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Gone caving: roles of the transcriptional regulators YAP and TAZ in skeletal development

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Abstract

Purpose of Review: The development of the skeleton is controlled by cellular decisions determined by the coordinated activation of multiple transcription factors. Recent evidence suggests that the transcriptional regulator proteins, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), could have important roles in directing the activity of these transcriptional programs. However, *in vitro* evidence for the roles of YAP and TAZ in skeletal cells has been hopelessly contradictory. The goal of this review is to provide a cross-sectional view on the state of the field and to synthesize the available data toward a unified perspective.

Recent Findings: YAP and TAZ are regulated by diverse upstream signals and interact downstream with multiple transcription factors involved in skeletal development, positioning YAP and TAZ as important signal integration nodes in an hourglass-shaped signaling pathway. Here, we provide a survey of putative transcriptional co-effectors for YAP and TAZ in skeletal cells. Synthesizing the *in vitro* data, we conclude that TAZ is consistently pro-osteogenic in function, while YAP can exhibit either pro- or anti-osteogenic activity depending on cell type and context. Synthesizing the *in vivo* data, we conclude that YAP and TAZ combinatorially promote developmental bone formation, bone matrix homeostasis, and endochondral fracture repair by regulating a variety of transcriptional programs depending on developmental stage.

Summary: Here, we discuss the current understanding of the roles of the transcriptional regulators, YAP and TAZ in skeletal development, and provide recommendations for continued study of molecular mechanisms, mechanotransduction, and therapeutic implications for skeletal disease.

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Introduction:

Édouard-Alfred Martel (1859–1938) is perhaps the most famous cave explorer of all time. He was obsessed with cartography, specifically underground cartography, i.e., the mapping of caves (Hunt 2018). Martel's innovation was to divide a cave into distinct cross-sections, or "coupés" (Figure 1).

In 1889, Martel became the first explorer to reach the bottom of the Gouffre de Padirac, a 100m-deep chasm in southwest France. Of the experience, he wrote: "The unknown draws us irresistibly forward. No man has gone before us in these depths, no one knows where we go nor what we see, nothing so strangely beautiful was ever presented to us, and spontaneously we ask each other the same question: are we not dreaming?" (Chevalier 1951).

In many ways, being a scientist is like being a 19th c. cave explorer: you walk into the dark with a backpack full of candles and begin to illuminate one cavern after another. But you also spend the vast majority of your time stumbling in the dark, stubbing your toes, making wrong turns. Once a fissure is illuminated, you don't set up camp in the light, but rather continue straight for the darkest corner with a cold draught and leave the light behind.

In this review article, we seek to provide a *coupé* of the cave system that represents the developmental biology of the skeleton, with specific focus on the transcriptional regulators, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). This is a story of numerous discoveries and some wrong turns and dead ends, but ultimately of new depths to be plumbed and caverns to be explored.

Development of the skeleton

The development of the skeletal elements occurs through two types of bone formation: intramembranous or endochondral ossification. Intramembranous ossification occurs by direct differentiation of mesenchymal cells into bone-depositing osteoblasts (Karsenty 2008), and is the dominant mechanism of bone formation in the craniofacial bones and the clavicles. The embryonic bone collar that forms the early cortical bone, in the long bones also occurs through intramembranous ossification of perichondrium-resident mesenchymal progenitors. In contrast, endochondral ossification (i.e., "through cartilage") occurs first through differentiation of condensed mesenchymal cells into chondrocytes, which form a cartilage *anlage*, or template, that will be remodeled and replaced by bone. The transition from cartilage to bone initiates with growth plate chondrocyte hypertrophy. The fate of these hypertrophic chondrocytes has been long debated (Shapiro et al. 2005). Evidence since the 1970s has pointed to the possibility of hypertrophic chondrocytes to transform into bone cells (Kahn and Simmons 1977; Roach 1992). Recent reports using inducible Cre-based lineage-tracing have shown direct chondrocyte transformation into bone cells during development (L. Yang et al. 2014; G. Yang et al. 2014; Zhou et al. 2014; Ono et al. 2014; Jing et al. 2015) and fracture repair (C. Bahney et al. 2014; C. S. Bahney et al. 2014; Hu et al. 2017). The process of bone development is therefore an exquisitely coordinated sequence of cellular decisions: proliferation, (trans-)differentiation, mobilization, etc.

Each of these cellular decisions is controlled by the coordinated activation of specific transcriptional programs. Thus, transcription factors are the master regulators of cell identity and decision making (Takahashi et al. 2007). The variety of transcription factors involved in skeletal development have been reviewed in detail elsewhere (Karsenty 2008), but new insights continue to emerge. A mechanistic understanding of how transcription factors regulate skeletal development is critical to understand the biology of the musculoskeletal system and to enable the development of therapeutic interventions for musculoskeletal disease. Careful work over the past decades has enhanced our understanding of the mechanisms by which transcription factors control skeletogenesis, yet many questions remain.

A key question is: how is the symphony of these transcription factors conducted during bone development? It is clear that different cells express different transcription factors to different degrees, but beyond expression, the regulation of transcriptional activity is critical and poorly understood. For example, chromatin organization and epigenetic mechanisms are important mediators of transcription factor activity in bone development, reviewed in (Wijnen and Westendorf 2019). However, transcription factors can also be regulated by transcriptional co-activators/co-repressors, which are proteins that bind to and form complexes with transcription factors and directly regulate their activity.

Yes-associated protein (YAP, also known as YAP1 and YAP65) and transcriptional co-activator with PDZ-binding motif (TAZ, also known as WWTR1) are transcriptional regulators whose primary function is to bind to other proteins, including many transcription factors (Sudol 1994). Orthologs of the Drosophila protein, Yorkie, YAP and TAZ share ~42% homology at the amino acid level (Kaan et al. 2017). Thus, YAP and TAZ can exhibit either convergent or divergent function, depending on context. For example, mice harboring a global deletion of YAP die early in embryogenesis (Morin-Kensicki et al. 2006), while TAZ knockout mice live to maturity, despite phenotypic deformities in various organ systems (Hossain et al. 2007). Both YAP and TAZ lack DNA-binding domains and cannot induce gene expression by themselves but require binding to co-effector transcription factors to drive or repress gene expression. The TEAD family of transcription factors (TEAD1–4) are the canonical transcriptional partners for YAP and TAZ. Complementarily, the TEAD proteins possess DNA-binding domains, but lack transcription activation domains, providing specificity for YAP/TAZ-TEAD (Vassilev et al. 2001).

YAP and TAZ also co-regulate many transcription factors that are involved in bone development, homeostasis, and repair. Here we have collected a list of transcription factors known to be involved in bone development or the function of skeletal cells and tabulated their known or putative regulation by YAP and/or TAZ (Table 1). The table is broken into two categories: I) transcription factors for which co-regulation by YAP/TAZ has been demonstrated in skeletal cells, II) transcription factors with known roles in skeletal cells for which YAP/TAZ binding has been studied in non-skeletal cell types.

The YAP/TAZ hourglass.

Multiple upstream cues converge on YAP/TAZ activation to regulate downstream outcomes. Therefore, the cellular role of YAP and TAZ is highly context-dependent and determined by

the integrated upstream cues and the available transcriptional co-effectors. This mode of regulation positions YAP/TAZ at the bottleneck of a biological signaling “hourglass,” where collective upstream inputs can generate different downstream outcomes via YAP/TAZ activation and transcription factor binding (Figure 2).

YAP/TAZ activity is controlled by their subcellular location: either inside or outside the nucleus. To function as transcriptional regulators, YAP and TAZ must bind transcription factors in the nucleus. Cytosolic retention therefore de-activates YAP/TAZ-driven transcriptional activity. YAP/TAZ subcellular localization is controlled by three primary, interconnected mechanisms: the Hippo pathway, mechanical cues, and biochemical cues. Collectively, these three upstream drivers of YAP/TAZ subcellular localization and the downstream transcriptional co-effectors regulate the function of YAP and TAZ within a cell.

Regulation of YAP/TAZ activity by the Hippo Pathway: YAP and TAZ are the terminal effectors of the Hippo pathway (Huang J et al 2005) (Huang et al. 2005), a kinase cascade that inhibits YAP and TAZ by sequential Serine phosphorylation. The majority of the core components of the Hippo pathway are conserved across species and can be categorized into core components, upstream regulators and downstream effectors (B. Zhao, Li, and Guan 2010). Briefly, the Hippo pathway initiates with the Sterile 20 (Ste20) family protein kinases MST1 and MST2, which become activated upon binding to and phosphorylation of Salvador. MST1/2 then phosphorylate LATS1 and/or LATS2, which are a part of the nuclear Dbf2-related (NDR) family of protein kinases. LATS kinases are also activated when phosphorylated by MPS One Binder Kinase activator-like 1A and 1B (MOB1A and MOB1B) proteins. LATS kinases phosphorylate YAP and TAZ and inhibit their activity as transcriptional coactivators. Phosphorylation of YAP by LATS 1/2 at Ser127 (or TAZ at S87) initiates a progressive phosphorylation of multiple Serine residues and facilitates binding to 14–3-3 proteins to sequester YAP/TAZ in the cytoplasm (B. Zhao, Li, and Guan 2010) and initiate ubiquitin-mediated proteasomal degradation by the E3 ubiquitin ligase β-TRCP. YAP can also be phosphorylated by other proteins that are not a part of the core Hippo pathway, which can lead to its degradation or stabilization depending on which residue has been phosphorylated (B. Zhao, Li, and Guan 2010). Non-phosphorylated YAP/TAZ translocate to the nucleus and form a complex with their co-effector transcription factors, bind to gene promoters or enhancers, and activate or repress the expression of target genes.

Regulation of YAP/TAZ by Growth Factors—YAP/TAZ activity is also orchestrated by growth factor signaling in both Hippo-dependent and -independent manners. For example, lysophosphatidic acid (LPA) and sphingosine 1-phosphosphate act through G12/13PCRs to inhibit LATS1/2 and promote nuclear translocation of YAP (Moya and Halder 2019). Independent of the Hippo pathway, YAP/TAZ activity can be regulated by alternative Wnt signaling via Frizzled (FZD) receptors (Park et al. 2015). Likewise, platelet-derived growth factor (PDGF) signaling can directly signal to YAP via Src Family kinases (SFK) that catalyze activating tyrosine phosphorylation of YAP (Smoot et al. 2018). Interaction of YAP/TAZ with growth factors is contextual, and different growth factors have been found to regulate YAP/TAZ/Hippo activity differentially depending on cell type and experimental or

physiologic context. Discretion is recommended when applying prior findings to a new cell type or context.

Mechanoregulation of YAP and TAZ—YAP and TAZ are also activated by mechanical cues. In 2006, Engler et al. demonstrated that mechanical properties of the extracellular matrix influence progenitor cell fate (Engler et al. 2006). Five years later, two independent groups linked these matrix stiffness-dependent cell lineage decisions to the mechanosensitive nuclear localization of YAP and TAZ (Dupont et al. 2011)(Wada et al. 2011). Both papers showed that YAP and TAZ translocate to the nucleus in response to matrix rigidity, but are sequestered in the cytosol in soft ECM environments (Dupont et al. 2011)(Wada et al. 2011). The Dupont et al. paper showed that this mechanoactivation of YAP/TAZ was necessary for the matrix stiffness-dependent switch between adipogenic and osteogenic differentiation of bone marrow stromal cells (Dupont et al. 2011). In this section, we will briefly summarize the current state of knowledge on the mechanisms by which YAP and TAZ are controlled by mechanical cues, including mechanosensation at the plasma membrane that leads to cytoskeletal remodeling and cytosolic signal transduction to effect YAP/TAZ nuclear localization (Figure 3).

Mechanosensation: YAP/TAZ-activating mechanical cues first reach the cell at the plasma membrane through cell-matrix, cell-cell, and cell-environment interactions. Forces between cells and their extracellular matrix are transduced in part by integrins, which are transmembrane adhesion molecules that couple the ECM to the cytoskeleton at focal adhesions (Sun, Guo, and Fässler 2016). Force production by the actomyosin cytoskeleton via talin-bound integrins (Sun, Guo, and Fässler 2016) induces conformational changes in nascent integrin engagements, promoting integrin clustering and increasing affinity for intracellular ligand binding (Sun, Guo, and Fässler 2016), (Horton et al. 2016). Recruitment of focal adhesion-stabilizing proteins, including vinculin, and paxillin (Martino et al. 2018) in turn initiate intracellular signaling cascades including Rho/ROCK and FAK/Src to promote further actomyosin contractility and subsequent YAP/TAZ nuclear localization. Forces between cells are transduced in part by cadherins, a family of transmembrane adhesion receptors that form adherens junctions, coupling the actomyosin cytoskeleton and transcription factor activation to intercellular mechanical force transduction (Cosgrove et al. 2016). Other forces exerted on cells by their environment, such as fluid shear stress, can be transduced in part by mechanosensitive ion channels, such as the Piezo channels. These mechanically gated, stretch-activated channels modulate the influx of ions, such as Ca^{2+}, into the cytosol (Pathak et al. 2014). Pathak and colleagues first demonstrated that YAP is activated downstream of Piezo activation in neural stem cells (Pathak et al. 2014), and recent data by Ellefson et al. show that myosin II activation produces membrane tension at focal adhesions to activate local Piezo1 channels, providing a direct mechanosensory link between actomyosin tension, Piezo-mediated Ca^{2+} flux, and YAP/TAZ activation (Ellefsen et al. 2019).

Mechanotransduction: Inside the cell, these mechanical cues are transduced by both physical and biochemical means to control the phosphorylation status and localization of

YAP and TAZ (Dupont 2016). Here, we will briefly discuss the actomyosin cytoskeleton and the prototypical mechano-activated Rho/ROCK and Src signaling pathways.

The actin-myosin cytoskeleton provides support, structure, and protection, and is the primary effector of motion in the cell. The actin cytoskeleton assembles from monomeric globular (G)-actin, which polymerizes to form filamentous (F)-actin (Aragona et al. 2013; Driscoll et al. 2015; Dupont et al. 2011). F-actin-bound non-muscle myosin II generates tensile forces in the actin cytoskeleton to regulate a variety of cell processes including polarity, cytokinesis, differentiation, and motility. In 2011, Sansores-Garcia and colleagues observed that altering F-actin dynamics in both drosphila and mamallian cells influenced Yorkie/YAP nuclear localization, such that increased F-actin organization promoted Yorkie/YAP activation (Sansores-Garcia et al. 2011). Actin dynamics are regulated in part by the Rho/ROCK pathway. Forces generated at the ECM activate the Rho GTPases, to activate ROCK, which phosphorylates and inactivates myosin light chain (MLC) phosphatase, promoting MLC activation (Maekawa et al. 1999; Aragona et al. 2013). Myosin activation induces tension generation, stress fiber formation, and recruitment of stabilizing proteins to the connections between the cell and the extracellular matrix at focal adhesions (Oakes et al. 2012). Remarkably, increased cytoskeletal tension causes YAP/TAZ nuclear localization (Aragona et al. 2013; Dupont et al. 2011).

The Src pathway is both a direct and indirect regulator of YAP/TAZ phosphorylation and activation. Src acts upstream of the Hippo pathway kinase, merlin, to promote LATS1/2-mediated YAP/TAZ phosphorylation and inactivation (Sabra et al. 2017), but can also directly phosphorylate YAP1 on three separate tyrosine residues (Y341/357/394) in its transcription activation domain, independent of the Hippo pathway (P. Li et al. 2016) (Elbediwy et al. 2018). In addition to responding to cytoskeletal tension, YAP/TAZ can also regulate the cytoskeleton in a feedback loop. Our recent data implicate YAP and TAZ in transcriptional feedback regulation of the cytoskeleton (Mason et al. 2019).

The mechanisms that control the nuclear shuttling of YAP/TAZ continue to emerge, but YAP/TAZ localization appears to be an equilibrium process that occurs without physical binding to a fixed component in either the nucleus or the cytoplasm (Ege et al. 2018). In addition to phosphorylation status, evidence suggests that physical deformation of the nucleus through the LINC complex is required for YAP/TAZ translocation (Driscoll et al. 2015), and recent data suggest that physical stretching of the nuclear membrane is necessary to open the nuclear pore complex to allow YAP translocation (Elosegui-Artola et al. 2017). However, protein shuttling through the nuclear pore complex may also be guided by importins and exportins which act through nuclear localization and nuclear export sequences (NLS; NES) (S. Wang et al. 2016; Ege et al. 2018). It was long thought that YAP/TAZ lacked defined NLS, but recent studies by Kofler, et al. identify an NLS in the transcription activation domain of TAZ, overlapping with the LATS phosphorylation site and a NES in the TEAD-binding domain (Kofler et al. 2018). Taken together, these observations establish YAP and TAZ as important transcriptional regulators that respond dynamically to mechanical cues and form a mechanistic link between physical stimuli and cell behavior.

Different types of mechanical stimuli in bone: Importantly, bone cells experience a variety of mechanical stimuli through development, disease, and repair. In the embryo, fetal movement and muscle forces are critical for the proper development of the skeleton (Hogg and Hosseini 1992). In adulthood, bone-lining osteoblasts are exposed to matrix strain (Martin et al. 2015) and bone marrow shear stress (Curtis et al. 2018). Within the osteocyte lacunar-canalicular system, osteocytes likewise respond to mechanical forces, predominantly as a consequence of fluid flow through the lacunar/canalicular system, as reviewed elsewhere (Weinbaum 2009), (Jenneke Klein-Nulend et al. 2013), (Schaffler et al. 2014). Whether YAP/TAZ mechanosignaling is necessary for bone physiology in vivo has not yet been studied.

Current understanding of the roles of YAP and TAZ in bone

Insights from *in vitro* studies—Although identified as critical regulators of mesenchymal progenitor cell differentiation, the evidence for positive vs. negative roles for YAP and/or TAZ during osteogenic differentiation *in vitro* is complicated. Studies have demonstrated both pro- and anti-osteogenic functions of both YAP and TAZ, depending on the context. Differences in experimental and cellular context may partially explain the conflicting evidence, but further study is necessary. Here, we discuss the existing evidence for both YAP and/or TAZ in both promoting and inhibiting *in vitro* osteogenic differentiation in model and primary skeletal cells.

A majority of the evidence for YAP and/or TAZ in inhibiting *in vitro* osteogenic differentiation is focused on YAP. YAP was first reported to suppress osteoblastic differentiation through sequestration and transcriptional repression of RUNX2 in ROS17/2.8 rat osteosarcoma cells (Zaidi et al. 2004). Sen and colleagues found that, in mouse bone marrow stromal cells (BM-MSCs), nuclear YAP inhibited RUNX2-mediated initiation of osteogenic differentiation while YAP nuclear export enhanced osteogenic differentiation (Sen et al. 2015). More recently, activator protein 2a (AP2a) was shown to recruit YAP and release the inhibition of RUNX2 by forming a YAP-AP2a protein complex, resulting in elevated osteogenic differentiation (Lin et al. 2019). Similarly, Basu-Roy and colleagues observed that SOX2 antagonized YAP expression to reduce osteogenic differentiation and maintain stemness in mOS-482 mouse osteosarcoma cells while YAP overexpression in primary mouse osteoblasts inhibited alkaline phosphatase activity and osteogenic differentiation (Basu-Roy et al. 2015). Seo and colleagues identified YAP as a target of SOX2 that antagonized activation of WNT/β-catenin target genes to inhibit osteogenic differentiation in both a model stem cell line (C3H10T1/2) and primary bone marrow stromal cells (Seo et al. 2013). With respect to TAZ, Park and colleagues implicated both YAP and/or TAZ as mediators of alternative WNT signaling via antagonizing WNT/β-catenin signaling, and found that either YAP or TAZ overexpression inhibited WNT/β-catenin signaling and osteogenesis (Park et al. 2015).

In contrast, YAP has also been found to promote osteogenic differentiation *in vitro*. YAP overexpression enhanced, while YAP depletion inhibited, osteogenic differentiation in MC3T3-E1 cells (B. Yang et al. 2019). In BM-MSCs, over-expression of a constitutively-active YAP mutant (YAP5SA) promoted osteogenic differentiation even under conditions

more favorable for adipogenesis (Dupont et al. 2011). Further, enhanced YAP activation by cytoskeletal contractility in differentiating BM-MSCs promoted the osteogenic capacity both in the context of topographical cues (X. Liu et al. 2019) and mechanical stimulation (Xue et al. 2017). Both pharmacological treatment and RNAi-depletion of YAP inhibited topographyinduced osteogenic differentiation in BM-MSCs (H. Pan et al. 2017). In addition to topographical cues, reductions in extracellular pH inhibited osteogenic differentiation by suppressing YAP in BM-MSCs (Tao et al. 2016). Finally, olfactomedin-like protein (OLFML1) negatively regulated mineralization in primary calvarial osteoblasts by inhibiting YAP nuclear translocation, consistent with a role for YAP promoting osteogenic differentiation *in vitro* (Murakami et al. 2018).

In contrast to YAP, evidence for TAZ is largely consistent and indicates a role for TAZ in promoting *in vitro* osteogenic differentiation. TAZ was first identified as a RUNX2 co-activator and inhibitor of the adipogenic nuclear receptor, PPARγ, in C2C12 cells (Hong et al. 2005; Hong and Yaffe 2006). More recent evidence in both C2C12 and C3H10T1/2 cells further found that TAZ promoted osteogenic differentiation through both RUNX2- (J. Feng et al. 2015; Mi Ran Byun, Sung, et al. 2014) and β-catenin- (M R Byun et al. 2014) dependent transcription. Similar work by Byun and colleagues observed that TAZ activation downstream of FGF2 and ERK mediated RUNX2-related osteogenic gene expression (Mi Ran Byun, Kim, et al. 2014). Similar to YAP, both topographical cues and mechanical stimulation affected TAZ-dependent *in vitro* osteogenic differentiation in BM-MSCs. For example, both nano-topographical surfaces (Qian et al. 2017; Hwang et al. 2017) and extracellular matrix stiffness (Hwang et al. 2015) promoted osteogenic differentiation through nuclear TAZ activation. Furthermore, simulated microgravity depolymerized F-actin and reduced TAZ nuclear translocation, which hindered osteogenic differentiation in BM-MSCs (Chen et al. 2016). Conversely, fluid shear stress stimulated TAZ nuclear localization and increased osteogenic differentiation (Kim et al. 2014). Lastly, pharmacological activation of TAZ enhanced osteogenic differentiation in adipose-derived stem cells (Zhu et al. 2018) while BM-MSCS from mice with heterozygous global deletion of TAZ exhibited defective *in vitro* osteogenic differentiation (Xiao et al. 2018).

In addition to their individual roles, a few studies have modulated both YAP and TAZ during *in vitro* osteogenic differentiation. For example, Park and colleagues found that RNAi-mediated depletion of YAP/TAZ in BM-MSCs reduced alkaline phosphatase activity and mineral deposition (Park et al. 2015). Similarly, dual RNAi-depletion of YAP and TAZ in BM-MSCs inhibited alkaline phosphatase activity under conditions favorable for osteogenesis (Dupont et al. 2011). Finally, heterozygous deletion of both YAP and TAZ in BM-MSCs inhibited osteogenic differentiation with reduced mineral deposition and downstream osteogenic gene expression (Tang et al. 2016).

Synthesizing these studies, we postulate that TAZ primarily promotes osteogenic differentiation *in vitro*, while YAP can either promote or inhibit osteogenesis, depending on the cellular and experimental context. Despite the emerging important roles of YAP and TAZ during osteogenic differentiation *in vitro*, continued careful and thorough interpretation of experiments modulating either YAP and/or TAZ is warranted. For example, the limitations of overexpression approaches that non-physiologically express otherwise tightly-regulated

transcriptional co-effectors should be taken into consideration. Further, dissecting the individual roles of YAP versus TAZ during osteogenic differentiation is necessary as current evidence suggests the potential for both divergent and convergent functions of YAP versus TAZ. Lastly, we caution against the use of YAP and/or TAZ expression or subcellular localization as markers or indicators of osteogenic differentiation, as these are insufficient to determine lineage commitment.

We further recommend that, while powerful and important for dissecting molecular mechanisms, *in vitro* studies must be supported and validated by *in vivo* approaches that enable the study of YAP/TAZ function in a physiologic context.

Insights from *in vivo* studies—A definitive understanding of YAP/TAZ function in bone will necessarily come from *in vivo* approaches. Because YAP and TAZ cannot bind DNA directly, and are capable of co-regulating multiple transcription factors, the transcriptional consequences of YAP and/or TAZ manipulation will depend on the transcription factor milieu present in a given cell. Thus, YAP and TAZ may have fundamentally distinct roles in one cell type compared to another. Here, we review the current literature on the *in vivo* roles of YAP and TAZ in mesenchymal progenitors, chondrocytes, osteoblasts, osteocytes, and osteoclasts. Other extra-skeletal cell types also contribute to the developmental niche and may likewise depend on YAP/TAZ signaling, but these are beyond the scope of this review.

Limb mesenchyme progenitors.: YAP/TAZ have important, but potentially divergent roles in the mesenchymal progenitors of the embryonic limb bud. Prx1-Cre targets these mesenchymal progenitors, and homozygous conditional ablation of both YAP and TAZ in Prx1-Cre mice produced embryonic lethality. However, mice with haploinsufficiency of YAP and homozygous TAZ deletion survived with increased postnatal bone mass (Xiong, Almeida, and O'Brien 2018). In contrast, Prx1-Cre deletion of YAP resulted reduced bone mass (Deng et al. 2016). Dermo1-Cre also targets limb mesenchymal progenitors, and Dermo1-conditional deletion of the Hippo kinases, MST1 and MST2, resulted in a mild developmental phenotype, but caused a significant defect in callus formation during fracture repair (Deng et al. 2016). Continued research will be necessary to dissect the mechanistic and combinatorial roles of YAP and TAZ in early skeletal development.

Chondroprogenitors: YAP and TAZ negatively regulate chondrogenesis *in vivo*. YAP-overexpression in Col2-expressing cells produced mice with a smaller skeleton and decreased bone volume due to delayed chondrocyte hypertrophy and reduced chondrocyte maturation (Deng et al. 2016). Conversely, Col2-conditional YAP deletion caused elongated growth plates and increased bone volume (Deng et al. 2016). Through complementary *in vitro* assays, Deng et. al. found that YAP positively regulated early chondrocyte proliferation by TEAD-dependent Sox6 expression and negatively regulated chondrocyte maturation via Runx2-dependent Col10a1 expression. Notably, YAP overexpression in Col2-expressing cells had more severe effects on fracture repair than development (Deng et al. 2016). Complementarily, conditional deletion of Mob1a/b in Col2-expressing cells resulted in chondrodysplasia from impaired chondrocyte maturation (Goto et al. 2018). Mob1a/b is a core component of the Hippo pathway whose deletion results in hyper-activation of YAP/

TAZ. However, Mob1a/b deletion-induced YAP/TAZ hyper-activity reduced early chondrocyte proliferation through transcriptional repression of Sox9 (Goto et al. 2018). Notably, this phenotype was largely rescued by the additional deletion of either YAP or TAZ. These data suggest that YAP and TAZ may be mutually compensatory in chondrogenesis, but explicit *in vivo* combinatorial gain- and loss-of-function experiments in chondrocytes will be required.

Osteoprogenitors: Though initially contradictory, the emerging evidence converges on positive roles for both YAP and TAZ in promoting osteoblast-lineage progression. YAP and TAZ most prominently immunolocalize in hypertrophic chondrocytes, osteoprogenitors, and osteoblasts during developmental bone formation (Kegelman et al. 2018). These expression patterns coincide with the localization of the transcription factor, Osterix/Sp7, which is critical to osteoblastogenesis (Rodda and McMahon 2006), motivating the use of Osterix-Cre for conditional YAP/TAZ deletion (Kegelman et al. 2018). We found that constitutive homozygous deletion of both YAP and TAZ from Osterix-expressing cells caused perinatal lethality due to asphyxiation, secondary to rib cage malformation (Kegelman et al. 2018). Importantly, mice with a single allele of YAP or a single allele of TAZ in Osterix-expressing cells survived, indicating mutual, but partial, compensation. Mice expressing only a single allele of either gene exhibited severe skeletal defects including spontaneous neonatal fractures, defects in collagen content and organization, and altered osteoblast/osteoclast-mediated bone remodeling (Kegelman et al. 2018). These data implicate both YAP and TAZ in functional bone development. Interestingly, post-natal deletion of both YAP and TAZ (doxycycline induced deletion at 3 weeks of age, and assayed at 12 weeks of age) exhibited only a modest bone phenotype, with increased osteoblast numbers and mineralizing surface percentage (Xiong, Almeida, and O'Brien 2018). Recent data provide insight into these discrepant phenotypes. Using post-natal fracture healing as a model to study YAP/TAZ roles in endochondral ossification, we found that constitutive deletion of YAP and/or TAZ from Osterix-expressing cells caused defects in both cartilage callus formation and callus mineralization due to a developmental defect in periosteal progenitor cell supply (Kegelman et al. 2020). However, inducible deletion after skeletal maturity impaired periosteal osteoprogenitor amplification and subsequent osteogenesis (Kegelman et al. 2020). Mechanical loading also promotes YAP/TAZ activation and endochondral bone regeneration through development-mimetic mechanisms (McDermott et al. 2019). Together, these data suggest that YAP/TAZ signaling in osterix-expressing cells has particularly important roles in bone development and in processes that partially reactivate developmental programs, such as fracture repair. Orthogonally, Li and colleagues observed that Osterix-conditional genetic deletion of MST1/2, the upstream Hippo kinases, inhibited bone accrual, formation and remodeling while stabilizing the key glucose transporter, Glut1, independent of YAP/TAZ regulation (W. Li et al. 2018).

Osteoblasts: Evidence for the roles of YAP and TAZ in mature osteoblasts is largely convergent. During osteoblastogenesis, immature, yet committed osteoblasts begin to express collagen I. Two established Collagen 1-dependent Cre drivers, Col1(3.6kb) (immature osteoblasts) and Col1(2.3kb) (committed osteoblasts) exist (Kalajzic et al. 2002; F. Liu et al. 2004), but neither has yet been used to assess osteoblast lineage-conditional

loss-of-function of YAP and/or TAZ. However, Col-1(2.3kb)-conditional over-expression of TAZ promoted bone formation, suggesting a similar role for TAZ in promoting osteoblasts as in osteoprogenitors (J.-Y. Yang et al. 2013). Although not specific to skeletal lineage cells, *in vivo* lentiviral delivery of TAZ alleviated osteoporotic symptoms in ovariectomized rats, further supporting a role for TAZ in promoting bone formation *in vivo* (Y. Zhang et al. 2016). As committed osteoblasts mature, osteocalcin expression increases, enabling Cre-mediated targeting in mature osteoblasts (M. Zhang et al. 2002). YAP deletion from Osteocalcin-expressing cells significantly reduced bone formation, impairing osteoblast proliferation induced by YAP co-activation of β-catenin (J.-X. Pan et al. 2018), supporting a role for YAP in promoting osteogenesis in vivo. In contrast, dual deletion of the upstream regulator MST1/2 from Osteocalcin-expressing cells inhibited bone accrual and formation consistent with a negative role for YAP in bone formation (W. Li et al. 2018). Nonetheless, deletion of both downstream YAP/TAZ target genes, CTGF and CYR61, from Osteocalcin expressing-cells resulted in reduced bone mass phenotypes (G. Zhao et al. 2018; Canalis et al. 2010), consistent with the evidence of osteoblast-specific genetic manipulations of YAP and TAZ.

Osteoclasts: Although the roles of YAP and TAZ in osteoclasts using cell-specific loss-of-function approaches has not been investigated directly, the Hippo pathway intersects with multiple signaling pathways that regulate osteoclastogenesis and osteoclast function (W. Yang et al. 2018). YAP/TAZ signaling in osteoblasts and osteocytes regulates the crosstalk to osteoclasts. For example, both deletion of YAP and/or TAZ in skeletal lineage cells and deletion of CYR61 from Osteocalcin-expressing cells increased osteoclast activity (Kegelman et al. 2018; G. Zhao et al. 2018). Similarly, DMP-1-conditional YAP/TAZ ablation promotes osteoclast activation (Xiong, Almeida, and O'Brien 2018; Kegelman et al. 2019), likely via paracrine signaling (Kegelman et al. 2019). Consistently, dual deletion of MST1/2 from Osteocalcin expressing-cells inhibited osteoclast formation (W. Li et al. 2018). However, deletion of YAP from Osteocalcin expressing-cells did not significantly impact osteoclast remodeling, potentially due to the compensatory effects of TAZ and/or the Cre model used (J.-X. Pan et al. 2018). As the roles for YAP and TAZ in regulating both osteoblastogenesis and osteoclastogenesis are beginning to emerge, a complete mechanistic understanding of their role in osteoblast/osteoclast-mediated bone remodeling during skeletal development remains incomplete.

Osteocytes: Similar to their role in osteoblasts, the evidence for the roles of YAP and TAZ in late stage osteoblasts and osteocytes is consistent. Late stage osteoblasts and early stage osteocytes express dentin matrix protein (DMP1). Both 10kb and 8kb DMP1-Cre models have been used to target gene deletion from osteocytes, but the osteocyte specificity depends on the sensitivity of the floxed alleles (O'Brien et al. 2008; Xiong et al. 2015; Rhee et al. 2011; Bivi et al. 2012). Regardless, dual YAP/TAZ deletion using either the 10kb-DMP1-Cre or the 8kb-DMP1-Cre reduced bone formation *in vivo,* with decreased osteoblast numbers and increased osteoclast activity (Kegelman et al. 2019; Xiong, Almeida, and O'Brien 2018). We further showed that dual deletion of YAP and TAZ using 8kb-DMP1-Cre impaired perilacunar/canalicular remodeling by regulating the expression of perilacunar/canalicular matrix remodeling enzymes including Ctsk, MMP13, and MMP14 as well as

Collagen 1a1, resulting in skeletal fragility due to impaired collagen organization in the bone matrix (Kegelman et al. 2019). Therefore, YAP/TAZ in late stage osteoblasts and osteocytes promote bone function *in vivo*. Since osteocytes are known as the primary mechanosensory cell in bone and YAP/TAZ are critical for mechanotransduction, the role of YAP and/or TAZ in osteocyte-mediated bone adaptation in the context of skeletal loading is an exciting emerging area, but has not yet been studied *in vivo*.

Directions for future research

YAP/TAZ signaling in bone mechanotransduction—Mechanical loading is extremely important in bone development and maintenance. Mechanical loading mediates many different pathways implicated as mechanistic mediators in bone cells. The focus of this review is YAP/TAZ but other mechanisms have been reviewed in (Tian, Wang, and Bikle 2017; Thompson, Rubin, and Rubin 2012; Regard et al. 2012; J Klein-Nulend, Bacabac, and Bakker 2012). YAP/TAZ can be activated by multiple cues in skeletal cells (Papachroni et al. 2009), including ECM stiffness (Dupont et al. 2011), strain or stretch (Codelia, Sun, and Irvine 2014; Cui et al. 2015), and fluid shear stress (Kim et al. 2014). Most evidence indicates that YAP/TAZ activation by mechanical cues promotes osteogenic differentiation (Khetan et al. 2013; Panciera et al. 2017; Low et al. 2014). Further, fluid shear stress in osteocytes activates Piezo1 calcium channels which were shown to act upstream of YAP/TAZ signaling *in vitro* (X. Li et al. 2019) and that in osteoblast stimulation by fluid shear caused YAP/TAZ activation in an integrin-dependent manner (Kaneko et al. 2014). Similarly, Piezo1 knockout mice exhibit decreased bone mass and mechanical load adaptation, while *in vitro* data show that Piezo1 signaling in osteocyte-like cells exposed to fluid shear stress requires YAP/TAZ (X. Li et al. 2019). However, the roles of YAP and TAZ in the mechanical load adaptation of bone has not yet been studied.

Implications for metabolic and developmental bone diseases—YAP and TAZ are known to drive aberrant cellular function in many diseases including atherosclerosis (K.-C. Wang et al. 2016), cancer (Zanconato, Cordenonsi, and Piccolo 2016), fibrosis (F. Liu et al. 2015; Mannaerts et al. 2015), cardiac hypertrophy (Xin et al. 2013), and muscular dystrophy (Iyer et al. 2019; Bertrand et al. 2014), but beyond their neoplastic activity in osteosarcoma (Fullenkamp et al. 2016), the role of YAP and TAZ in bone disease is unknown. Due to the altered mechanical environment in disease pathogenesis, the mechanotransductive effects of YAP and TAZ are implicated in driving abnormal cellular function (Panciera et al. 2017). Further synthesis of the upstream signals and the downstream targets of YAP/TAZ signaling in bone is therapeutically important to understand the disease pathology and potential therapeutic interventions. Accordingly, the emerging roles of YAP and TAZ in skeletal lineage cells implicate YAP and TAZ in both developmental and metabolic skeletal diseases.

Dysfunctional YAP and/or TAZ signaling in mice mimicked characteristics of human cases of developmental bone diseases such as skeletal dysplasia (Lemyre et al. 1999) and osteogenesis imperfecta (OI) (Forlino and Marini 2016; van Dijk et al. 2011; Rauch and Glorieux 2004; Kegelman et al. 2018). As a heterogeneous group of inheritable diseases, the severity of OI ranges from mildly increased fracture risk to perinatal lethality (Forlino and Marini 2016). OI is characterized by increased bone fragility and deformity as well as

collagen matrix disorganization (van Dijk et al. 2011). Osterix-conditional YAP/TAZ knockout mice also mimicked several established mouse models of OI (Khillan et al. 1991; Pereira et al. 1993; Chipman et al. 1993) with spontaneous fractures, disorganized collagen, and altered osteoblast/osteoclast-mediated remodeling. More recently, a novel transgenic mouse model of OI type I with mutations in the *COL1A1* gene demonstrated downregulation of YAP expression in bone, potentially implicating a feedback loop between upstream matrix activation and downstream transcriptional regulation (Y. Liu et al. 2019). Although the emerging evidence of YAP/TAZ in skeletal lineage cells resembles OI pathogenesis, loss of function mutations directly in either YAP or TAZ are unlikely to play a casual role in human OI. Nonetheless, YAP/TAZ coordinate multiple signaling axes, including TGFβ (Varelas et al. 2008, 2010) and WNT-β-catenin (Heallen et al. 2011; Azzolin et al. 2014), with known roles in bone disease.

In addition to developmental disease, the emerging roles of skeletal cell YAP and/or TAZ in regulating bone remodeling implicate a potential link to metabolic skeletal diseases, specifically related to the coordination of osteoblast/osteoclast and osteocyte-intrinsic remodeling. Metabolic skeletal disorders and diseases primarily related to abnormal bone remodeling include Paget's disease and osteoporosis (X. Feng and McDonald 2011). In the context of both diseases, aberrant cellular function in osteoblasts, osteoclasts, and osteocytes causes altered bone remodeling, resulting in low bone mass, structural deterioration and/or deformities (X. Feng and McDonald 2011). YAP/TAZ deletion in mature osteoblasts and/or osteocytes resulted in low bone mass with increased osteoclastic remodeling, similar to these diseases (Kegelman et al. 2019; Xiong, Almeida, and O'Brien 2018). While targeting osteoblast/osteoclast-mediated bone remodeling is under clinical investigation, therapies targeting bone quality to treat metabolic bone remodeling disease are currently emerging. Treating bone quality to improve bone strength relates improving the integrity of the osteocyte lacunar/canalicular network. Both increased age (Vashishth et al. 2000) and reduced TGFβ signaling (Dole et al. 2017) cause defects in osteocyte lacunar/canalicular network associated with skeletal fragility. Correspondingly, YAP/TAZ deletion from osteocytes affected both bone quantity and quality via defects in perilacunar/canalicular remodeling, resulting in bone fragility (Kegelman et al. 2019). Evidence for YAP/TAZ signaling in both regulating osteoprogenitor cell function in skeletal development and coordinating osteoblast-osteoclast as well as osteocyte-mediated bone remodeling suggests a more mechanistic understanding of how YAP/TAZ signaling affects bone function could contribute new insights into the heterogeneity and/or etiology of both metabolic and developmental skeletal diseases.

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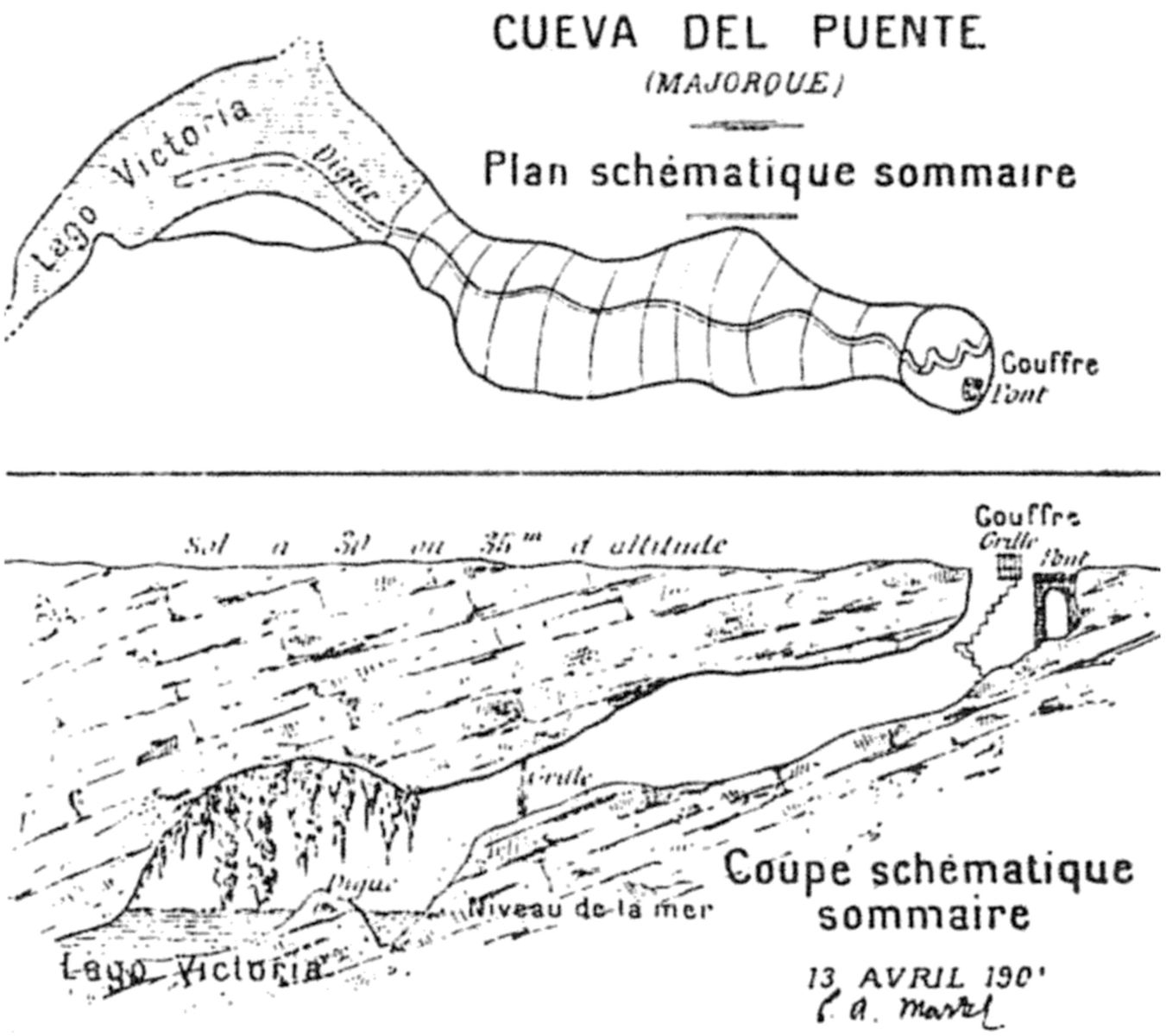

Figure 1.

Coupé of “Bridge Cave”, on the island of Mallorca, drawn by Édouard-Alfred Martel in 1901. Uncovering the mechanisms that control the development of the skeleton is like exploring a cave.

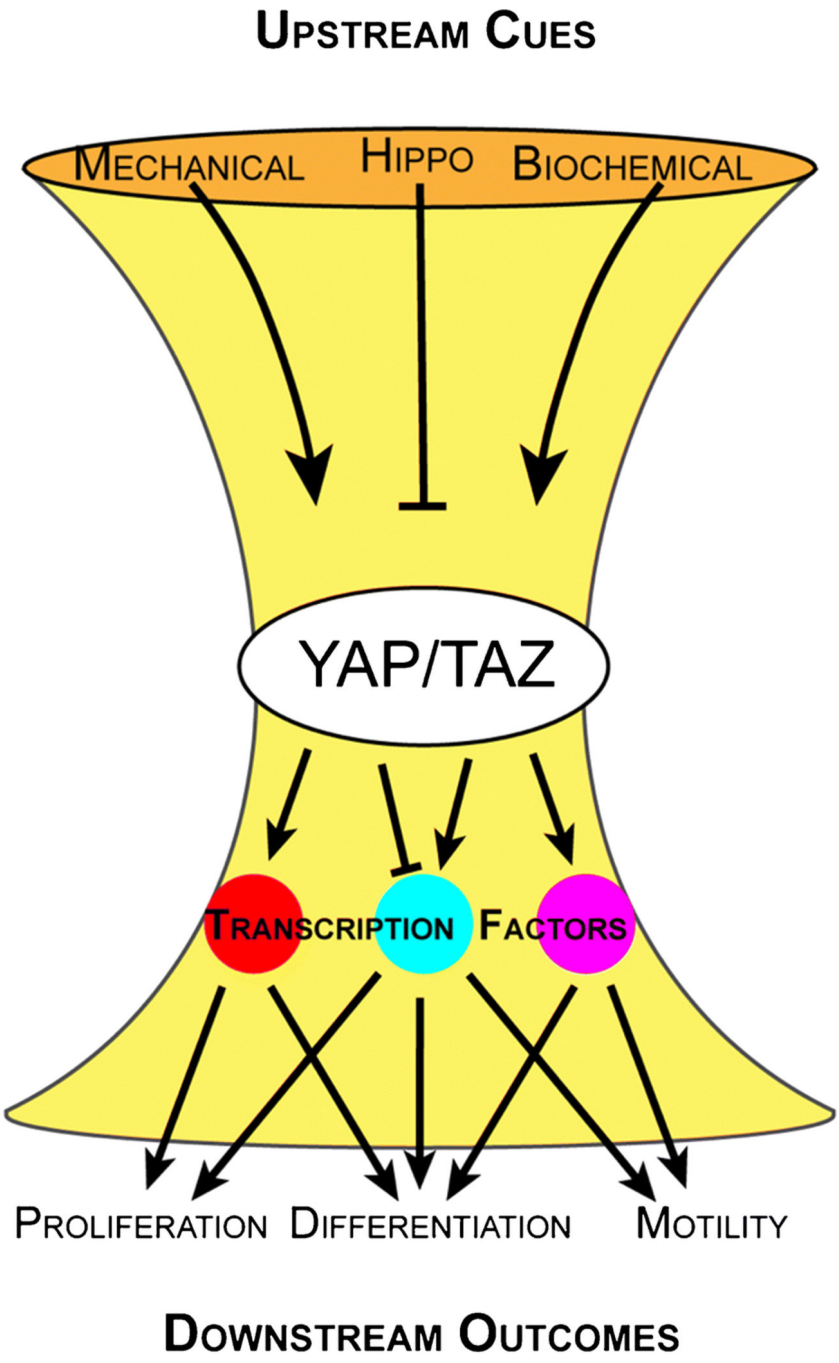

Figure 2. The YAP/TAZ hourglass: a schematic of YAP/TAZ regulation and function.

YAP and TAZ are controlled by three primary inputs: mechanical cues, the Hippo pathway, and biochemical cues. Upon activation, YAP/TAZ bind to a variety of transcription factors in the nucleus to regulate diverse downstream outcomes.

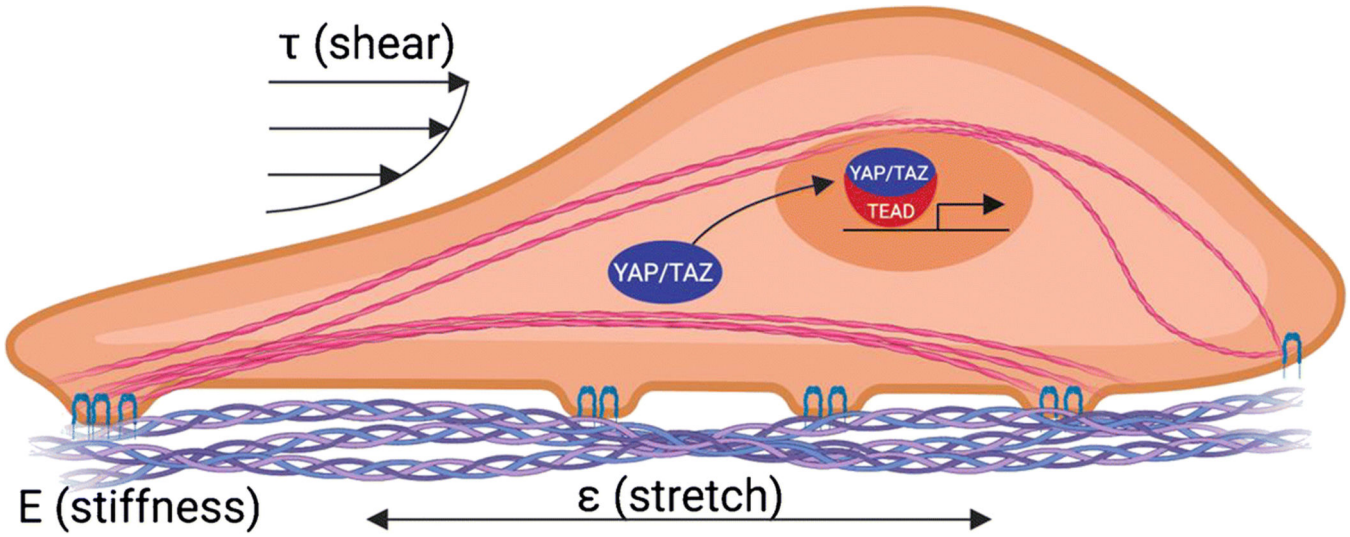

Figure 3. Illustration of YAP/TAZ activation in response to mechanical cues.

YAP and TAZ translocate to the nucleus in response to cytoskeletal tension induced by various mechanical stimuli, including matrix stiffness (E), stretch (ε), and fluid shear stress (τ), among others.

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Table 1:

Putative YAP/TAZ-interacting transcription factors

	Transcription factor	Cells	YAP binding activity	Role in skeletogenesis
Skeletogenesis	RUNX2	Osteoblasts	PPxY motif in Runx proteins binds to WW domain of YAP1[1].	Master regulator of osteogenesis required for osteoblast proliferation[2].
	Snail/Slug	Skeletal stem/stromal cells	N terminal domain of Snail/Slug (SNAG domain) binds to WW domain of YAP1[3].	Promote skeletal stem cell renewal[3].
	β-catenin	Osteoblast lineage cells/ BMSCs	Exact mechanism of interaction is unknown[4].	Involved in orchestrating endochondral or intramembranous ossification by specifying chondrogenic versus osteogenic fate[5].
	TEAD		C terminal region of TEAD binds to TEAD binding domain of YAP1[6].	YAP/TAZ TEAD complex promotes osteogenic and collagen-related gene expression[7].
Involved in other cells	CREB	Neuroblastoma cells	CREB binds to N terminal of YAP1[8].	CREB family of activators is involved in regulating chondrocyte proliferation in developing bone[9].
	HIF1-α	Hepatocellular carcinoma cells	Exact mechanism of interaction is unknown[10,11].	Regulates chondrogenesis by regulating Sox9 expression in hypoxic prechondrogenic cells[12].
	SOX5	Non-small cell lung cancer cells	Exact mechanism of interaction is unknown[13].	In combination with Sox6, Sox5 is required for proper development of endochondral bones[14].
	Notch	Mouse aortic smooth muscle cells	Notch intracellular domain binds to first WW domain of YAP1[15].	Notch signaling is essential for normal progression of hypertrophic chondrocyte differentiation into bone[16].
	RUNX3	Mammary epithelial cells	PPxY motif in Runx proteins binds to WW domain of YAP1[17].	Essential for chondrocyte maturation[18].
	Pax3	Neural crest derived cells or HEK293 cells	Pax3 homeodomain is involved in binding activity[19].	Downregulation of Pax3 is required for normal differentiation of cranial neural crest cells[20].
	SMAD1/5/8	HEK293 cells	Phosphorylation by CDK8/9 at S206 facilitates binding of SMAD1 to YAP[21].	SMAD signaling is suppressed by YAP to impair chondrogenesis[22].
	SMAD2/3	HEK293 cells	SMAD2 MH1 domain binds to TAZ coiled-coil domain[23].	SMAD2 is indespensible during early development and SMAD3 is required for articular chondrocyte homeostasis[24].
	SMAD7	COS-7 cells	SMAD7 PY motif interacts with WW domain of YAP1[25].	SMAD7 inhibits chondrocyte differentiation during endochondral ossification at different steps[26].
	p73	Mouse embryonic carcinoma (P19) cells	WW domain of YAP1 interacts with the PPPPY motif of p73 protein[27].	p73 is essential for vitamin D mediated osteoblast differentiation[28].
	ErbB-4	HEK293 cells	WW domain of YAP1 interacts with the PPxY motif closest to the C-terminal of ErbB-4 receptor[29,30].	ErbB signaling is involved in chondrocyte maturation and periosteal osteoblast differentiation[31].
	EGR1	Prostate cancer cells	WW domain of YAP1 interacts with the PPXY motif of EGR1[32].	EGR1 is involved in maintaining the chondrocyte extracellular matrix[32,33].

	Transcription factor	Cells	YAP binding activity	Role in skeletogenesis
	C/EBPα	Liver cancer cells	WW domain of YAP1 interacts with the PPXY motif of C/EBPα[34].	C/EBPα is involved in terminal differentiation of osteoclasts[35].
	TBX5		WW domain of TAZ interacts with the C-terminal region of TBX5[36].	TBX5 is involved in forelimb bud formation and outgrowth[37].