Z., Chen, T., and Hong, W. (2015) YAP and TAZ take center stage in cancer. *Biochemistry* 54, 6555–6566

7. Rozycki, M., Bialik, I. F., Speight, P., Dan, Q., Knudsen, T. E., Sztro, S. G., Yuen, D. A., Szárszi, K., Pedersen, S. F., and Kapus, A. (2016) Myocardium-related transcription factor regulates Nox4 protein expression: linking cytoskeletal organization to redox state. *J. Biol. Chem.* 291, 227–243

8. Sztro, S. G., Narimatsu, M., Lu, M., He, X., Sidiqi, A. M., Tolosa, M. F., Chan, L., De Freitas, K., Bialik, I. F., Majumder, S., Boo, S., Hinz, B., Dan, Q., Advani, A., John, R., et al. (2016) YAP and TAZ are mechanoregulators of TGFβ-Smad signaling in renal fibrogenesis. *J. Am. Soc. Nephrol.* 27, 3117–3128

9. Speight, P., Kofler, M., Szász, K., and Kapus, A. (2016) Context-dependent switch in chemo/mechanocontraduction via multilevel cross-talk among cytokine-regulated MRTF and TAZ and TGFβ-regulated Smad3. *Nat. Commun.* 7, 11642

10. Speight, P., Nakano, H., Kelley, T. J., Hinz, B., and Kapus, A. (2013) Differential topological susceptibility to TGFβ in intact and injured regions of the epithelium: key role in myofibroblast transition. *Mol. Biol. Cell.* 24, 3326–3336

11. Liu, F., Lagares, D., Choi, K. M., Stopfer, L., Marinkovic, A., Vrbanac, V., Probst, C. K., Hiemer, S. E., Sisson, T. H., Horowitz, J. C., Rosas, I. O., Stanley, S. A., Fredenburgh, L. E., Feghali-Bostwick, C., Varelas, X., Tager, A. M., and Tschumperlin, D. J. (2017) TAZ activation drives fibroblast activation and fibrosis. *Am. J. Physiol. Cell Physiol.* 32, L344–L357

12. Iorgenson, A. J., Choi, K. M., Sicard, D., Smith, K. M., Hieier, S. E., Varelas, X., and Tschumperlin, D. J. (2017) TAZ activation drives fibroblast spheroid growth, expression of profibrotic paracrine signals, and context dependent ECM gene expression. *Am. J. Physiol. Cell Physiol.* 312, C277–C285

13. Bertero, T., Cottrill, K. A., Annis, S., Bhat, B., Gochuico, B. R., Osorio, J. C., Rosas, I., Haley, K. J., Corey, K. E., Chung, R. T., Nelson Chau, B., and...
TGFβ induces p38-dependent, MRTF-mediated TAZ expression

Chan, S. Y. (2015) A YAP/TAZ-miR-130/301 molecular circuit exerts systems-level control of fibrosis in a network of human diseases and physiologic conditions. *Sci. Rep.* 5, 18277

Martin, K., Pritchett, J., Llewellyn, J., Mullan, A. F., Athwal, V. S., Dobie, R., Harvey, E., Zeef, L., Farrow, S., Streuli, C., Henderson, N. C., Friedman, S. L., Hanley, N. A., and Piper Hanley, K. (2016) PAK proteins and YAP-1 signalling downstream of integrin β1 in myofibroblasts promote liver fibrosis. *Nat. Commun.* 7, 12502

Meng, Z., Moroishi, T., and Guan, K. L. (2016) Mechanisms of Hippo pathway regulation. *Genes Dev.* 30, 1–17

Zhang, H., Liu, C. Y., Zha, Z. Y., Zhao, B., Yao, J., Zhao, S., Xiong, Y., Lei, Q. Y., and Guan, K. L. (2009) TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition. *J. Biol. Chem.* 284, 13355–13362

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Yu, J., Lin, J. D., Wang, C. Y., Chinnaiyan, A. M., Lai, Z. C., and Guan, K. L. (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev.* 22, 1962–1971

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giullitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Biccini, S., Elvassore, N., and Piccolo, S. (2011) Role of YAP/TAZ in mechanotransduction. *Nature* 474, 179–183

Aragona, M., Panciara, T., Manfrin, A., Giullitti, S., Michelini, F., Elvassore, N., Dupont, S., and Piccolo, S. (2013) A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. *Cell* 154, 1047–1059

Manca-Capelli, S., Paramasivam, M., Dutta, S., and McCollum, D. (2014) Angiomotins link F-actin architecture to Hippo pathway signaling. *Mol. Biol. Cell* 25, 1676–1685

Balder, G., Dupont, S., and Piccolo, S. (2012) Transduction of mechanical and cytoskeletal cues by YAP and TAZ. *Nat. Rev. Mol. Cell Biol.* 13, 591–600

Chen, J., and Harris, R. C. (2016) Interaction of the EGF receptor and the Hippo pathway in the diabetic kidney. *J. Am. Soc. Nephrol.* 27, 1689–1700

Barry, E. R., and Camargo, F. D. (2013) The Hippo superhighway: signaling crossroads converging on the Hippo/Yap pathway in stem cells and development. *Curr. Opin. Cell Biol.* 25, 247–253

Mauviel, A., Nallet-Staub, F., and Varelas, X. (2012) Integrating develop-

signaling to control cell fate in human embryonic stem cells. *Cell Rep.* 5, 1611–1624

Zhao, L., Jiang, S., and Hanthash, B. M. (2010) Transforming growth factor β1 induces osteogenic differentiation of murine bone marrow stromal cells. *Tissue Eng. Part A* 16, 725–733

Olson, E. N., and Nordheim, A. (2010) Linking actin dynamics and gene transcription to drive cellular motile functions. *Nat. Rev. Mol. Cell Biol.* 11, 353–365

Miralles, F., Posern, G., Zaromytidou, A. L., and Treisman, R. (2003) Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* 113, 329–342

Duffield, J. S. (2014) Cellular and molecular mechanisms in kidney fibrosis. *J. Clin. Invest.* 124, 2299–2306

LeBlu, V. S., Tadjiri, G., O’Connell, J., Teng, Y., Cooke, V. G., Woda, C., Sugimoto, H., and Kalluri, R. (2013) Origin and function of myofibroblasts in kidney fibrosis. *Nat. Med.* 19, 1047–1053

Masszi, A., Fan, L., Rosivall, L., McCulloch, C. A., Rotstein, O. D., Mucsi, I., and Kapus, A. (2004) Integrity of cell-cell contacts is a critical regulator of TGF-β1-induced epithelial-to-mesenchymal transition: role for β-catenin. *Am. J. Pathol.* 165, 1955–1967

Fan, L., Sebe, A., Peretti, Z., Masszi, A., Thirone, A. C., Rotstein, O. D., Nakano, H., McCulloch, C. A., Szászki, K., Mucsi, I., and Kapus, A. (2007) Cell contact-dependent regulation of epithelial-mesenchymal transition via the rho- rho kinase-phospho-myosin pathway. *Mol. Biol. Cell* 18, 1083–1097

Masszi, A., Speight, P., Charbonney, E., Lodýga, M., Nakano, H., Szászki, K., and Kapus, A. (2010) Fate-determining mechanisms in epithelial-myo-

fibroblast transition: major inhibitory role for Smad3. *J. Cell Biol.* 188, 383–399

Brown, K. A., Pietenpol, J. A., and Moses, H. L. (2007) A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-β signaling. *Cell. Biochem.* 101, 9–33

Matsuzaki, K. (2013) Smad phospho-isoforms direct context-dependent TGF-β signaling. *Cytokine Growth Factor Rev.* 24, 385–399

Liu, C. Y., Zha, Z. Y., Zhou, X., Zhang, H., Huang, W., Zhao, D., Li, T., Chan, S. W., Lim, C. J., Hong, W., Zhao, X., Xiong, Y., Lei, Q. Y., and Guan, K. L. (2010) The Hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCL/β-TrCP E3 ligase. *J. Biol. Chem.* 285, 37159–37169

Huang, W., Xu, X., Liu, C., Zha, Z., Zhang, H., Jiang, Y., Xiong, Y., Lei, Q. Y., and Guan, K. L. (2012) The N-terminal phosphodegron targets TAZ/ WWP1 protein for SCFβ-TrCP-dependent degradation in response to phosphatidylinositol 3-kinase inhibition. *J. Biol. Chem.* 287, 26245–26253

Caraci, F., Gili, E., Calafaro, M., Failla, M., La Rosa, C., Crimi, N., Sortino, M. A., Nicoletti, F., Copani, A., and Vancheri, C. (2008) TGF-β1 targets the GSK-3β/β-catenin pathway via ERK activation in the transition of human lung fibroblasts into myofibroblasts. *Pharmacol. Res.* 57, 274–282

Dibble, C. C., and Cantley, L. C. (2015) Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol.* 25, 545–555

Evelyn, C. R., Wade, S. M., Wang, Q., Wu, M., Iñiguez-Lluhí, J. A., Merajver, S. D., and Neubig, R. R. (2007) CCG-1423: a small-molecule inhibitor of Rhoa transcriptional signaling. *Mol. Cancer Ther.* 6, 2249–2260

Hinson, J. S., Medlin, M. D., Lockman, K., Taylor, J. M., and Mack, C. P. (2007) Smooth muscle cell-specific transcription is regulated by nuclear localization of the myocardin-related transcription factors. *Am. J. Physiol. Heart Circ. Physiol.* 292, H1170–H1180

Lee, M., San Martin, A., Valdivia, A., Martín-Garrido, A., and Griendling, K. K. (2016) Redox-sensitive regulation of myocardin-related transcription factor (MRTF-A) phosphorylation via p38 in vascular smooth muscle cell differentiation marker gene expression. *Plos One* 11, e0153199

Panayiotou, N., Miralles, F., Pavlović, R., Diring, J., Flynn, H. R., Skehel, M., and Treisman, R. (2016) Phosphorylation acts positively and negatively to regulate MRTF-A subcellular localisation and activity. *Elife* 5, e15460

Ronkina, N., Laféra, J., Kotyaryov, A., and Gaestel, M. (2016) Stress-dependent phosphorylation of myocardin-related transcription factor (MRTF-A) by the p38(MAPK)/MK2 axis. *Sci. Rep.* 6, 31219

Moorey, R. J., Burnette, B. L., Brustkern, S. J., Daniels, J. S., Hirsch, J. L., Hood, W. F., Meyers, M. J., Minh, S. J., Pierce, B. S., Saabye, M. I., Schin-
TGFβ induces p38-dependent, MRTF-mediated TAZ expression

dler, J. F., South, S. A., Webb, E. G., Zhang, J., and Anderson, D. R. (2010) A benzo thiophene inhibitor of mitogen-activated protein kinase-activated protein kinase 2 inhibits tumor necrosis factor α production and has oral anti-inflammatory efficacy in acute and chronic models of inflammation. J. Pharmacol. Exp. Ther. 333, 797–807

55. Roglani, P., Calzetta, L., Cavalli, F., Matera, M. G., and Cazzola, M. (2016) Pirfenidone, nintedanib and N-acetylcysteine for the treatment of idiopathic pulmonary fibrosis: a systematic review and meta-analysis. Pulm. Pharmacol. Ther. 40, 95–103

56. Nanthakumar, C. B., Hatley, R. J., Lemma, S., Gauldie, J., Marshall, R. P., and Macdonald, S. J. (2015) Dissecting fibrosis: therapeutic insights from the small-molecule toolbox. Nat. Rev. Drug Discov. 14, 693–720

57. Cucoranu, D., Clempus, R., Dikalova, A., Phelan, P. J., Ariyan, S., Dikalov, S., and Sorescu, D. (2005) NAD(P)H oxidase 4 mediates transforming growth factor-β1-induced differentiation of cardiac fibroblasts into myofibroblasts. Circ. Res. 97, 900–907

58. Bondi, C. D., Manickam, N., Lee, D. Y., Block, K., Gorin, Y., Abboud, H. E., and Barnes, I. L. (2010) NAD(P)H oxidase mediates TGF-β1-induced activation of kidney myofibroblasts. J. Am. Soc. Nephrol. 21, 93–102

59. Martin-Garrido, A., Brown, D. I., Lye, A. N., Dikalova, A., Seidel-Rogol, B., Lassègue, B., San Martin, A., and Griendling, K. K. (2011) NADPH oxidase 4 mediates TGF-β-induced smooth muscle α-actin via p38MAPK and serum-response factor. Free Radiic. Biol. Med. 50, 354–362

60. Goetttsch, C., Goettsch, W., Muller, G., Seebach, I., Schnittler, H. J., and Morawietz, H. (2009) Nox4 overexpression activates reactive oxygen species and p38 MAPK in human endothelial cells. Biochem. Biophys. Res. Commun. 380, 355–360

61. Nosault, C., Stewart, A., Gualdrini, F., East, P., Horswell, S., Matthews, N., and Treisman, R. (2013) Smad2 and myocardin–SRF cofactor MAL.

62. Yu, O. M., Miyamoto, S., and Brown, J. H. (2016) Myocardin-related transcription factor A and yes-associated protein exert dual control in G protein-coupled-receptor- and RhoA-mediated transcriptional regulation via NET1 in the transforming growth factor-β1/β2/β3 signaling and its role in cancer progression and metastasis. Cancer Metastasis Rev. 35, 553–568

63. Seb, A., Masszi, A., Zulyš, M., Yeung, T., Speight, P., Rotstein, O. D., Nakano, H., Musci, I., Szászi, K., and Kapus, A. (2008) Rac, p38 and regulate cell contact-dependent nuclear translocation of myocardin-related transcription factor. FEBS Lett. 582, 291–298

64. Abdalla, M., Goc, A., Segar, L., and Somanath, P. R. (2013) Akt1 mediates α-smooth muscle actin expression and myofibroblast differentiation via myocardin and serum-response factor. J. Biol. Chem. 288, 33483–33493

65. Yu, J., Kim, W. Y., Hur, J., Kim, H., Nam, S. A., Choi, A., Kim, Y. M., Park, S. H., Chung, C., Kim, J., Min, S., Myung, S. J., Lim, D. S., and Kim, Y. K. (2016) The Hippo-Salvador signaling pathway regulates renal tubulointerstitial fibrosis. Sci. Rep. 6, 31931

66. Zhang, T., Zhang, J., You, X., Liu, Q., Du, Y., Gao, Y., Shan, C., Kong, G., Wang, Y., Yang, X., Ye, L., and Zhang, X. (2012) Hepatitis B virus X protein regulates oncogene Yes-associated protein by CREB to promote growth of hepatoma cells. Hepatol. Mol. Biol. 26, 2051–2059

67. Wu, H., Xiao, Y., Zhang, S., Ji, S., Wei, L., Fan, F., Geng, I., Tian, J., Sun, X., Qin, F., Jin, C., Lin, J., Yin, Z. Y., Zhang, T., Luo, L., et al. (2013) The Erk transcription factor GABP is a component of the hippo pathway essential for growth and antioxidant defense. Cell Res. 3, 1663–1677

68. Xiang, L., Gilkes, D. M., Hu, H., Takano, N., Luo, W., Lu, H., Bullen, J. W., Samanta, D., Liang, H., and Semenza, G. L. (2014) HIF-1α hypoxia-inducible factor 1 mediates TAZ expression and nuclear localization to induce the breast cancer stem cell phenotype. Oncotarget 5, 12509–12527

69. Cho, H. H., Shin, K. K., Kim, J. Y., Song, J. S., Kim, J. M., Bae, C. Y., Kim, C. D., and Jung, I. S. (2010) NF-κB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression. J. Cell. Physiol. 223, 168–177

70. Bian, H., Lin, J. Z., Li, C., and Farmer, S. R. (2016) Myocardin-related transcription factor A (MRTF-A) regulates the fate of bone marrow mesenchymal stem cells and its absence in mice leads to osteopenia. Mol. Metab. 5, 970–979

71. Davis-Dusenberg, B. N., Chan, M. C., Reno, K. E., Weisman, A. S., Layne, M. D., Lagna, G., and Hata, A. (2011) Down-regulation of Kruppel-like factor-4 (KLF4) by microRNA-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-β and bone morphogenetic protein 4. J. Biol. Chem. 286, 28097–28110

72. Onwuegbusi, B. A., Rees, J. R., Lao-Sirieix, P., and Fitzgerald, R. C. (2007) Selective loss of TGFβ Smad-dependent signalling prevents cell cycle arrest and promotes invasion in oesophageal adenocarcinoma cell lines. PloS one 2, e177

73. Masszi, A., and Kapus, A. (2011) Smadding complexity: the role of Smad3 in epithelial-myoﬁbroblast transition. Cells Tissues Organs 193, 41–52

74. Drabscy, B., and ten Dijke, P. (2012) TGF-β signaling and its role in cancer progression and metastasis. Cancer Metastasis Rev. 31, 553–568

75. Charbonney, E., Speight, P., Masszi, A., Nakano, H., and Kapus, A. (2011) β-Catenin and Smad3 regulate the activity and stability of myocardin-related transcription factor during epithelial-myoﬁbroblast transition. Mol. Biol. Cell 22, 4472–4485